목재공학 28(1) : 48~54
Mokchae Konghak 28(1) : 48~54, 2000

Thiamin Requirements for Vegetative Growth and Fruit Body Formation of *Lentinula edodes* *1

Gab-Gyun Shin*2 · Sadatoshi Meguro*3 · Shinsaku Kawachi*3

ABSTRACT

The effects of thiamin on vegetative mycelial growth and fruit body formation of *Lentinula edodes* were investigated in basal peptone-glucose liquid medium in relation to the uptake of thiamin. Thiamin was essential for fruit body formation, and the minimum requirements for thiamin were estimated to be approximately 10 μg/L. The vegetative mycelial growth was little influenced by the addition of thiamin in the range of 1.5 μg~1.5 mg/L. While the mycelium was successively transferred to fresh peptone-glucose-agar medium three times, the repression of mycelial growth was not significant. Even in cases using vitamin-free casamino acid or glutamic acid as a nitrogen source instead of peptone, a thiamin deficiency for mycelial growth did not occur as a result of transferring the mycelia to fresh media. Almost all of the thiamin contained in the media accumulated in the mycelia during the first 3 weeks of a 9-week incubation. These results suggest that only trace amounts of thiamin are required for vegetative mycelial growth in *Lentinula edodes* and that almost all thiamin added to a basal medium will be used for fruit body formation.

Keywords: *Lentinula edodes*, Thiamin, Fruit body formation, Mycelial growth

- 요 약 -

표고버섯의 균사성장과 자실체 형성에 있어서 티아민의 효과를 펩톤·글루코스 기본 액체배지를 이용하여 조사하였다. 티아민은 표고버섯의 자실체 형성을 위해서는 필수 인자이며 최소 요구량은 10 μg/L 정도이다. 영양 생장기에는 티아민 농도 1.5 μg~1.5 mg/L의 범위 내에서는 영향을 거의 받지 않았다. 티아민을 첨가하지 않은 펩톤·글루코스·한천배지에 3회 계대 배양을 실시해도 티아민에 의한 균사성장의 차이는 나타나지 않았다. 이상의 결과, 티아민은 표고버섯의 균사성장에는 극히 소량이 요구되지만, 거의 대부분의 티아민은 자실체 형성에 사용된다는 것을 알았다.

INTRODUCTION

We have previously reported that the fruit body of the basidiomycete *Lentinula edodes* (Berk.) Sing., 'Shiitake' is formed in a basal peptone-glucose liquid medium with the addition of yeast extract (Matsuo *et al.* 1992; Mohamed *et al.* 1992). The activity of the yeast extract,

*1 Received Jan. 5, 2000
*2 Institute for Agricultural Resource Utilization, GyeongSang National University, Chin-ju, Korea
*3 Wood Resources Division, Faculty of Agriculture, Miyazaki University, Miyazaki 889-21, Japan

however, is drastically lowered by heating under alkaline conditions, suggesting that the fruit body-inducing substance in the yeast extract could be alkaline-labile thiamin. With the addition of thiamin instead of yeast extract, the fruit body was also formed in a basal peptone-glucose liquid midium (Shin et al. 1997). It has therefore been concluded that thiamin must be the active substance in yeast extract for the fruiting of *Lentinula edodes*.

Because *Schizophyllum commune* and some other fungi have been found to require thiamin for vigorous growth, thiamin has been generally accepted as a growth factor for many fungi (Robbins 1938). The effects of thiamin on fruit body formation have been investigated for which the fruit body is easily formed in chemically defined agar or liquid media various such as *Schizophyllum commune* (Raper and Krongelb 1958; Oyama *et al.* 1976), *Favolus arcularius* (Kitamoto and Kasai 1968a, 1968b), *Psilocybe panaeoliformis* (Kitamoto *et al.* 1980), or *Coprinus lagopus* (Madelin 1956). For all of these fungi, thiamin is an essential element for fruiting.

There have been few reports of the thiamin requirements for edible mushroons, except for *Flammulina velutipes* (Yamada and Aoyama 1986). In particular, the thiamin requirements for fruiting of *Lentinula edodes* remain unknown because of the difficulties to achieve a fruit body in synthetic media.

In this paper, the effects of thiamin on vegetative mycelial growth and fruit body formation for *Lentinula edodes* in basal peptone-glucose liquid media were investigated in relation to thiamin uptake.

