チアミンによるシイタケ子実体誘導機構の解明

（研究課題番号　08660209）

平成8年度～平成9年度科学研究費補助金（基盤研究(C)(2)）

研究成果報告書

平成10年3月

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研究成果要旨

酵母抽出物中に含まれるシイタケ子実体形成誘導物質を明らかにしようとして，酵母抽出物をSephadex G-25を用いたゲルクロマトグラフィーによって，260nmの吸収を基にⅠ-Ⅴの5つのフラクションに分画した。５つのフラクションの内，酵母抽出物と同等の活性を示したフラクションⅡ中の活性物質について検討した。フラクションⅡを100倍に希釈してもなお83％の子実体形成率を示したが、アルカリ性で加熱処理をすると著しく活性が減少した。アルカリ性で極めて不安定なことで知られるチアミンが酵母抽出物1ｇ中に0.58ｍｇ存在し、その約85％に相当する0.49ｍｇが、フラクションⅡに遊離型で存在していた。そこで，アルカリ処理やパームチッドを用いてフラクションⅡ中のチアミンを除くと、子実体はまったく形成されず、チアミンを単独で基本液体培地に添加すると子実体が形成された。これらの結果より、酵母抽出物に含まれる活性物質の主体はチアミンと結論された。

本研究では、チアミンの添加によってシイタケ子実体が誘導される原因を明らかにするために、チアミンの栄養菌糸成長および子実体形成に及ぼす影響を，チアミンの菌糸体内への取り込みと関連付けながら検討した。シイタケの菌糸体重量は，チアミンの添加によって，培養4週間目の菌糸塊形成以降は明らかな増加が見られた。しかし，培養約3週間までの栄養成長段階には，チアミンの菌糸成長に及ぼす効果は認められず，シイタケはチアミン無添加培地においても，速度は遅いものの停滞することなく培養9週間目まで菌糸成長を持続した。チアミンの栄養菌糸成長に及ぼす効果を，約1.5 mg～約1.5 μg /liter の濃度範囲で詳細に検討したが，チアミンの影響はまったく認められなかった。さらにこのことを確かめるために，チアミンを添加していない種々の寒天培地を用いてシイタケの継代培養を繰り返したが，チアミンの欠乏によると思われる栄養菌糸成長の明らかな抑制は見られなかった。一方，シイタケの子実体形成に関しては少なくとも15μｇ / liter以上のチアミンの添加

が必要であり，それ以下では全く形成されないことが分かった。したがって，シイタケの子実体形成には比較的高濃度のチアミンを要求するものの，栄養世代の菌糸成長にはチアミンを要求しないものと考えられた。

次に、シイタケによる液体培地からのチアミンの取り込みを検討した。培養2週間目には、培養開始時に培地に存在したチアミンの約60％が菌糸体内へ取り込まれることが分かった。3週間目以降は菌糸体中のチアミン蓄積量はほぼ一定となり、一方培養液中のチアミンは培養4週間以降ではほとんど検出されなかった。これらの結果から，培地中のチアミンは，培養期間全9週間のうち，初期のわずか3週間でそのほぼ全量が培地から菌糸体内へ取り込まれることが明らかとなった。一方，培養初期に取り込まれたチアミンの約60％は遊離型のまま存在し、エステル類の割合は少なかった。しかし、培養時間が経過するにつれ遊離型のチアミン量は減少し、逆にチアミン二リン酸エステルが徐々に増加する傾向が見られた。さらに、シイタケ原基が形成される培養4週間目には生理活性の高い二リン酸エステルの割合は最高となったが、その後減少することが分かった。

以上の検討により、チアミンはシイタケの栄養菌糸成長を促進することにより、子実体の形成を速めるというような間接的なものではなく、栄養菌糸成長から生殖成長への転換期において直接的かつ必須の役割を果たしていると考えられた。

研究組織・経費・成果発表

研究組織

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研究経費

平成8年度	1,600,000円
平成9年度	700,000円
計	2,300,000円

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Active constituent in Yeast Extract for Fruit Body Formation of *Lentinula edodes*

