Elucidating the Flavour Synthesis Pathway of *Agaricus bisporus*

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Abstract: The mushroom *Agaricus bisporus* has a strong and unique flavour that is mainly due to the release of volatile eight-carbon compounds. However, despite the economic importance of this mushroom and the crucial role of flavour in the sales of fresh produce, very little is known on the biochemical mechanism of 8-carbon volatile synthesis and its regulation. Eight-carbon volatiles have also been shown to be involved in the control of the phase change from mycelial to reproductive growth modes, and the perception by insects of the presence of mushroom fruitbodies. Eight-carbon volatiles are derived from the oxygenation and the cleavage of the polyunsaturated fatty acid, linoleic acid. This reaction has similarities to the plant system, but also major differences. Examination of the enzymic mechanisms and the fatty acid chemistry suggested that the enzyme involved in the oxygenation step could be a lipoxygenase (as found in plants) or a heme-dioxygenase, similar to the recently isolated linoleate diol synthase from *Gaeumannomyces graminis*. We adopted a multidisciplinary approach to characterise the mushroom flavour biogenesis pathway. The range of molecular tools we used (PCRs, cloning, libraries screening and functional genetics) enabled us to isolate several candidate genes, and chemical analysis (Solid phase microextraction, GC-MS, thin-layer chromatography) provided information needed to understand the chemistry of eight-carbon volatile formation.

Key words: Linoleic acid, oxidation, flavour, 1-octen-3-ol, hydroperoxides, volatile compounds

1 Introduction

Out of the main factors affecting quality, like texture, colour and maturity, mushroom flavour is maybe the least understood. Very little is known on how it is produced, or regulated. Essential to the consumer, it represents a complex set of interactions between flavour active chemicals and the human senses of taste and smell. Volatile flavour compounds also possess a wide range of biological activities from crop protection to initiation of pin formation. Flavour is highly variable, depending on the mushroom strain, the maturity, and the storage of the harvested crop, as well as being easily altered by processing like cooking, freezing or drying.
Non-volatile compounds like sugars, amino acids and monosodium glutamate-like (MSG like) components take part in producing the delicate and unique mushroom flavour,[1-3] but 8-carbon volatile compounds are its main components, especially the aliphatic alcohol 1-octen-3-ol.[4] Depending on the extraction method, these 8-carbon volatiles account for 44.3 to 97.6% of the total volatile fraction. Their respective threshold values and aromas are listed in Table 1.
Discovered in 1936 by Murahashi,[5] 1-octen-3-ol is also called "mushroom alcohol". It is produced by enzymatic oxidation of the main fungal fatty acid: linoleic acid (18:2, Δ9,12). The resulting hydroperoxide is then processed by a cleavage enzyme, to produce 1-octen-3-ol. The identity of the enzyme involved in the oxidation step is still unknown in fungi and several hypotheses have been proposed.
Tressl[6, 7] described a biochemical pathway involving a lipoxygenase, a non-heme, iron-containing enzyme. This pathway illustrates the formation of 1-octen-3-ol from linoleic acid through a 13-hydroperoxide (13-HPOD) step, this hydroperoxide being later cleaved to 1-octen-3-one and 10-oxodecenoic acid. An alcohol

oxidoreductase would then process the 1-octen-3-one into 1-octen-3-ol.
However, Wurzenberger and Grosch[8-10] later showed that a 10-HPOD, and not a 13-HPOD, was the intermediate in 1-octen-3-ol formation. They proposed a new pathway for 1-octen-3-ol formation, involving a lipoxygenase-like enzyme oxidising linoleic acid into a 10-HPOD, this one being later cleaved in 1-octen-3-ol and 10 oxodecenoic acid by a hydroperoxide lyase. Hydroperoxide lyase (HPOL) is an enzyme from the cytochrome P450 family and is involved in flavour production in plants. Its activity has recently been re-investigated by Grechkin & Hamberg[11] who revealed that HPOL is not a lyase, but an isomerase producing a hemiacetal that undergoes spontaneous decomposition to produce aldehydes. No HPOL encoding gene has yet been found in the fungal kingdom, adding to the difficulty in understanding the mushroom flavour biogenesis pathway.

