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Polyol metabolism in the mycelium and fruit-bodies during development of *Flammulina velutipes*

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Changes of polyol contents in the mycelium and fruit-bodies of *Flammulina velutipes* were measured. The results suggested that arabinitol is accumulated in the fruit-bodies as the end-product after its translocation from the mycelium, while mannitol in the fruit-bodies is converted into fructose by the action of mannitol dehydrogenase (MDH). The development of fruit-bodies was promoted by feeding of mannitol to the mycelial colony. A ^{14}C tracer experiment indicated that half of mannitol translocated from mycelium to fruit-bodies was utilized for fruit-body development. NAD-linked MDH and D-arabinitol dehydrogenase (D-ADH) were detected in both mycelium and fruit-bodies. The activities of MDH and ADH in the mycelium reached their maximum levels in the initial stage of fruit-body development and decreased thereafter. In contrast, the activity of MDH in the fruit-bodies showed a peak in the middle stage of development. The activity of ADH in the fruit-bodies was less than half of that of MDH. MDH showed a lower Km value for mannitol (1.3×10^{-3} M) than for fructose (6.0×10^{-2} M). The Km value of ADH for arabinitol was extremely high (1.3×10^{-1} M).

Key Words——arabinitol dehydrogenase; *Flammulina velutipes*; fruit-body; mannitol; mannitol dehydrogenase.

Mannitol and arabinitol are common components of polyols in the fruit-bodies of basidiomycetes (Lewis and Smith, 1967). Mannitol in *Agaricus bisporus* (Lange) Imbach is produced from fructose by mannitol dehydrogenase (MDH) using NADPH as coenzyme (Ueng et al., 1976). Arabinitol arises *via* the reduction of either xylulose or ribulose with NAD-linked dehydrogenase (ADH) (Niederpruem et al., 1965). However, little is known about the physiological function of polyols in basidiomycetes. Mannitol and arabinitol might be the carbohydrates translocated directly into fruit-bodies from the mycelium in *Flammulina velutipes* (Curt.: Fr.) Sing. (Kitamoto and Gruen, 1976). It was assumed that these polyols might be utilized as the substrates of *F. velutipes* fruit-body development (Gruen and Wu, 1972). On the other hand, Rast (1965) has suggested that mannitol is not used as a reserve carbohydrate during normal growth of *A. bisporus* because it accumulates the polyol with age. However, these hypotheses are not based on experimental results. Here, we examined on the metabolic function of polyols in fruit-body development of an edible wood-rotting mushroom, *F. velutipes*.

Materials and Methods

Organism The strain used in this study was *F. velutipes* 721B1, which was the stock culture of the Laboratory of Microbial Biotechnology, Tottori University.

Culture conditions Liquid medium contained, in 1 L, a decoction from 400 g of peeled, diced potato and 20 g of glucose. Twenty ml of liquid medium poured in a 100-ml Erlenmeyer flask was sterilized at 102°C twice for 30 min each time. Each flask was inoculated with a block of mycelium-agar ($3 \times 3 \times 3$ mm) cut from near the margin of a mycelial colony grown on a potato dextrose agar plate for 12 d at 25°C in the dark. The experimental cultures were maintained at 23°C in the dark. After 2 weeks, the flasks were exposed for fruiting to continuous illumination of 200 lx from white fluorescent lamps at 18°C to induce fruiting.

Measurement of polyol in mycelium and fruit-bodies Two grams of fresh mycelia or fruit-bodies were homogenized with a glass homogenizer at 1,500 rpm with 10 ml of 80% ethanol for 3 min. The resulting suspension was heated in a hot-water bath for 30 min, and ethanol was removed in an evaporator. The residue was treated with ion-exchange resins (Amberlite IR-120 and IR-45), then condensed, freeze-dried, and treated with trimethylsilyl (TMS). Finally, the samples were analyzed with a gas chromatograph (Hitachi, model 163).