Materials and Methods

Organism

A commercial dikaryotic strain of *Lentinula edodes*, Mori 465 (Mori Sangyo), was maintained at 5℃ on potato-glucose-agar(PDA) slants.

Media and cluture conditions

The basal liquid medium (PG media) consisted of glucose, 50 g; polypeptone, 2.5 g; KH_2PO_4, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $CaCl_2 \cdot 2H_2O$, 0.5 g; $FeCl_2 \cdot 6H_2O$, 10 ㎎; $MnCl_2 \cdot 4H_2O$, 7.2 ㎎; $ZnCl_2$, 4 ㎎; $CuSO_4 \cdot 5H_2O$, 1 ㎎; and 1 liter of distilled water.

In addition, agar was added at 2.5 g/L to PG medium to make a peptone-glucose-agar medium (PGA media); vitamin-free casamino acid (Difco) was added at 2.5 g/L instead of peptone in the PGA medium to make a casamino acid-glucose-agar medium (CGA media); and glutamic acid was added at 2.5 g/L instead of peptone in the PGA medium to make a glutamic acid-glucose-agar medium (GGA medium). 100 ㎖ portion of the liquid medium was dispensed into a 500 ㎖ Erlenmeyer flask and autoclaved at 121℃ for 30min. The mycelial inoculum, an agar disk of 5 mm diameter cut from the previous colonized plate, was placed at the center of the liquid medium. The mycelial cultures were incubated statically for 9 weeks at 25℃ and 60% relative humidity under approximately 200 lux of light with a 12h light and dark cycle. Thiamin was added at various concentrations to the PG medium before autoclaving.

Although the optimal concentration of yeast extract for fruit body formation of *Lentinula edodes* was 2.5 g/L, fruit body was produced even at concentrations as low as 25 mg/L of yeast extract (Matsuo *et al.* 1992). The content of thiamin in 1g of yeast extract was 0.58 ㎎ as a thiamin hydrochloride (Shin *et al.* 1997). The concentration of thiamin in 25 ㎎/L of yeast extract was therefore calculated to be 14.5 ㎍/L. *Lentinula edodes* was incubated for 9 weeks in a basal peptone-glucose liquid medium supplementing by 14.5 ㎍/L thiamin (PGT media).

A 30 ml portion of agar medium previously autoclaved was poured into 9 cm diam. Petri

dishes, and, after solidification, was inoculated with a mycelial disk cut from a colonized PDA plate. After 11days of incubation, the mycelial disk was cut from the colonized plate and transferred to fresh medium. The transfer was repeated again in the same manner. Incubation was at 25℃ and 60% relative humidity in the dark.

Determination of thiamin

The grown mycelium was separated by filtration and washed several times with distilled water through a glass filter. The mycelium obtained was frozen and dried by a freeze-drier (YAMATO, DC-35).

After measuring mycelial weight, the mycelia were extracted with 50 mℓ of 0.1 M HCl in a water bath at 100℃ for 30min. This solution was treated with takadiastase B (Sankyo) and then with a Permtid column. The sample solution was injected and then separated on a Shimadzu STR ODS-II (15 cm×4.6 cm) column with acetonitrile: 0.1 M $NaH_2PO_4 \cdot 2H_2O$ (3 : 97 by volume ratio) at 0.5 mℓ/min using a Shimadzu LC-6A HPLC system. Thiamin was determined by the post-column fluorescence method. The excitation and emission wavelengths for thiamin were 375 nm and 450 nm, respectively. Other details were almost the same as reported previously (Shin *et al.* 1997).

Results

Effects of thiamin on mycelial growth in liquid media

Mycelial growth in PGT media was slower than that in basal media supplemented by yeast extract, but small mycelial aggregates were formed on the surface of the colony after about 4 weeks and then they grew up to primordia. Fruit body formation began after about 7weeks. Fruiting occurred in 60% of cultures within 9 weeks after inoculation. In basal liquid media without thiamin, neither primordia nor fruit bodies were produced.