Introduction

Phenomenon of fruit body formation in basidiomycetes is regarded as a model system for studies of differentiation and morphogenesis mechanisms of fungi. Fruit body inducing substances are very useful in clarifying the mechanism of fruit body formation. Among basidiomycetes, the fungi which grow well on simple culture media and complete their life cycle in as short as a few weeks, are frequently used for these investigations. Uno and Ishikawa (1973) detected the activity to induce the fruit body formation of *Coprinus macarorhizus* in the fruit body extracts of the same fungus and identified the active substance as 3', 5'-cAMP. It was also reported by Kawai and Ikeda (1982) that a cerebroside prepared from *Schizophyllum commune* had the fruit body inducing activity on the same fungus. The similar activity of anthranic acid, p-aminobenzoic acid, and cyclooctasulfur was reported with *Favolus arcularius* (Murao et al. 1984; Hayashi

et al. 1985). *Streptmyces* strain B-412 similar to *Streptmyces rubiginosus* produced the substance which induced the formation of incomplete fruit body of *Favolus arcularius* under a dark condition. This active substance was named basidifferquinone by Azuma et al. (1990).

On the other hand, there are only few reports about the inducing or stimulating-substance for the fruit body formation of shiitake (*Lentinula edodes* (Berk.) Pegler) because of difficulty of fruiting in a synthetic or semisynthetic medium. Leatham and Stahmann (1984) reported that Ni and Sn stimulated the fruit body formation of *Lentinula edodes*. Kawamura et. al. (1983) showed that the fruit body formation of this fungus in peptone-glucose liquid medium was promoted by the addition of a lignin precursor such as ferulic acid and vanillin. Terashita et al. (1981) observed that the addition of a protease-inhibitor (S-PI) obtained from a *Streptomyces* strain cased a decrease in the period required for maturation of the fruit bodies in *Lentinula edodes*.

We previously examined the effects of various compounds on the fruit body formation of *Lentinula edodes* by using a peptone-glucose basal liquid media (Matsuo et al. 1992). *Lentinula edodes* was incubated statically in a basal liquid medium at 25℃ under illumination, but no fruit body

was formed within 70 days after inoculation . When yeastextract was added to a basal liquid medium, normal fruit bodies were formed in all cultures tested after about 36 days of incubation and without shifting to cooler temperature. These results suggested that the inducer for fruit body formation of a basidiomycetes *Lentinula edodes* would be contained in the yeast extract.

In analogy with peptone and malt extract, yeast extract (an autolysate of brewer's yeast) is widely used as a nutrient in natural and semisynthetic media. It is well known that yeast extract contains various growth-factors such as vitamins, amino acids, and nucleic acids which accelerate the mycerial growth.

This investigation aimed to identify the active constituent in yeast extract for the fruit body formation of *Lentinula edodes.*

Materials and methods

Organism

Commercial dikaryotic strain of *Lentinula edodes*, Mori 465 (Mori Sangyo) was maintained at 5Åé on potato dextrose agar slants.

Media and culture conditions

Basal liquid medium consisted of the following components: 50g glucose; 2.5g polypeptone; 1.0g KH_2PO_4; 0.5g $MgSO_4 \cdot 7H_2O$; 0.5g $CaCl_2 \cdot 2H_2O$; 10mg $FeCl_2 \cdot 6H_2O$; 7.2mg $MnCl_2 \cdot 4H_2O$; 4mg $ZnCl_2$; 1mg $CuSO_4 \cdot 5H_2O$. The initial pH was adjusted to 5.7. The culture volume used for various experiments was 100 mL. The liquid medium was dispensed into a 500-ml Erlenmeyer flask and autoclaved at 121 ℃ for 30 min. An agar disk of 5 mm diameter cut from the previous colonized plate was floated on the center of the liquid medium. The mycelia cultures were incubated statically for 70 days at 25 ℃ and 60 % relative humidity under about 200 lux of light from daylight fluorescent bulbs with a 12 h light and 12 h dark cycle. Other details were almost the same as before (Matsuo et al. 1992).

Yeast extract was added at a concentration of 2.5 g/L before autoclaving. Thiamin hydrochloride solution was added through 0.20-μm size filter to the basal liquid medium previously autoclaved at 121 ℃ for 30 min. Yeast extract and polypeptone were prepared by Difco and Nihon Seiyaku, respectively.

Gel chromatography

The solution (2 mL) containing 0.5g of yeast extract was applied to a Sephadex G-25 fine (Pharmacia Biotech) column

(1.6×100cm). The column was eluted at a flow rate of 0.8 mL/min with a distilled water. The column effluent was monitored by the absorbance at 260 nm.

