CHAPTER 36

EXTRACELLULAR ENZYMES AS TARGETS FOR STRAIN IMPROVEMENT IN *AGARICUS BISPORUS*

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1. INTRODUCTION

The cultivation of *Agaricus bisporus* represents the largest proportion of mushroom production in the world. In common with the production of other edible fungi this output is dependent on bioconversion of low cost waste lignocellulosic residues into a useful human foodstuff (Wood, 1989).

Agaricus bisporus production is carried out in a two-stage process. The first is the controlled composting of cereal straw with suitable nitrogen sources such as manures or inorganic nitrogen fertilisers. The second stage is the **partial** utilisation of the compost by the mycelium of the fungus. This is followed by various cultural manipulations to promote production of fruit bodies (Wood & Smith, 1987).

Extensive nutritional and enzymological studies have revealed which nutrient fractions of the compost are utilised by the fungus and the likely extracellular enzymes used (Wood *et al.*, 1990; Wood *et al.*, 1991). The fungus has been shown to be able to degrade both plant and microbial biopolymers such as lignin, cellulose, hemicelluloses, protein and microbial cell walls (Durrant *et al.*, 1991; Manning & Wood, 1983; Fermor & Wood, 1981). The secreted enzymes produced by *Agaricus* include both hydrolases and oxidases. These include laccases, cellulases, hemicellulases, proteases and other enzymes.

When cropping is terminated the quantity of substrate finally utilised by the fungus is about 25-30% of the starting dry matter present after the preparation of compost. This is partitioned into mycelial biomass, respiratory products such as CO_2 and fruit body biomass (Wood, 1979; Wood & Smith, 1987). There is thus a considerable quantity of non-degraded organic material still available from the compost after fruit body production has finished.

Scope exists, therefore, for approaches aimed at increasing the efficiency of compost utilisation. One route would be to increase the level of extracellular enzyme secretion into the compost and then to determine if this change could effect an increase in mycelial biomass and greater fruit body yield.

As part of a programme aimed at this approach to strain manipulation, two extracellular enzymes of *Agaricus bisporus*, laccase and cellulase, have been studied in considerable detail. Both

of their activities are strongly regulated in the substrate during fruit body development (Wood & Goodenough, 1977). Their regulatory control indicates that these enzymes are associated with both the nutritional and developmental physiology of the fungus. Studies have been carried out on the production, enzymic properties, physiological role, protein structure, antigenicity and molecular genetics of both enzyme activities (Wood & Thurston, 1991; Raguz *et al.*, 1992; Perry *et al.*, 1993a & b). The aim of this work is to provide suitable enzyme targets in the life cycle that could be manipulated by genetic engineering methods, particularly by the use of DNA transformation techniques. Transformed strains would then be analysed for any useful effect of increased enzyme level on mycelial biomass and fruit body yield, and regulation of the "flushing" response. Additionally, the analysis of the molecular genetics of these enzymes could provide useful DNA sequences to assist the development of a workable DNA transformation system for the fungus.

2. LACCASE

2.1. Regulation, Structure and Biosynthesis

Laccase activity is strongly regulated during fruit body production on compost. Its activity increases during colonisation and then declines rapidly when fruit bodies develop (Wood & Goodenough, 1977). The use of axenic cultures and strains of *Agaricus* genetically blocked in fruiting shows that regulation of this decline in activity occurs at the stage of fruit body **enlargement**, not initiation. A useful by-product of this work was the observation that laccase activity can be used (in cultures before fruiting occurs) to estimate the yield of mycelial biomass (Wood, 1979). Analysis of the loss of activity showed this was due primarily to inactivation followed by enzyme protein loss.

The molecular properties of laccase protein from both liquid and compost cultures have been extensively analysed (Wood, 1980a, b; Wood *et al.*, 1991; Perry *et al.*, 1993a). The enzyme is abundant, forming over 2% of cell protein and its various molecular forms have been purified from malt extract medium and from compost cultures at maximal activity (high activity, HA form) and low activity (low activity, LA form).

The purified liquid culture form is a laccase of 100 kDa molecular weight, with substrate specificities, kinetic properties and inhibitor profile similar to other fungal laccases but with lower copper content and is yellow. The HA laccase from compost has considerable enzyme and structural similarities to the liquid culture form of the enzyme, but has more copper and consequently is blue. The LA form differs considerably, is nearly copper deficient and has a very different amino acid profile.

The HA and LA forms show considerable differences on SDS-PAGE analysis. Various antibodies have been prepared to native enzyme forms, denatured forms, and to an N-terminal 15 amino acid sequence of the major polypeptide (Perry *et al.*, 1993a). The enzyme forms present in liquid and compost cultures have been analysed by Western blotting techniques. The different band patterns revealed are probably due to the production of partially cleaved enzyme molecules associated with differing extents of glycosylation.

Studies on biosynthesis in liquid culture show that a single major excreted protein product of 68 kDa is made. The higher molecular weight of purified enzyme may be due to dimerisation. Poly(A) containing mRNA from these cultures translates an immunoprecipitable product of 57 kDa, consistent with a carbohydrate (glycosylation) level of 15% for the final form of the exported enzyme.

Biosynthesis of laccase protein in compost cultures shows that maximal biosynthesis occurs at the veil break stage when enzyme activity is rapidly **declining**.