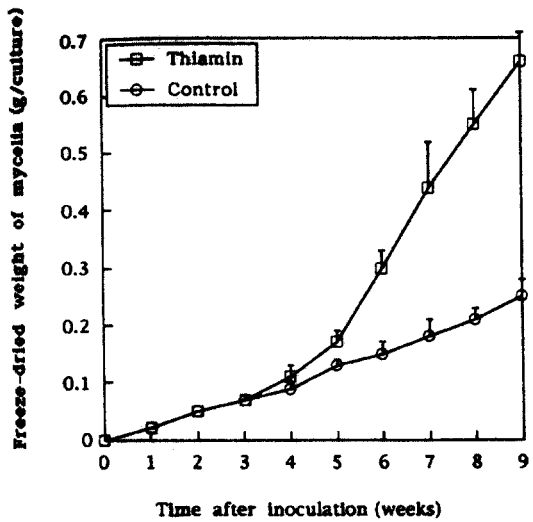

Fig. 1. Effect of thiamin on mycelial growth in peptone-glucose media. Thiamin was added to basal peptone-glucose liquid media at 14.5 μg/L before autoclaving. *Lentinula edodes* was statically incubated for 9 weeks. The freeze-dried weight of mycelia including mycelial aggregates and primordia formed after about 4 weeks, and fruit bodies formed after about 7 weeks. Data represents the mean and SD of three separate experiments. Ten replicate cultures were tested for each experiment.

The mycelial weight increased significantly after about 4 weeks of incubation in PGT media, as shown in Fig. 1. The effects of thiamin on mycelial weight, however, were not observed until third week. Even in the media without thiamin, mycelia continued to grow up to 9th week of incubation.

Effects of subculture on mycelial growth

The mycelial growth rates were also investigated by the usual technique of subculturing in peptone-glucose-agar (PGA) media. *Lentinula edodes*, which had been maintained on potato-glucose-agar (PDA) media, was subcultured

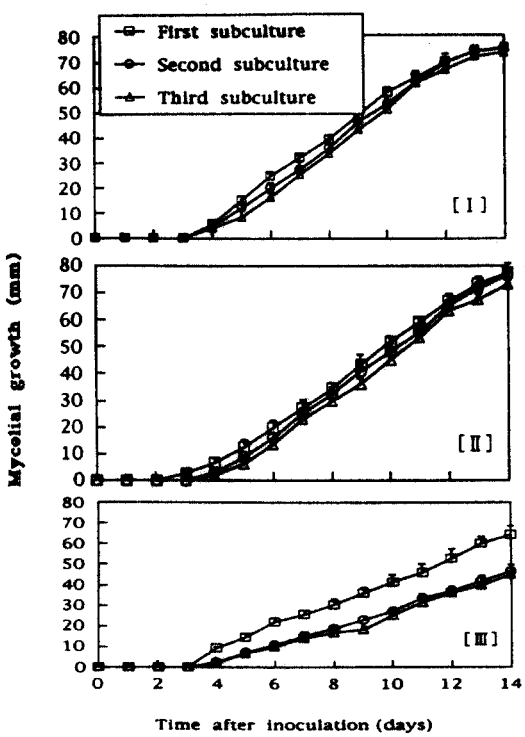

Fig. 2. Effect of subcultures on mycelial growth in : (Ⅰ) Peptone-glucose-agar media, (Ⅱ) casamino acid-glucose-agar media, and (Ⅲ) glutamic acid-glucose-agar media. First subculture : Mycelial disk of *Lentinula edodes* cut from colonized PDA plats were incubated in each medium. Second subculture : After 11 days of incubation, mycelial disk was cut from colonized plate and transferred to fresh media. Third subculture : The transfer was repeated again with the same manner. Mycelial growth is expressed as a colony diameter of mycelia after 11 days of incubation. Data represents the mean and SD of ten replicate cultures.

three times in PGA media. The decrease in mycelial growth, however, was insignificant in each succeeding transfer, as shown in Fig. 2(Ⅰ). While PGA media supplemented by thiamin were used for the second or third transfer, the mycelial growth rate was essentially the same as that in media free from thiamin.

Lentinula edodes was also subcultured three times in a CGA medium containing a casamino acid which was specially purified for a vitamin assay in place of the peptone used in PGA media. The inhibition of mycelial growth was also insignificant in each subculture, as shown in Fig. 2(Ⅱ).

When investigated in the chemical-defined media (GGA media) containing glutamic acid instead of the peptone used in PGA media, the mycelial growth was significantly inhibited in the second subculture, as shown in Fig. 2(Ⅲ). However, the further inhibition of mycelial growth did not occur in response to a successive transfer. Because the mycelial growth was not recovered even when GGA media supplemented with thiamin were used for the second transfer, the deficiency of thiamin did not occur to be responsible for the inhibition of mycelial growth by subculturing.