Table 1.Eight carbon volatile compounds found in mushrooms[12]

Compound	Relative concentration (%)	Threshold value (p.p.m)	Aroma
1-octen-3-ol	33	0.010	Mushroom like
1-octen-3-one	0.02	0.004	Boiled mushroom
Trans-2-octen-1-ol	6	0.040	Medical, oily, sweet
Trans-2-octenol	0.05	0.003	Sweet, phenolic
3-octanol	1	0.018	Cod liver oil
3-octanone	4	0.050	Sweet, fruity, musty
Octanol	0.3	0.48	Detergent, soap
1-octen-3-yl acetate	0.05	0.09	Mushroom like, soapy
1-octen-3-yl propionate	0.4	0.022	Mushroom like, fruity

Recently, a variety of functions have been attributed to fatty acid metabolites, such as anti-fungal activity in *Laetisaria arvalis*,[13] or sporogenic functions in *Aspergillus nidulans*[14] the latest establishing a genetic connection between fatty acid oxidation and sporulation.
With the recent discovery of a novel oxidising enzyme from the wheat pathogen *Gaeumannomyces graminis*, a new hypothesis has been formulated. This enzyme, linoleate diol synthase (LDS), combines a dioxygenase and an isomerase activity. It uses linoleic acid as a substrate to produce a 8-HPOD, which is then converted to a diol.[15, 16] The gene coding this enzyme shows high similarity to the prostaglandin endoperoxide synthase H (PGHs) family, present in animals.[17] LDS is a novel kind of fungal fatty acid dioxygenase from an ascomycete and provides us with a new model for the formation of a 10-HPOD and 1-octen-3-ol.
Eight-carbon volatile formation is unique to fungi and is likely to involve a fungal specific enzyme pathway. It is evident that lipid and fatty acid metabolism have been under-investigated in the fungal kingdom, with very few genes and enzymes yet identified. This adds to the difficulty in understanding unique systems such has eight-carbon compounds production. Having to rely heavily on animal and plant resources, from sequence information to model biochemical pathways, complicates the characterisation of such unique systems.
This paper presents the work achieved so far to further our understanding of flavour biogenesis in *Agaricus bisporus*. We are studying the mechanism of flavour biogenesis using molecular tools as well as chemical analysis to gain a full understanding of this biochemical pathway.

2 Materials and Methods

2.1 Mushroom strain and growth conditions

A. *bisporus*, strain A15, was grown at the mushroom unit at HRI according to commercial practice.

2.2 Volatiles analysis

Volatiles were extracted by SPME (Solid Phase Microextraction). Mushroom samples were either whole mushroom, cut mushroom or blended mushroom. Mushrooms were blended in MES buffer pH 7.0 in a 2:1 ratio. In homogenates, 1-hepten-3-ol at a final concentration of 1µg/mL was added as an internal standard for later quantification. Mushroom samples or homogenates were placed in gas tight jars, left 10 minutes for equilibration of volatiles in the headspace, at a constant temperature of 25°C (and under constant stirring for the homogenates). A 50/30 µm DVB/Carboxen/PDMS fibre (Supelco) was used with the SPME fibre holder device. It was conditioned in the GC injection port at 270°C for at least three hours. The needle of the sample holder was then inserted in the vial and the fibre was exposed to the headspace for 10 minutes. The fibre was withdrawn and inserted in the GC inlet (230°C, 1 minute) where the analytes were thermally desorbed.

2.3 GC-MS analysis conditions

The gas chromatographic analysis was performed on a 6890 GC system (Agilent Technologies, USA) equipped with a 5973 Network MSD mass spectrometer. The compounds were run on a 30m x 0.25 mm 19091N-133 Innowax column (Agilent Technologies). Once the analytes were desorbed, the column was maintained at 40°C for 1 minute, increased at 10°C/min to 90°C, further increased at 6°C/min to 160°C and finally maintained at 160°C for 3 minutes. Helium was used as the carrier gas at a velocity of 36 cm/sec.

2.4 Western blots of proteins

Mushroom proteins were extracted in 50 mM Tris-HCl pH 7.5, 0.5% Triton X100. Mushrooms from stage 1 to 7 were blended in cold buffer in a ratio of 2:1 (buffer volume: mushroom weight) and centrifuged at 20,000g for 20 minutes. An aliquot (20µL) of each sample's supernatant was added to loading dye and loaded on 10% SDS-polyacrylamide gel. The proteins were then transferred on to nitro-cellulose in 48 mM Tris, 39mM glycine, 20% methanol, pH 9.2. The protein blots were send to the laboratory of Dr Jose J. Sanchez-Serrano, Madrid, Spain where they were probed with antibodies raised to the potato lipoxygenases LOX H1 and LOX H3.

2.5 DNA extraction

Small-scale DNA extractions were performed using the QIAGEN plant DNA extraction mini kit, following the manufacturer's instructions. For large-scale extraction, the facile DNA extraction protocol described by Challen et al [18] was used.

2.6 RNA extraction and RT-PCR

Small-scale RNA extractions were performed using the QIAGEN RNA extraction mini kit according to the manufacturer's instructions. The RT-PCR step was performed using Thermoscript from Invitrogen. RNA was primed either with oligo d(T) or gene specific primers following the manufacturer's instruction.