Acid and alkaline hydrolysis of fraction Ⅱ

The fraction Ⅱ of yeast extract was diluted 10^2 times with a distilled water. The diluted sample was adjusted to pH 2.0 with concentrated HCl and to pH 13 with concentrated NaOH solution. These solutions were autoclaved at 121 ℃ for 30 min, then adjusted again to pH 5.7 with concentrated. HCl or NaOH solution before adding to the basal liquid media through 0.20-μm size filters.

HPLC determination of thiamin

Thiamin was determined by the method of Kimura et al. (1980). Yeast extract (0.5g) and each fraction of that was added to 50 mL of 0.1 M HCl. This sample solution was heated in a water bath at 100 ℃ for 15 min. This solution was adjusted to pH 4.5 with 4 M sodium acetate and was mixed with 2 mL of 1 % Takadiastase B (Sankyo) solution. The sample solution was then incubated at 37 - 40 ℃ for 12 h in a shaking water bath. After heating in a water bath at 100 ℃ for 15 min, the sample solution was loaded onto a permutite column (Vitachange, Wako Pure

Chemical) and eluted with a boiling 25 % KCl-0.1N HCl solution. The elute is made of exactly 25 mL in a volumetric flask.

The sample solution was injected and separated on a Shimadzu STR ODS-Ⅱ(15 cm×4.6 mm) column with 0.1 M $NaH_2PO_4 \cdot 2H_2O$ at 0.5 mL/min using a Shiadzu LC-6A HPLC System. $K_3Fe(CN)_6$- NaOH solution was applied at 0.5 mL/min by a proportioning pump and mixed with the column effluent to convert thiamin into fluorescent derivative, thiochrome. The peaks were detected using a Shimadzu RF-550 luminescence spectrometer connected to the chromatograph. Excitation and emission wavelengths for thiamin were 375 nm and 450 nm, respectively.

When thiamin, thiamin monophosphate, diphosphate and triphosphate were determined separately, the treatment with 1 % Takadiastase solution was omitted.

Results

Gel chromatography of yeast extract

Yeast extract was applied to a Sephadex G-25 column and the effluent was monitored by the absorbance at 260 nm. Two large peaks (at about 60 mL and about 140 mL) and three of small peaks (at about 165 mL, about 220 mL

and about 270 mL) of elution volume were observed as shown in Fig. 1. On the basis of these five peaks of UV absorption, yeast extract was divided into five fractions; Ⅰ: 50 - 100 mL, Ⅱ: 101 - 160 mL, Ⅲ: 161 -210 mL, Ⅳ: 211 - 260 mL, Ⅴ: 261 - 290 mL of elution volume. Each fraction was assayed for activity to induce fruit body formation of *Lentinula edodes* in a peptone-glucose basal liquid media. Every fraction of yeast extract was found to give the inducing activity for the formation of fruit body, as shown in Fig. 2 . Fraction Ⅰ (higher molecular region) and Fraction Ⅴ (lower molecular region) of yeast extract showed about 60 % and 50 % of fruiting, respectively. The addition of fraction Ⅱ caused 100 % of fruiting and both the yield and the day required for fruiting were as close as those of the original yeast extract. These results suggested that the main inducing factor of yeast extract would be contained in fraction Ⅱ.

The fruit body inducing effect of fraction Ⅱ

In order to confirm activity of fraction Ⅱ of yeast extract to induce fruit body formation, *Lentinula edodes* was incubated in a basal liquid medium containing various concentrations of fraction Ⅱ. The induction of fruit body formation was consistently observed at 10^2 times dilution of the fraction Ⅱ, as shown in Table 1.

Fig.1. Gel chromatography of yeast extract through Sephadex G-25 fine column.
Yeast extract was divided into five fractions from I to V on the basis of five peaks of UV absorption.

I)50 ~ 100mL, II)101 ~ 160mL, III)161 ~ 210mL
IV)211 ~ 260mL, V)261 ~ 290mL of elution volume.