Effects of thiamin on mycelial growth and fruit body formation

In order to confirm the effects of thiamin on fruit body formation, *Lentinula edodes* was incubated for 9weeks in basal liquid media supplemented by thiamin at various concenations before autoclaving. The results are shown in Fig. 3(I). The level of fruit body formation was still maintained at over 60% at 14.5 μg/L, which corresponds to only 1/100 of the original concentration of thiamin, but levels decreased significantly below about 5 μg/L (1/300), and fruit bodies were not formed at 1.45 μg/L (1/1000). Because approximately 20% of the thiamin added to PG media denatured under autoclaving at 121℃ for 30min, the concentration of thiamin at the begining of the incubation decreased from 14.5 μg/L to 12.1 μg/L.

These results suggest that thiamin is an essential element for the fruit body formation of *Lentinula edodes* and that the minimum thiamin requirement can be estimated to be approximately 10 μg/L.

The effects of thiamin on mycelial weight

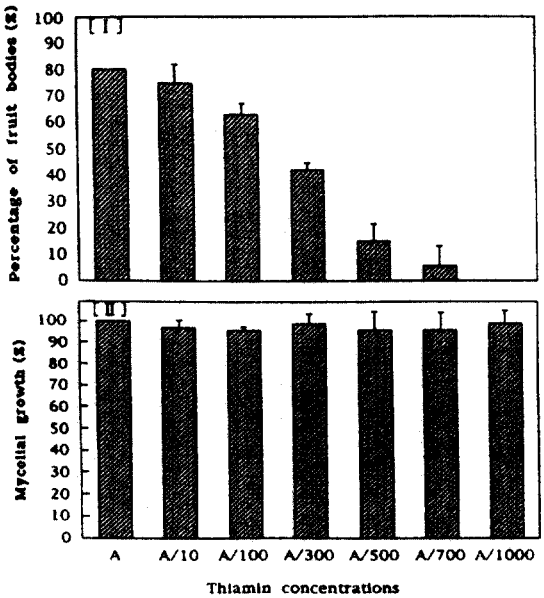

Fig. 3. Effect of thiamin concentration in the region of 1.45mg-1.45 μg/L on : (I) fruit body formation, (II) mycelial growth. The symbol A in the figure means 1.45mg/L which is the thiamin concentration contained in 2.5g/L yeast extract. (I): Fruit body formation is expressed as a percentage of cultures with fruiting until ninth week of incubation. (II): Mycelial growth is expressed as a percentage of mycelial weight for that incubated in the media with 1.45mg/L thiamin after 21 days of incubation. Data represents the mean and SD of two separate expriments. Ten replicate cultures were tested for each experiment.

during the vegetative growth stage until the third week of incubation were also investigated. The vegetative mycelial growth was little influenced by the addition of thiamin in the range of 1.5 μg~1.5 mg/L, as shown in Fig. 3 (II).

Uptake of thiamin

Lentinula edodes was incubated in 100 ml of PG basal media containing 1.45 μg/L of thiamin, and the thiamin content was determined both in

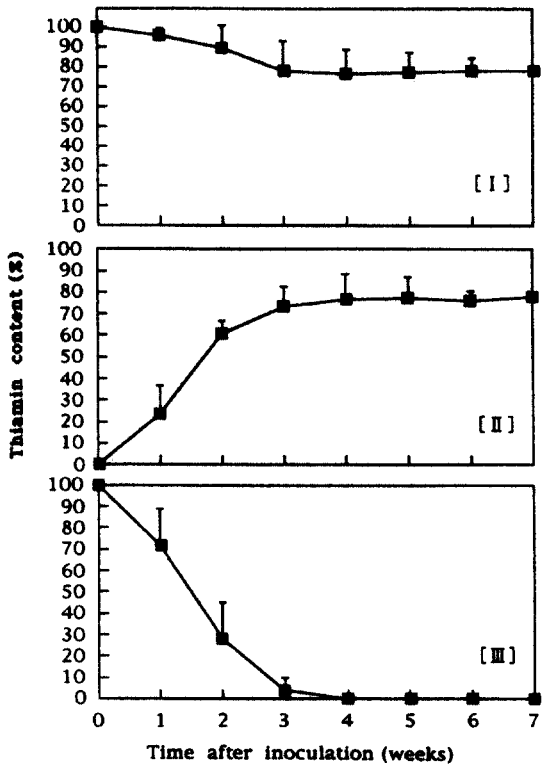

Fig. 4. Uptake of thiamin into the mycelia from the media. I: Total thiamin content, II: thiamin content in the mycelia, III: thiamin content in the culture filtrate. The thiamin content in 100 ml of media was 12.1 μg at the begining of incubation, which is shown as a 100%. Data represents the mean and SD of two separate experiments.