2.7 PCR using degenerate primers

PCR reactions were performed on cDNA. The reaction comprised 2µL of cDNA obtained from the previous step, 2.5 µL of forward and reverse primers (20 µM stock solutions), 18 µL of DEPC treated H_2O and 25µL of Readymix red Taq polymerase (Sigma). The thermo-cycling conditions (Omnigene Thermal cycler, Hybaid) were: 95°C, 5 minutes, for one cycle; 94°C for 1 minute; annealing temperature (according to the primers Tm)

for 2 minutes; 72°C for 3 minutes. This was repeated for 30 cycles, followed by a final cycle at 72°C for 5 minutes. An aliquot (45µL) of the PCR reaction was run on a 1% agarose gel.

2.8 PCR library screening

The genomic library was constructed from *A. bisporus* strain C54-carb8 by Challen et al.[19] A DNA pool was created from each 96-well plate. This DNA was screened by PCR, using primers designed after a candidate gene fragment obtained by degenerate PCR. An aliquot (5µL) of the 1/10 diluted pool DNA was mixed with 1. 25µL of forward and reverse primer (20 µM stock solutions), 5 µL DEPC treated H_2O and 12.5 µL of Readymix red Taq polymerase (Sigma). The thermo-cycling conditions were 95°C for 2 minutes, for 1 cycle, 94°C for 1 minute, 65°C for 2 minutes, 72°C for 1 minute. This was repeated for 35 cycles, and followed by a final cycle at 72°C for 5 minutes. Each entire reaction was run on a 1% agarose gels.

2.9 RACE (Rapid Amplification of cDNA Ends) PCR

RNA was reverse transcribed to cDNA as described above. The cDNA was tailed at the 5'end (polyA) with terminal deoxynucleotidyl-transferase (TdT).[20] The PCR and nested PCR steps were performed as described by Frohman et al. [20]

2.10 Cloning and sequencing of PCR fragments

Samples (2µL) of fresh PCR product was used for cloning using the Invitrogen TA cloning kit, following the manufacturer's instructions. The cloned insert was sequenced using the vector's M13 primers (reverse and forward primer) and ABI PRISM Big Dye terminator (AB applied biosystems). The thermo-cycling conditions were 96°C for 10 seconds, 50°C for 5 seconds, 60°C for 4 minutes. This was repeated for 25 cycles, and followed by a final cycle at 60°C for 5 minutes. The samples were sequenced at Warwick HRI genomic centre using an ABI PRISM 3100 Genetic analyser.

2.11 Southern blots, probe production and hybridisation

For Southern blots, at least 8 µg of restricted genomic DNA was loaded on each gel lane. DNA was transferred by alkali blotting (as described by Americium, Hybond-N+ manual) on Highland-N+ membranes (Americium). DNA from colony lifts was transferred on Hybond-N membranes. Probes were produced using the Reed Primer II Kit (Americium), using [32P] DTP. For homologous probing, membranes were pre-hybridised at 68°C overnight, the probe was added and hybridisation was carried out at 68°C. The membranes were washed twice with 1x SSC, 0.1% SDS, for 30 minutes, and twice with 0.1x SSC, 0.1% SDS, for 30 minutes, at 68°C. Heterologous hybridisation was carried out in the same conditions, at a temperature of 65°C and replacing the second wash by 0.2x SSC, 0.1% SDS.
The hybridisation conditions were altered for the heterologous screening of the cDNA libraries arrayed on Highland N+ membranes. All steps were performed at 65°C, and only two washes were carried out, for 30 minutes each, the first with 3x SSC, 0.1% SDS and then 1x SSC, 0.1% SDS solutions.

3 Results and Discussion

3.1 Volatiles analysis

All the mushroom samples were freshly harvested and subjected to the analysis in the shortest time possible.

Tissues were collected if necessary, with care to produce a little damage as possible.
Solid Phase Microextraction[21] is a simple and powerful tool to use to obtain volatile data from different samples. As a solvent free method, a variety of different matrices can be subjected to volatile analysis using this technique. Its main advantage concerning this study was to extract the volatiles contained in the headspace, reflecting more closely the true flavour of the sample, as perceived by the consumer. In this study, it allowed sampling of the headspace of whole, cut or blended mushrooms. A representative capillary GC chromatogram from the homogenate headspace of a stage 3 mushroom is shown in Figure 1. Volatile profiles have been established for each stage of development, enabling to show a decrease in octenol emission according to maturity. We were able to identify the main eight-carbon volatile compounds from the GC chromatogram: 1-octen-3-ol, 1-octen-3-one, 3-octanone, 3-octanol.