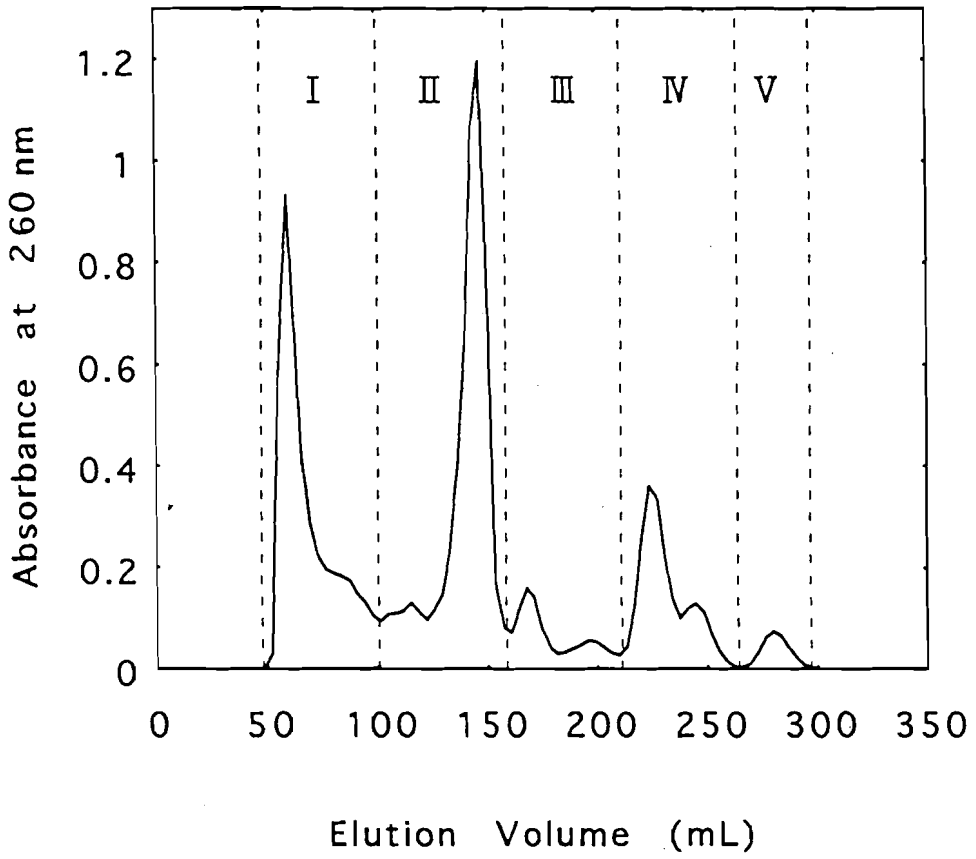
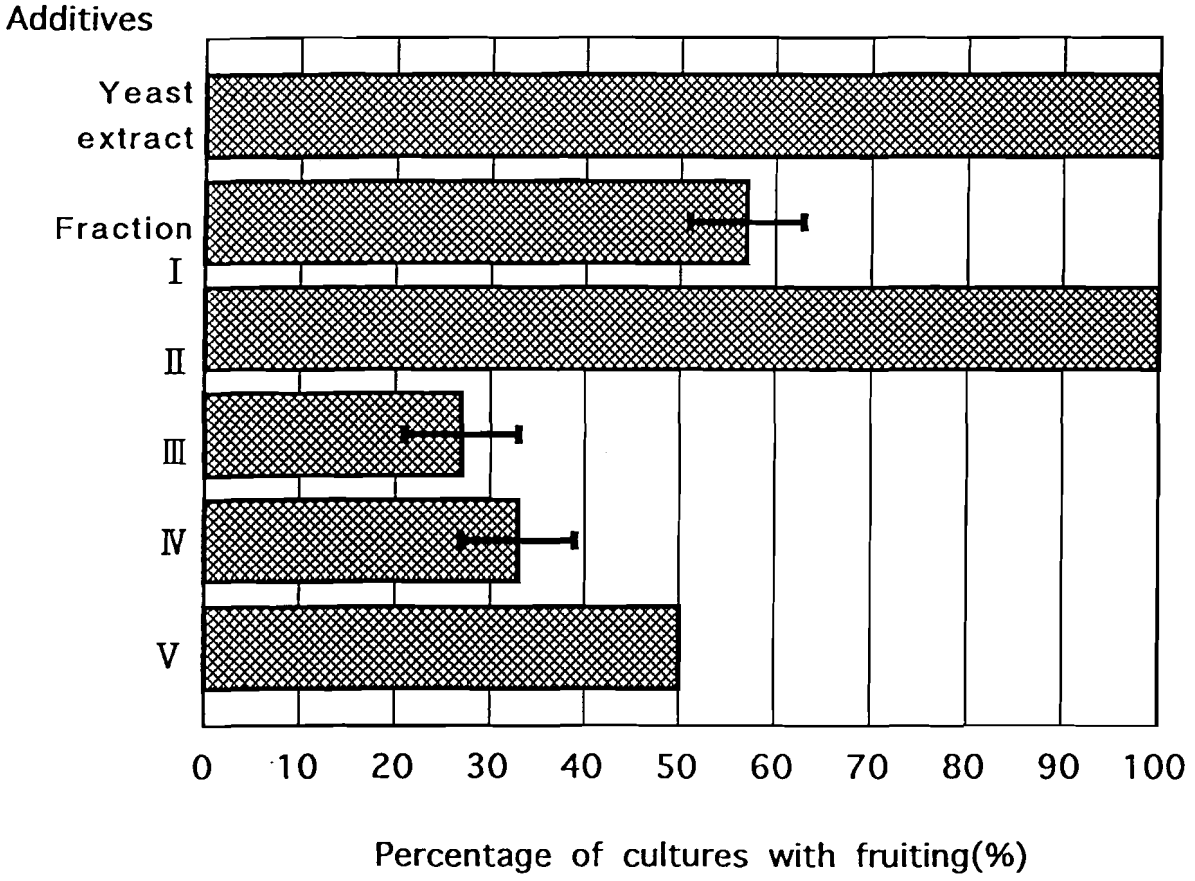


