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Note

Characterization of proteins expressed abundantly in the fruit-body of *Flammulina velutipes*

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Chronological changes of protein expression in the vegetative mycelium of *Flammulina velutipes* and expression of these proteins in the fruit-body were investigated by two-dimensional polyacrylamide gel electrophoresis. Four proteins (FBA1-4) expressed abundantly in the fruit-body were found to have different expression patterns in the vegetative mycelium after the fruiting treatment. FBA1-4 had similar amino acid sequences and displayed a high similarity with the deduced amino acid sequence of the *C1* cDNA, which has an Arg-Gly-Asp (RGD) cell-attachment sequence. This suggests that FBA1–4 may have cell-to-cell attachment activity.

Key Words—amino acid sequencing; *Flammulina velutipes*; fruit-body formation; RGD cell attachment sequence; 2D-PAGE.

Fruit-body formation is the most conspicuous cell differentiation process in basidiomycetous mushrooms. In intensive studies of fruit-body formation in *Schizophyllum commune* Fr.: Fr., several genes involved in fruit-body formation have been isolated. The *Sc1* and *Sc4* belong to a family of genes encoding hydrophobins, which are excreted into the cell walls of fruit-body hyphae where they form an insoluble complex (Schuren and Wessels, 1990; Wessels et al., 1991). The *Sc7* encodes protein that is apparently loosely associated with the hyphal wall (Schuren et al., 1993). The *Frt1* gene has a conserved sequence found in nucleotide-binding proteins (Horhon and Rarer, 1995). The cDNA clones *priB*, *Aa-Pri1*, and *FDS*, which are involved in fruit-body formation, have been isolated from *Lentinus edodes* (Berk.) Sing, *Agrocybe aegerita* (Brig.) Quel, and *Flammulina velutipes* (Curt.: Fr) Sing, respectively (Endo et al., 1994; Espinar and Labarere, 1997; Azuma et al., 1996). On the other hand, many proteins expressed during fruit-body formation have been detected by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) (De Vries and Wessels, 1984; Salado and Labarere, 1989; Miura et al., 1994). However, the amino acid sequences of these proteins separated by 2D-PAGE were not reported and no further information is available.

The experiments reported below were undertaken to investigate the proteins expressed during fruit-body formation of *F. velutipes*. Chronological changes of proteins expressed in the vegetative mycelium during fruit-body formation were investigated by 2D-PAGE analysis. The expression in fruit-bodies of the proteins expressed in the vegetative mycelium was also investigated. Four proteins that were expressed abundantly in the fruit-body but showed different expression patterns in the vegetative mycelium after the fruiting treatment were amino acid-sequenced. The relation between the amino acid sequence of these proteins and the deduced amino acid sequence of a previously sequenced cDNA (named *C1*) isolated in our laboratory from *F. velutipes* (Azuma et al., 1996) is discussed.

The strain (*F. velutipes*: Fv-4) used in this work was obtained from the Hokkaido Forest Products Research Institute Asahikawa, Japan. Discs of 5 mm in diam were punched out from mycelium grown on potato dextrose agar (PDA) plates for 5 d at 23°C in the dark, and each disc was inoculated on a sawdust medium. The sawdust medium packed in the test tube (21 × 180 mm) contained the sawdust from beech (*Fagus crenata* Blume) and wheat bran (sawdust : wheat bran = 4 : 1 v/v) with a water content of 65%. Cultures were incubated at 23°C in the dark for 1 mo. For fruit-body formation, they were transferred to a room with continuous illumination at 500 lux with a fluorescent lamp at 16°C (fruiting treatment). Primordia were formed at 12 d, pileus and stipes were observed at 14 d, and gills were observed at 21 d after the start of fruiting treatment.

For protein analysis, mycelium on the sawdust medium was sampled every day for 21 d after the start of fruiting treatment, frozen in liquid nitrogen, and stored at −80°C. Fruit-bodies were frozen in liquid nitrogen and stored at −80°C, and fruit-bodies formed after 21 d of fruiting treatment were used for protein analysis. Samples were sonicated in an extraction buffer (0.01 M Tris-HCl, pH 7.4, 5 mM $MgCl_2$) on ice and centrifuged (4°C, 10,000 × *g*, 15 min). Supernatants were mixed with an equal volume of the sample buffer (9 M urea, 1% ampho-

rine 3.5–9.5, 3% 2-mercaptoethanol), and urea was added to a final concentration of 9 M. Protein concentration was determined by the Bradford method (Bradford, 1976) with bovine serum albumin (BSA) as the standard. The protein concentration in all samples was adjusted to 2.0 mg/ml for 2D-PAGE analysis.