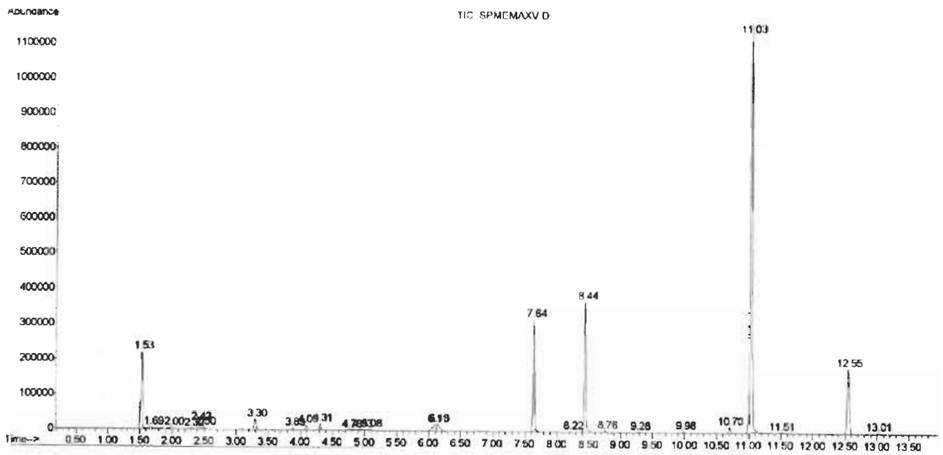

Figure 1. Volatile profile, mushroom homogenate
Retention times (in minutes): 1-octene (2.43); 3-octanone (7.64); 1-octen-3-one (8.44); 3-octanol (9.98) ; 1-octen-3-ol (11.03)

Damaged mushrooms were obtained by cutting and blending whole, freshly harvested fruitbodies. It was possible to establish than 8-carbon volatile emission is proportional to damage level. This suggests that the substrate of the reaction, linoleic acid, and the enzyme(s) responsible for eight-carbon volatile formation are stored in different cell compartments, and brought in contact by damage. The reaction was so rapid that it is not likely to be produced by transcriptional activation caused by damage.
It was also possible to observe a difference in volatile profiles between cut and blended mushroom. If octenol is the major volatile for blended mushrooms, then the ketone, 3-octanone, was the main one for cut mushrooms. It partly reveals the complexity of the biochemical interactions taking place between the different flavour components, depending on the level of damage applied.
We could also observe that gill tissue is more active than cap, stipe or skin tissue for the production of 1-octen-3-ol.

3.2 Identification of candidate genes involved in 10-HPOD formation

Preliminary studies on Western blots of proteins from mushrooms (stage 1 to 7) revealed the presence of a lipoxygenase-like enzyme (about 72 kDa) in *A. bisporus* (Figure 2). It was detected using an antibody raised against a fusion peptide of the lipoxygenase H3 from potato.[22, 23]
The presence of such an enzyme in *Agaricus* is of great interest, since lipid metabolism has not been extensively investigated yet. However, further studies are required to establish if it is involved in octenol production

or not. We have decided to follow both directions: studying both the lipoxygenase genes and the LDS-like genes, in order to understand how octenol is produced.

Figure 2. Western blot of mushroom proteins, probed with the antibody raised against LOXH3
Lane 1: Marker; lane 2 to 8: mushroom proteins, stage 1 to 7; lane 9: Marker; lane 10: Marker.

Using LDS as a model gene, we performed multiple sequence alignment with related sequences in order to design degenerate primers to the conserved regions of those gene sequences. The primers were used on both *A. bisporus* and *G. graminis* cDNA. Each PCR fragment produced was cloned and sequenced, in order to examine identity by performing BLAST searches. This approach yielded a fragment showing similarities to LDS, in *A. bisporus*. This fragment was then used to design new primers in order to screen a previously created *A. bisporus* genomic DNA library.[19]
The release of new fungal genomes sequences, allowed more BLAST searches and multiple sequence alignments to be performed. Several probes from *Phanerochaete chrysosporium* genes similar to LDS were designed and used to heterologously screen Southern blots of restricted genomic DNA of *A. bisporus*, and three cDNA libraries from *A. bisporus*, corresponding to stages 1, 4 and 2-days storage. The library screening process revealed a large number of positive clones. These clones were sequenced and the sequence information produced is being stored efficiently in a new database system. The degenerate primers tested on *G. graminis* DNA also produced two more candidates.
We now have a range of candidate genes likely to perform the oxidation of linoleic acid in the production of eight-carbon volatiles. The function of those candidate genes is being investigated by heterologous expression in baculovirus and should provide us with a clear picture concerning flavour biogenesis, but also fatty acid metabolism in mushrooms.
The molecular approach generated considerable amounts of new sequence information that will be greatly profit to this project, as well as others, since the lack of genomic sequence is still a hurdle in most gene searching exercises. The multidisciplinarity of the project was extremely valuable, as combining biochemical analysis with molecular searches enabled us to better understand how flavour is produced, as the observation made during the volatile analysis are capital in supporting the molecular evidence gathered.

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