Radioisotope experiments An isolated fruit-bodies were cultured at 25°C for 4 h in the 0.1 M potassium phosphate (pH 7.5) containing 1% mannitol (^{14}C-mannitol: 2×10^4 Bq), then washed with 0.1 M potassium phosphate buffer (pH 7.5). The fruit-body was then homogenized with 80% ethanol with a glass homogeniz-

er and centrifuged at 12,000 rpm for 15 min. The resultant supernatant was analyzed by ascending paper chromatography on Whatman No. 51 paper with ethyl ketone-acetic acid-water saturated with boric acid (9 : 1 : 1 v/v/v) (Hammond and Nichols, 1976). A sample of 100 μl was spotted on the paper, and the chromatography was run three times for 10 h each time. The paper was cut in 5-mm strips from the origin to the front, and each strip was placed into a scintillation vial containing 10 ml of scintillation fluid. Radioactivity was determined with a liquid scintillation spectrometer (Nuclear Chicago, model Unilux II).

Preparation of enzyme extracts Mycelium and fruit-bodies were cut with a scalpel from liquid cultures, washed with distilled water, and stored in a freezer at −20°C. Frozen mycelium or fruit-bodies were homogenized with cold 0.05 M glycine-KOH buffer (pH 9.0) containing 2 mM EDTA and 2 mM glutathione (reduced form: GSH), or 0.05 M potassium phosphate buffer (pH 7.6) containing 2 mM EDTA and 2 mM GSH in a glass homogenizer at 1,500 rpm for 5 min. After centrifugation at 15,000 rpm for 15 min, the supernatant was dialyzed in a Centriflo CF25 (Amicon Corp. USA) at 2,800 rpm. The upper solution remaining on the Centrifro corn was used as the dialyzed crude extract for enzymatic experiments.

Enzyme assays MDH and ADH activities were assayed by spectrophotometrically. For MDH activity, the reaction mixture contained 100 μmol of D-mannitol, 2 μmol of NAD(P), 2 μmol of GSH, 2 μmol of EDTA, 75 μmol of glycine-KOH buffer (pH 9.6), and an appropriate amounts of the crude extract in a total volume of 1.0 ml. The mixture was incubated at 25°C, and the increase per min of absorbance at 340 nm was measured with a spectrophotometer (Shimadzu UV-2100S with temperature-controlled cell housing). ADH activity was assayed under the same conditions with 400 μmol of D-arabinitol in place of D-mannitol. The reaction mixture for the reverse reaction was composed of 200 μmol of D-fructose (D-ribulose or D-xylulose), 0.24 μmol of NAD(P), 2 μmol of GSH, 2 μmol of EDTA, 75 μmol of potassium phosphate buffer (pH 7.0), and an appropriate amount of crude extract in a total volume of 1.0 ml. The mixture was incubated at 25°C and the decrease of absorbance at 340 nm per min was measured. One unit of enzyme was defined as an amount of enzyme causing the increase or the decrease of 1 μmol of NAD(P)H ($\varepsilon = 6.2 \times 10^3$) per min. Protein was determined by the Lowry method (Lowry et al., 1951), with bovine serum albumin as the standard.