2D-PAGE was performed by the method of Hirabayashi (Hirabayashi, 1981) with modification. The first dimension involved iso-electricfocusing (IEF) on 2% agarose gel (diameter 1.5 mm, length 180 mm) with a pI range of 3.5–9.5. Protein samples (50 μl) and standard markers (5 μl) were applied to the gel, and electrophoresis was carried out at a constant voltage of 400 V for 15 h, then for a further 1 h at 800 V. For the second dimension, running gel with a 10–20% concentration gradient and a 4% stacking gel (160 × 180 × 1.5 mm) was used. The PAGE was carried out at a constant current of 30 mA for 5 h. The gel was soaked in a Coomassie brilliant blue (CBB) gel-staining solution (2.5% CBB-R250, 20% methanol, 7.5% acetic acid) overnight and destained with 50% methanol. Destaining was stopped with 10% acetic acid.

For the amino acid sequencing, electroblotting was carried out using a semi-dry method. Gel and a polyvinylidene difluoride (PVDF) membrane were equilibrated with a transfer buffer (30 mM Tris, 17 mM boric acid, 0.055% SDS, 20% methanol) for 20 min. Protein spots were blotted onto a PVDF membrane at a constant voltage at 15 V for 15 min, 20 V for 15 min, and 25 V for 30 min. The membrane was soaked in a CBB membrane-staining solution (0.1% CBB-G250, 40% methanol, 1.0% acetic acid) for 1 min, then destained with 50% methanol. Spots were cut out from the membrane and sequenced using a protein sequencer ABI 477A (Applied Biosystems).

Proteins were separated by two-dimensional agarose-polyacrylamide gel electrophoresis and detected by CBB staining (Fig. 1). There were four proteins (named FBA1-4) expressed abundantly in the fruit-body that showed different expression patterns in the vegetative mycelium after the fruiting treatment. FBA1 (32 kDa and pI 5.2) and FBA2 (31 kDa and pI 5.3) were expressed abundantly in the vegetative mycelium of 0 d (before fruiting treatment, Fig. 1a). FBA1 exhibited the maximum expression in the vegetative mycelium of 5 d (Fig. 1b), then decreased. FBA2 was expressed abundantly in the vegetative mycelium until 5 d, and decreased similarly to FBA1. At 21 d, low expression of FBA1 and FBA2 were observed in the vegetative mycelium (Fig. 1d). FBA3 (30 kDa and pI 5.1) was expressed