the mycelia and the culture filtrate every week after inoculation. As shown in Fig. 4, about 60% of the thiamin from the media had already been taken into the mycelia after only 2 weeks of incubation. Almost all of the thiamin originally existing in the media was found in the mycelia at the third week; the thiamin content in the mycelia stayed fairly constant after 3 weeks. We could not detect any thiamin in the culture filtrate after 4 weeks under these experimental conditions. These results suggest that almost all thiamin contained in the media accumulated in the mycelia during the first 3 weeks of the

9-week incubation.

Discussion

Raper and Krongelbha(1958) investigated the effects of thiamin on the mycelial growth and fruit body formation of *Schizophyllum commune* in glucose-asparagine media. They showed that thiamin was a necessary factor for fruiting in *S. commune* and that the thiamin requirement was variable for different strains in the region of 7.5~30 μg/L. *S. commune* was also repored to require 30~100 μg/L of thiamin for fruiting in glucose-asparagine media (Oyama *et al.* 1976). The thiamin requirement for the fruiting of *Favolus arcularius* in maltose-casamino acid-agar media, and that of *Coprinus lagops* in alanine-glucose-agar media have been found to be 3 and 10 μg/L, respectively (Kitamoto and Kasai 1968a, 1968b; Kitamoto *et al.* 1980).

Flammulina velutipes, which is one of the economically important edible mushroom in Japan, requires over 10 μg/L of thiamin for fruiting in asparagine-glucose-agar media (Yamada and Aoyama 1986). We have previously reported that *Lentinula edodes* can form a fruit body in basal peptone-glucose liquid media by adding 12.3 μg/L thiamin instead of fraction II of yeast extract (Shin *et al.* 1997). In this experiment, *Lentinula edodes* achieved 60% fruiting with the addition of thiamin at 14.5 μg/L (the concentration after autoclaving was 12.1 μg/L). Therefore, it seems that the thiamin requirement of *Lentinula edodes* for fruiting is at almost the same level as those of the fungi described above.

Though the mycelial growth of *Schizophyllum commune* is also promoted by thiamin, the thiamin requirement for mycelial growth is lower than that for fruiting (Raper and Krongelb 1958). Oyama *et al.* (1976) and others have reported that thiamin has little effect on the vegetative growth of *Schizophyllum commune*. Ishikawa (1967) has investigated the effects of thiamin on the mycelial growth of *Lentinula edodes* by using ammonium tartarate-glucose liquid media. He has reported that the dry weight of mycelia increases by about three times with the addition of thiamin at 100 μg/L. In this experiment, there was no significant difference in the mycelial weight of *Lentinula edodes* between the liquid media with and without thiamin. While we tried Ishikawa's media instead of PG media, the mycelial growth of Mori 465 strain was not affected by the addition of thiamin. Thus, we might guess that the thiamin requirement of *Lentinula edodes* is variable for different strains.

It has been assumed that the relatively abundant vegetative growth of *Coprinus lagopus* and *Flammulina velutipes* in media without thiamin is due to the thiamin contained in the agar itself, and in the inoculum, respectively (Kitamoto and Kasai 1968a; Madelin 1956). If the inoculum contains thiamin, the thiamin taken into the culture would decrease and finally be extinguished by successive transfers to fresh media free from thiamin. Therefore, the mycelia of *Lentinula edodes* was successively transferred to fresh peptone-glucose-agar media three times. The repression of mycelial growth, however, was insignificant. Peptone is made from a natural substance, so it may possibly contains trace amounts of thiamin as an impurity. However, even in the case of using vitamin-free casamino acid of glutamic acid instead of peptone, the deficiency of thiamin for mycelial growth did not occur as a result of the transfers.

From the determination of the thiamin contents both in mycelia and culture filtrate, it was found that almost all thiamin contained in the media accumulated in the mycelia during vegetative mycelial growth. These results suggest that only trace amounts of thiamin are required for the vegetative mycelial growth of *Lentinula edodes.* Because *Lentinula edodes* requires approximately 10 μg/L thiamin for fruiting,

almost all the thiamin added to the basal media must be used for fruit body formation.

Acknowledgements

This work was supported in part by financial aid from the Scientific Research Fund(No. 08660209) of the Japanese Ministry of Education, Science, and Culture.

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