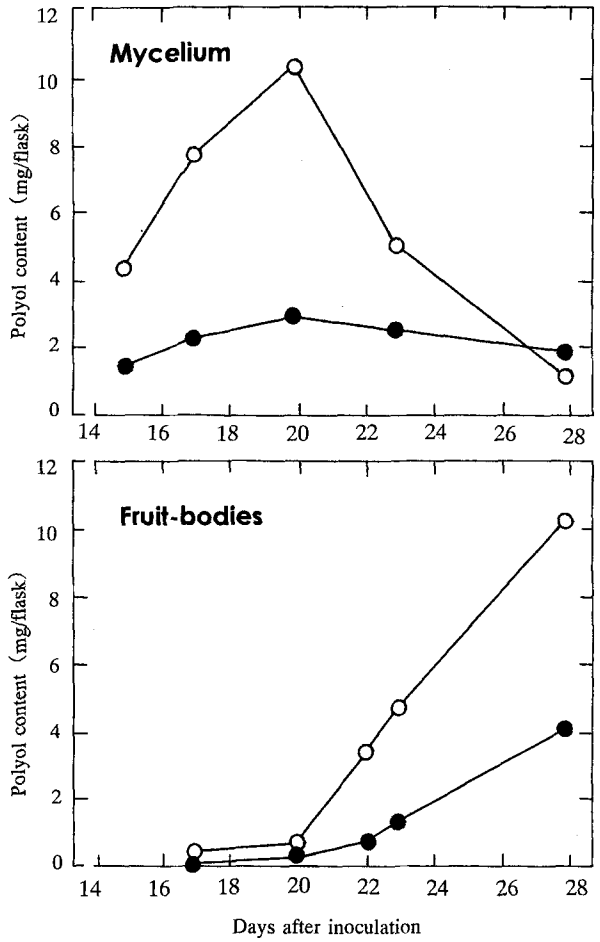

Fig. 1. Changes in amounts of polyols in the mycelium and fruit-bodies during development of *Flammulina velutipes*. ●: Mannitol, ○: Arabinitol.

Results

Changes in polyol contents in mycelium and fruit-bodies

Table 1. Effect of feeding of various carbohydrates into the medium on the growth of mycelium and fruit-bodies of *Flammulina velutipes*.

Carbohydrate added (1%)	Dry weight (mg/flask)			Stipe length (mm)	Pileus diameter (mm)
	Frui-bodies	Mycelium	Total		
None	202.3	274.4	449.7	85.5	13.3
Glucose	294.7	336.5	631.2	97.4	11.7
Trehalose	300.9	274.1	575.0	87.8	11.2
Mannitol	243.4	331.2	574.6	105.6	13.5

The samples were harvested 28 d after inoculation. Dry weights of fruit-bodies were measured by weighing all mature fruit-bodies. The stipe length and pileus diameter were measured for the largest fruit-body in the flask. Each datum is the average of five replicates.

during development The liquid cultures produced fruit-body primordia about 17–18 d after inoculation, and the fruit-bodies matured about 28 d after inoculation. Many primordia usually formed in one cluster in each culture, but only one to three developed into mature fruit-bodies.

Figure 1 shows the changes of polyol contents in mycelium and fruit-bodies during fruiting stage. Arabinitol accumulated in vegetative mycelium in the vegetative growth stage, then decreased markedly with the vigorous fruit-body development that occurred about 20 d after inoculation. The decrease of arabinitol in the mycelium corresponded quantitatively with the increase in fruit-bodies, suggesting that most of this polyol was synthesized in the mycelium, then translocated into fruit-bodies, where it accumulated as an end-product. Mannitol was contained at relatively low concentration in the mycelium, while its final accumulation in fruit-bodies was somewhat higher. Further, the specific activity of NAD dependent mannitol dehydrogenase (NAD-MDH) was four times higher than that of arabinitol dehydrogenase (NAD-ADH) in the mycelium (Table 3), although the mannitol content in the mycelium was less than one-third of arabinitol contents in the 20-d-old colony. From these results, we suggest that mannitol may be continuously synthesized in the mycelium and translocated into fruit-bodies, where it is metabolized to fructose by the action of NAD-MDH.

Feeding effect of some carbohydrates to mycelial colony To determine whether the mannitol in fruit-bodies could be metabolized as a growth substrate of fruit-bodies, a feeding experiment was conducted with the liquid cultures of *F. velutipes*. Trehalose, mannitol, or glucose at a final concentration of 1% was added to the culture filtrate of a 14-d-old (mycelial) culture, then fruiting was induced as described in Materials and Methods. As shown in Table 1, the addition of mannitol increased the fruit-body yield about 1.2 times over the control (no addition), while the trehalose and glucose increased the yield about 1.5 times. Therefore, the mannitol in the culture filtrate was incorporated into mycelium and translocated into fruit-bodies, where it might be converted to growth substrate for fruit-body development.