Fig.2. Effects of yeast extract and fractions of it on the fruit body formation of *Lentinula edodes*.
Yeast extract and fractions were added to the basal peptone-glucose liquid media before autoclaving. The concentration of yeast extract was 2.5g/L.
Data represents the means±SD of three separate experiments.
Ten replicate cultures were tested for each experiment.



The stability of fraction diluted 10^2 times was examined for heat, acid and alkaline hydrolysis. The results are shown in Table 2. Fraction Ⅱwas hydrolyzed at pH 2.0 to give 60 % of the fruiting, as compared with 80 % of that hydrolyzed at pH 5.7. The fruit body inducing activity of the sample hydrolyzed at pH 13.0 was drastically decreased to only about 10 % of the fruiting. These results suggested that the fruit body inducing factor in fraction Ⅱ must be the substance which was relatively stable for acid, but instable for alkaline hydrolysis to decompose easily.

The fruit body inducing effect of thiamin

Of the nutrients contained richly in yeast extract such as amino acids, nucleic acids, vitamins and minerals, thiamin is well known to be considerably stable in acidic condition but instable in alkaline condition (Burden et al. 1990; Dwivedi et al. 1973). Therefore, thiamin in the yeast extract and the five fractions were determined by the post-column fluorescence method (Kimura et al. 1980). Despite the Takadiastase treatment being omitted, thiamin mono-, di-, and tri-phosphate could not be detected in the yeast extract; thus showing the thiamin contained in the yeast extract produced by Difco predominantly existed in a free-form. The content of thiamin in 1 g of yeast extract was 0.58 mg

Table 1. Effect of concentration of fraction Ⅱ of yeast extract on the fruit body formation of *Lentinula edodes*.

Concentration of fraction Ⅱ	Concentration of thiamin in fraction Ⅱ (μg/L)	Percentage of cultures with fruiting (%)
1	12.3×10^{2}	100
1×10^{-1}	12.3×10	100
1×10^{-2}	12.3	80±14
0.3×10^{-2}	3.7	15±7
0.2×10^{-2}	6.2	0

Note: Values are expressed as means±SD of two separate experiments. Ten replicate cultures were tested for each experiment. Linear ($P<0.05$) response between 1×10^{-2} and 0.2×10^{-2} ; no differences ($P>0.05$) among $1-1\times10^{-2}$.
Fraction Ⅱ was added to the basal peptone-glucose liquid media before autoclaving at pH 5.7, 121℃ for 30 min.

Table 2. Effects of some chemical treatments of fraction Ⅱ and thiamin on the fruit body formation of *Lentinula edodes*.