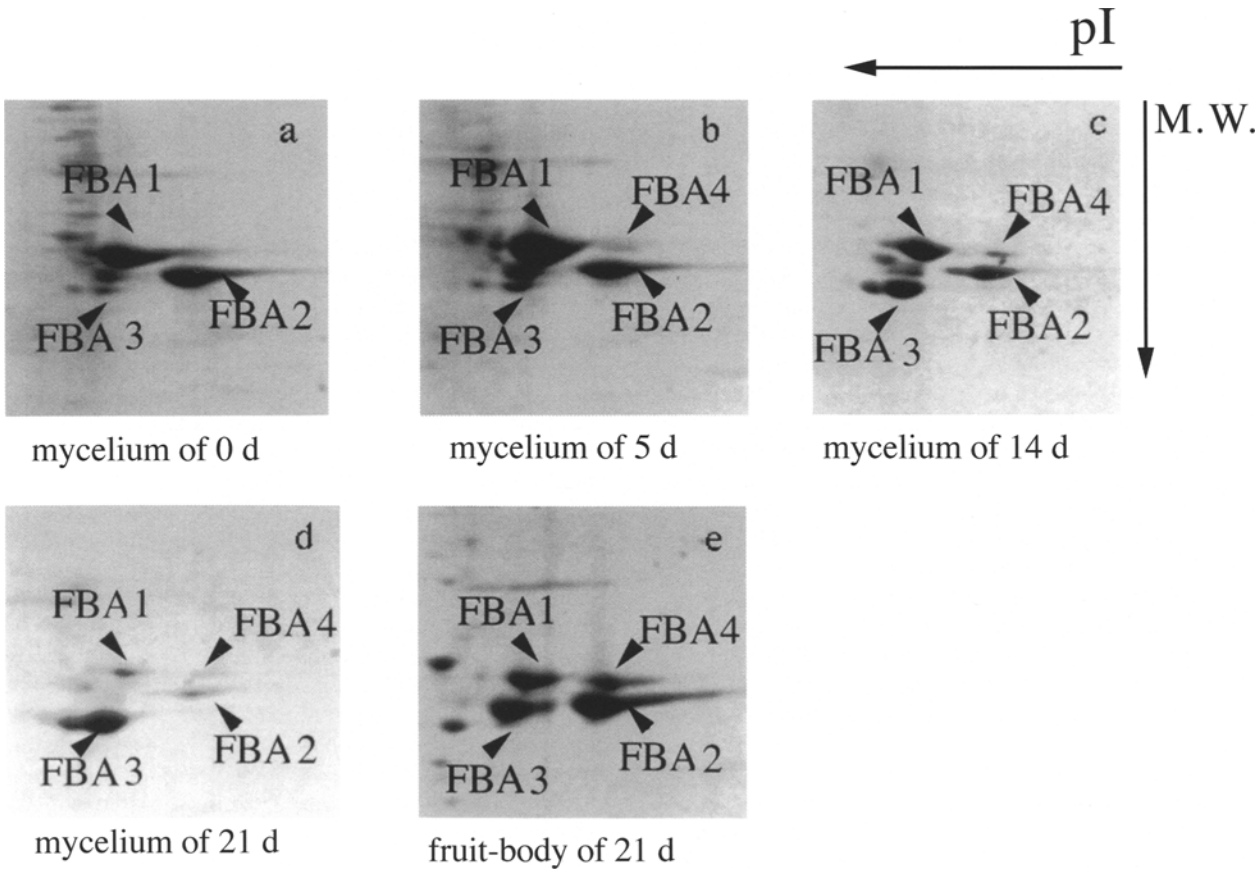

Fig. 1. 2D-PAGE analysis with CBB staining of proteins that are expressed in the vegetative mycelium during fruit-body formation and the fruit-body of *Flammulina velutipes*. a) Vegetative mycelium just before fruiting treatment (0 d). b) Vegetative mycelium of 5 d after the start of fruiting treatment. c) Vegetative mycelium of 14 d after the start of fruiting treatment. d) Vegetative mycelium of 21 d after the start of fruiting treatment. e) Fruit-body of 21 d after the start of fruiting treatment.

Table 1. The N-terminus amino acid sequences of FBA1-4 and the deduced amino acid sequence of the *C1* cDNA.

	molecular mass (kD)	pI	sequence
FBA1	32	5.2	PQVKTTWEDLANLGWPIEEVYK ***** ********** ***
FBA2	31	5.3	PQVXTIIEDLINLGWPIQQV *** * *** *********
FBA3	30	5.1	QVKTSWEDLANLGWPIXQV **************** **
FBA4	32	5.3	XQVKTSIEDLANLGWPIE ***** **********
C1	30[a]	5.2[a]	PQVKTSWEDLANLGWPIQQVYK

X, unidentified amino acid.
*, amino acid matches the deduced amino acid sequence of *C1* cDNA
a) Molecular mass and pI of *C1* are calculated from the deduced amino acid sequence.

at lower level than FBA1 or FBA2 in the vegetative mycelium of 0 d (Fig. 1a), but increased thereafter. After 21 d of the fruiting treatment, FBA3 was expressed abundantly in both the vegetative mycelium (Fig. 1d) and the fruit-body of 21 d (Fig. 1e). FBA4 (32 kDa and pI 5.3) was not detected in the vegetative mycelium of 0 d (Fig. 1a), but was expressed abundantly in the fruit-body of 21 d (Fig. 1e). FBA4 first appeared in the vegetative mycelium after 5 d of the fruiting treatment (Fig. 1b) and increased slightly at 14 d (Fig. 1c), but its expression in the vegetative mycelium after 21 d of the fruiting treatment was minimal (Fig. 1d). This pattern of expression suggests that FBA4 was required in the fruit-body more directly than FBA1-3.

Table 1 shows N-terminus amino acid sequences of FBA1-4. The N-terminus amino acid sequence of FBA1-4 display 75–93% similarity to one another, and 84–100% similarity to the deduced amino acid sequence of a cDNA clone named *C1*, which was isolated from mycelium of *F. velutipes* and sequenced previously in our laboratory (Azuma et al., 1996). The *C1* cDNA was isolated from a cDNA library as a clone expressed constantly in the mycelium during fruit-body formation of *F. velutipes*. The nucleotide sequence and the deduced amino acid sequence of *C1* cDNA deposited in the DNA Data Bank of Japan (DDBJ accession No. AB030006). The molecular mass and the pI value of the C1 protein were estimated to be 30 kDa and 5.2, respectively. It is considered that the genes encoding FBA1-4 and the *C1* gene belong to the same gene family. However, their expression patterns were not identical to each other, and more information is needed to reveal the relations among FBA1-4 and the *C1* gene.

The nucleotide sequence of the *C1* cDNA was compared with sequences in the DNA and protein databases. One cDNA clone (DDBJ accession No. AB012289-1), isolated form *F. velutipes*, was found that 100% homology to the *C1* cDNA, but no further information was obtained. A characteristic motif analysis revealed that the deduced amino acid sequence of the *C1* cDNA has several phosphorylation sites, two amidation sites, two myristoylation sites, and a glycosylation site. Furthermore, the deduced amino acid sequences of the *C1* cDNA contain the Arg-Gly-Asp (RGD) cell attachment sequence observed in fibronectin (Pierschbacher and Ruoslahti, 1984) and laminin (Schulze et al., 1996), which are so called "cell adhesion molecules". A gene (*mfbA*) that had the RGD sequence and glycosylation site was reported in *Lentinus edodes* (Berk.) Sing. (Kondoh et al., 1995). The MFBA protein has expansion and adhesion activity in mouse melanoma cells (Kondoh et al., 1995) and *Saccharomyces cerevisiae* cells (Yasuda and Shishido, 1997). In consequence, the RGD sequence in the deduced amino acid sequence of the *C1* cDNA suggests the possibility that FBA1-4 have cell to cell attachment activity.

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