Incorporation and metabolism of ^{14}C mannitol to fruit-bodies To test the metabolism of mannitol in fruit-bodies, an excised 25-d-old fruit-body was cultured in a solution containing 1% mannitol (2×10^4 Bq of ^{14}C mannitol) for 4 h, and the distribution of radio activity into different carbohydrate fractions was traced. As shown in Table 2, most of the radioactivity (93.6%) was detected in mannitol, and the remaining 6.4% was distributed among fructose, glucose, trehalose, and sugar phosphate fractions. This result demonstrated that the mannitol was taken into fruit-bodies, incorporated into the glycolytic pathway *via* fructose, and converted into various carbohydrates and their phosphate esters. The rate of mannitol incorporation into fruit-bodies was calculated to be 1.98 mg/d, from the data in Table 2, and the incorporated mannitol corresponded to about 50% of the total mannitol found in fruit-bodies. The turnover of mannitol was estimated to be 0.6 mg/d per 100 mg based on the data of Table 2.

Table 2. Incorporation and distribution of ^{14}C-mannitol into sugar fractions of fruit-bodies in *Flammulina velutipes*.*

Fraction	Radioactivity (dpm)	Distribution of ^{14}C-radioactivity (%)
Mannitol	7,087	93.6
Fructose	44	0.58
Glucose	21	0.27
Trehalose	69	0.91
Sugar phosphates	351	4.62
Total	7,572	100

* Rate of incorporation of ^{14}C-mannitol was 1.98 mg/d from replacement medium containing 1% mannitol and 0.1 M potassium phosphate buffer (pH 7.5).
Each datum is the average of five replicates.

Determination of enzymatic types of MDH and ADH in mycelium and fruit-bodies The involvement of different enzymatic types of MDH and ADH was examined by determining their specific activities in the mycelium (14-d-old) and the fruit-bodies (25-d-old). The results are shown in Table 3.

NAD-MDH was very active in both mycelium and fruit-bodies, but NADP-MDH or mannitol phosphate dehydrogenase was inert in both. NAD-linked D-arabinitol dehydrogenase showed moderately high specific activity in both mycelium and fruit-bodies, but no NADP-ADH or L-arabinitol dehydrogenase activity was detected in either part of the colony.

Changes in MDH and ADH activities in mycelium and fruit-bodies during development The changes in specific activities of MDH and ADH in the mycelium and fruit-bodies were examined. As shown in Fig 2, MDH activity levels in the mycelium were somewhat higher than those of ADH, and both reached maximum in the initial stage of fruit-body development, declining thereafter. This suggests that mannitol and arabinitol are most actively synthesized from the vegetative growth stage to the initial stage of fruiting in the mycelium and translocat-

Table 3. Distribution of mannitol, mannitol 1-phosphate and arabinitol dehydrogenases in *Flammulina velutipes*.

Substrate	Coenzyme	Specific activity ($\times 10^{-3}$ U/mg protein)	
		Mycelium*	Fruit-bodies**
D-Mannitol	NAD	0.039	0.104
D-Mannitol	NADP	<0.003	0.007
D-Mannitol 1-P	NADP	<0.001	<0.001
D-Arabinitol	NAD	0.010	0.011
D-Arabinitol	NADP	<0.001	<0.001
L-Arabinitol	NAD	<0.001	<0.001
L-Arabinitol	NADP	<0.001	<0.001

* Mycelium at 14 d after inoculation. ** Fruit-bodies at 25 d old.
Each datum is the average of five replicates.