The physiological role of laccase is unknown. Its abundance indicates a key role. It may be associated with the ability to release recalcitrant or bound nutrients linked to phenolic polymers, and thus contributes to the 'selectivity' of mushroom compost as a growth medium for the fungus (Wood & Smith, 1987).

2.2. Molecular Genetics

Two distinct laccase genes were identified when an expression library was screened with anti-laccase antibody (Perry *et al.*, 1993b). Identification of certain of these clones as laccase genes was confirmed by demonstration of two characteristic copper binding motifs in the deduced amino acid sequence and by alignment of deduced sequence with amino acid sequence of a cyanogen bromide fragment of pure laccase protein.

One cDNA clone was used to identify further cDNA clones and a genomic clone. The coding sequences showed two related but non-identical genes were present. The two genes, lcc1 and lcc2 are non-allelic as they did not segregate in Southern blot analysis of DNA from the uninucleate spores of a 4 spored basidium. Both laccase genes encode a sequence expressed as a 2.3 kb mRNA specifying a 520 residue polypeptide including a 19 amino acid residue signal peptide not present in the mature protein. The laccase genes mapped to the 2 smallest *Agaricus* chromosomes separable by electrophoretic karyotyping (Lodder *et al.*, 1993).

3. CELLULASE

3.1. Regulation, Structure and Biosynthesis

Regulation of endocellulase activity is associated also with fruit body development (Wood & Goodenough, 1977). Endocellulase activity increases and decreases in parallel with harvested fruit body biomass in each 'flushing' cycle (Claydon *et al.*, 1988). By use of different harvesting techniques such as continuous picking, or allowing large fruit bodies to reach senescence on the production beds it was shown that fruit body biomass positively regulates the quantity of substrate located extracellular endocellulase activity (Claydon *et al.*, 1988). It is also known that various **intracellular** fruit body carbon metabolising enzymes undergo cyclic variation in activity associated with flushing (Hammond, 1985; Wells *et al.*, 1987). This regulatory pattern has allowed the formulation of a simple physiological model to account for the observed enzyme activity changes (Wood *et al.*, 1988). It is assumed that the physiological role of endocellulase is to act on cellulose in the compost. This produces soluble carbohydrates which can be assimilated by the mycelium. When fruit bodies subsequently form on the mycelium the levels of intracellular mycelial carbon compounds are depleted due to translocation of these to the developing fruits. This depletion activates a putative signalling system which eventually results in more cellulase being produced to replenish carbohydrate levels in the mycelium. When fruit bodies are harvested there is no requirement for increased cellulase level and enzyme activity is down regulated.

Endocellulase activity has been purified from bulk liquid cultures grown on cellulose as sole carbon source (Manning & Wood, 1983). Purification was achieved by successive ultrafiltration, ion exchange and preparative gel electrophoresis steps.

Unpurified and purified liquid culture enzyme activity and compost extracts show five activity bands of increasing mobility when stained by zymogram techniques on cellulose with Congo Red counterstaining. These purified bands have been used to prepare an anticellulase antibody. Each

isoform of native endocellulase resolves into further multiple bands using isoelectric focusing, and each isoform has different structural properties. Analysis by SDS-PAGE gels shows that each band differs quantitatively in polypeptide composition but that they all share certain common bands. The isoforms 1, 2 contain predominantly polypeptides of 56 and 40 kDa, isoforms 4 and 5 of 22 kDa. Amino acid sequence analysis shows that the 56 and 40 kDa bands share a common N-terminal sequence of up to 15 amino acids.

Biosynthesis of endocellulase protein in compost cultures parallels expression of enzyme activity, with maximum biosynthesis occurring at the senescent stage of maximal fruit body biomass, and synthesis declines rapidly on harvesting of fruit bodies. Successive flushes are associated with new rounds of enzyme biosynthesis.

3.2. Molecular genetics

The anticellulase antibody has been used to isolate cellulose induced genes. RNA was prepared from cellulose grown mycelium. *In vitro* translation was then used to identify three main polypeptides of 38, 58 and 60 kDa. No such products were found from mycelium grown on fructose. The same antibody was used to screen an expression library, and two related cDNA clones were isolated. One clone selected for an mRNA that translated a 38 kDa product. A gene, cel 1, was sequenced from a genomic fragment hybridising to this cDNA clone. Sequence analysis shows this gene encodes for a cellulase, as shown by identification of a cellulose binding domain, and a separate proline-threonine-serine rich region known as the 'hinge' region by homology with other glycanases. The remaining core region is not homologous to any other -glycanase so far sequenced. A further gene cel 3, has been identified, and is being sequenced.

4. FUTURE WORK

This is aimed at studying gene expression during growth on liquid or compost cultures to determine if regulation of laccase or cellulase activity is associated with transcriptional regulation. Sufficient genomic DNA sequence has been obtained to allow identification of regulatory regions upstream to the coding region for both sets of enzyme protein genes. This information should aid development of a workable efficient DNA transformation system by allowing homologous genes and their associated upstream regulatory regions to be placed in suitable transforming vector DNA sequences. Once this tool is in place it may be possible to use further genetic manipulation technology specifically to amplify or delete the production of laccase and cellulase to examine the possibility of producing novel mushroom strains with improved agronomic qualities.

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