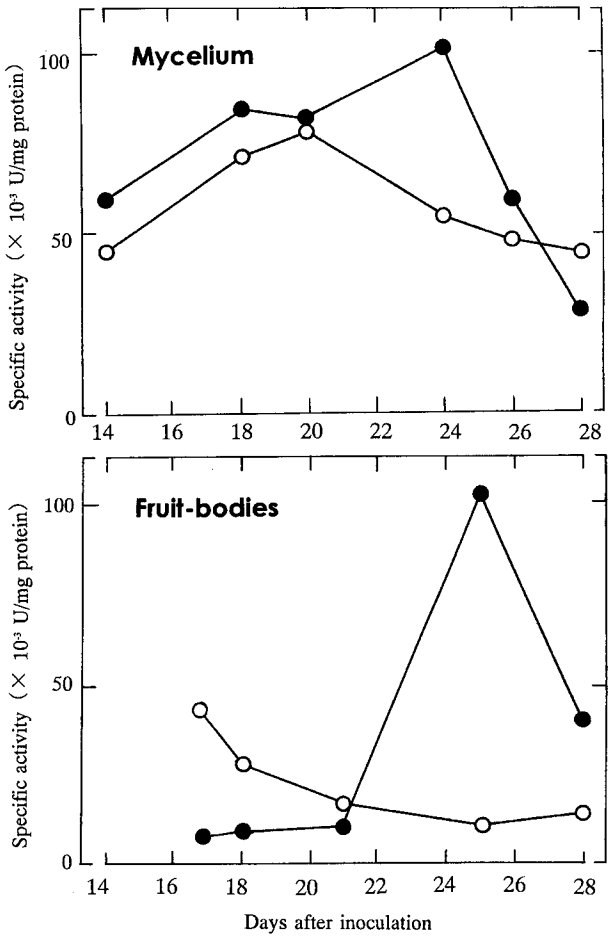

Fig. 2. Changes in activities of mannitol dehydrogenase (MDH) and arabinitol dehydrogenase (ADH) in the mycelium and fruit-bodies during development of *Flammulina velutipes*. ●: MDH, ○: ADH.

ed into fruit-bodies. In the fruit-bodies, MDH activity in the fruit-bodies was much higher than ADH activity. It reached the maximum level in the middle stage of fruit-body development and decreased thereafter. ADH activity was less than a half of the average level of MDH and showed a clear decrease with the development of fruit-bodies. These results suggest that the turnover of mannitol into fructose takes place in this part of the colony, but that arabinitol is not metabolized into the corresponding sugar product during fruit-body development.

Michaelis parameters of MDH and ADH Table 4 shows the Michaelis parameters of MDH and ADH in *F. velutipes*. MDH showed a lower Km value for mannitol (1.3×10^{-3} M at optimal pH of 9.1) than for fructose (6.0×10^{-2} M at optimal pH of 7.0). It was therefore suggested that MDH could catalyze the conversion of mannitol to fructose *in vivo* in fruit-bodies. In contrast, the Km of ADH for arabinitol was extremely high value (1.3×10^{-1} M at the optimal pH of 9.8). The values for the substrates for polyol synthetic reactions were lower (ribulose, 1.3×10^{-2} M; and xylulose, 1.0×10^{-2} M). The

Table 4. Michaelis parameters of mannitol and arabinitol dehydrogenases from *Flammulina velutipes**.

Substrate	Km (M)	Reaction pH**
MDH		
D-Mannitol	1.3×10^{-3}	9.1
NAD	3.2×10^{-4}	9.1
D-Fructose	6.0×10^{-2}	7.0
NADH	2.8×10^{-5}	7.0
ADH		
D-Arabinitol	1.3×10^{-1}	9.8
NAD	2.0×10^{-4}	9.8
D-Ribulose	1.3×10^{-2}	7.0
D-Xylulose	1.0×10^{-2}	7.0
D-Arabinose	2.7×10^{-2}	7.0
NADH	9.2×10^{-5}	7.0

* The crude extract was prepared from fruit-bodies at 25 d old.
** The reactions were carried out at the optimum pH of the forward and the reverse reactions of each enzyme.

Km values of MDH and ADH for the coenzymes NAD and NADH were in the order of 10^{-4} or 10^{-5}.

Discussion

Polyols such as mannitol and arabinitol are the carbohydrates translocated from mycelium into fruit-bodies in basidiomycete mushrooms (Hammond and Nichols, 1976; Kitamoto and Gruen, 1976). They are present in fruit-bodies at average levels of a few percent. However, their metabolism and functions in basidiomycetes are the subject of varying opinions. In *A. bisporus*, mannitol is produced from fructose by dehydrogenase using NADPH as coenzyme (Ruffner et al., 1978; Morton et al., 1985). Dutch and Rast (1972) suggested that the oxidation of NADPH during mannitol synthesis, rather than the polyol itself, could act in regulating the growth of *A. bisporus* by controlling glucose oxidation *via* hexose monophosphate pathway (HMP). On the other hand, Nyunoya and Ishikawa (1980a) reported that the fruiting of *Coprinus cinereus* (Schaeff.: Fr) S. F. Gray was repressed by 2-deoxyglucose, which is known to inhibit some of the glycolytic enzymes, such as glucose 6-phosphate isomerase (PGI) and glucose 6-phosphate dehydrogenase (G6PDH). Further, a *pgi* mutant of *C. cinereus* deficient in PGI activity has been isolated (Nyunoya and Ishikawa, 1980b). The dikaryotic mycelium of this mutant (*pgi/pgi*) showed higher G6PDH activity than the wild type and an elevated intracellular glucose 6-phosphate (G6P) level, but it failed in fruiting in a conventional fruiting medium. However, the addition of a reduced amount of glucose with a compensatory amount of fructose into the medium partially restored fruiting (Nyunoya et al., 1984). This modification of the culture medium decreased the intracellular G6P to almost the

same level as in the wild type. Moreover, the addition of mannitol could completely restored the fruiting ability without affecting the level of G6P. The mechanism of the polyol effect on fruiting in this mutant strain remains to be established.

We have demonstrated that the feeding of mannitol into the colonies of *F. velutipes* increased the stipe length, pileus diameter, and the yield of fruit-bodies. The mannitol in *F. velutipes* was produced by the dehydrogenase using NADH as coenzyme. In the vegetative mycelium of *F. velutipes*, the flux of the HMP pathway was much higher than that of the Embden-Meyerhof-Parnas (EMP) pathway (Kitamoto et al., 1982). In *F. velutipes*, NADP-dependent MDH was not detected. This result is clearly contrasts to that in *A. bisporus*. In the *Agaricus* mushroom, only NADP-MDH has been detected (Edmundowicz and Wriston, 1963). The ^{14}C mannitol labeling experiments suggested that about half of the mannitol translocated from mycelium to fruit-bodies metabolized to carbohydrates and sugar phosphates *via* fructose.

Arabinitol was detected at higher levels than mannitol in mycelium of *Schizophyllum commune* Fr.: Fr. (Cotter and Niederpruem, 1971), *F. velutipes* (Kitamoto and Gruen, 1976), and *Favolus arcularius* (Fr.) Ames (Kitamoto et al., 1978), although arabinitol contents in fruit-bodies were lower than mannitol in fruit-bodies in most species (Kitamoto et al., 1981). Arabinitol may arise *via* the reduction of either xylulose or ribulose by NAD-linked dehydrogenase (Niederpruem et al., 1965). Hammond (1979) has postulated a physiological role of the polyol accumulated in *Agaricus* fruit-bodies as an osmoticant for developing tugor pressure in these tissues for support, and to promote water-flow from the mycelium. The polyols contained in *F. velutipes* might perform the same osmotic function.

The Km value of MDH in the *F. velutipes* fruit-body was 1.3×10^{-3} M for mannitol and 6.0×10^{-2} M for fructose. This value for mannitol was approximately one-tenth of the value of known dehydrogenase from fungi (Lewis and Smith, 1967). The evidence presented here indicates that MDH of *F. velutipes* acts for the production of mannitol in the mycelium and the metabolism of mannitol in the fruit-bodies after translocation. In contrast, arabinitol in the fruit-bodies might not be metabolized, judging from the Km values of for ADH 1.3×10^{-1} M and the *in vivo* concentration of arabinitol of 3.0×10^{-2} M.

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