Additives	Concentration of thiamin (μg/L)	Percentage of cultures with fruiting (%)
(a) fraction Ⅱ hydrolyzed		
at pH 5.7, 121 ℃ for 30 min	9.1	80±14
at pH 2.0, 121 ℃ for 30 min	11.8	60±0
at pH 13.0, 121 ℃ for 30 min	trace	10±0
(b) fraction Ⅱ passed through the permutite column	ND	0
(c) thiamin	12.3	60±0
(d) thiamin plus fraction Ⅱ hydrolized at pH 13.0 121 ℃ for 30 min	12.3	60±0

Note: Values are expressed as means±SD of two separate experiments. Ten replicate cultures were tested for each experiment. Fraction Ⅱ was diluted 10^2 times with a distilled water. Thiamin, and fraction Ⅱ subjected to some chemical treatments were added through 0.20-μm size filter to the basal liquid media previously autoclaved. ND, not detected.

as a thiamin hydrochloride. It was found that a 0.49 mg (about 85 % of total thiamin) was contained in the fraction Ⅱ. Thiamin was also detected in fraction Ⅰ at the amount of 0.06 mg (about 10 % of total thiamin), but not in fractions Ⅲ, Ⅳ, and Ⅴ. Since the yeast extract was added to the basal liquid medium at the concentration of 2.5 g/L in this experiment, the concentration of thiamin contained in fraction Ⅱ was 1.23 mg/L. Fraction Ⅱ was still effective for inducing the fruit body formation even at 10^2 times dilution. Thus the concentration of thiamin was calculated to be 12.3 μg/L.

When fraction Ⅱ diluted 10^2 times was autoclaved at pH 2.0 for 30 min, the concentration of thiamin was 1.18 mg/L which was only a about 4 % loss of thiamin contained originally in it. In the case of the hydrolysis of fraction Ⅱ at pH 13.0, only a trace amount of thiamin could be detected and the fruit body inducing effect was lowered to 10 %, as shown in Table 3. However, the activity of fraction Ⅱ came back to 60 % by addition of thiamin to be at 12.3 μg/L after autoclaving under an alakline condition. Fraction Ⅱ, which was previously passed through a permtid column to eliminate the thiamin solely, completely lost fruit body inducing activity. It was also found that the fruit body of

Lentinula edodes was able to be formed in the basal peptone-glucose liquid media only by the addition of thiamin instead of fraction Ⅱ of yeast extract.

From these results, we concluded that the fruit body inducing factor contained in the fraction Ⅱ of yeast extract must be thiamin.

Discussion

Although each of the five fractions of yeast extract divided by gel chromatography exhibited activity at different levels, all of fractions were able to induce the fruit body formation of *Lentinula edodes*. These results suggest that the fruit body inducing factor contained in yeast extract was not a single one, rather some substances which had different molecular weights must have contributed to the fruit body formation of *Lentinula edodes*. Fraction Ⅰ which was the higher molecular weight region of yeast extract, contained thiamin at 10 % of the total amount. Iwashima et al. (1979) submitted the possibility that a thiamin-binding protein in *Escherichia coli* participated in the thiamin transport system. In this experiment, thiamin phosphates could not be detected in yeast extract produced by Difco. Therefore, it seemed that thiamin (Mw=301) could have existed in a wide

range of molecular weight from fraction I to II by means of the binding with some kinds of high molecular weight proteins. If this theory is correct, the fruit body inducing substance contained in fraction II would also be thiamin. Addition of fraction II induced 100 % fruiting which was the same as that of the original yeast extract. When fraction II was diluted 10^2 times inducing activity was decreased to 80 % fruiting. When thiamin was added to the basal liquid media at the same concentration as that contained in the 10^2 times diluted fraction II (12.3 μg/L), the fruit body formation was decreased to only 60 %. These results suggested that other components contained in yeast extract such as amino acids, nucleic acids, and vitamins contributed somewhat to the fruit body formation and were not as effective as thiamin. Alanin, glutamic acid, aspartic acid, leucin, and methionine in the amino acids and adenine and cytosine in the nucleotic acids were reported to promote the mycelial growth of *Lentinula edodes* (Isikawa 1967).

It is known that *Favolus arcularius* requires light irradiation for the initiation and development of fruiting in analogy with many other fungi. It was reported that anthranilic acid, p-aminobenzoic acid, and cyclooctasulfur were able to induce the formation of fruit body under dark condition (Murao et al. 1984; Hayashi et al. 1985). These results show that these

substances were able to replace the stimulus of light to induce fruit body formation. On the other hand, we reported that the light irradiation for a considerably long period during the incubation was essential for both the primordium and the fruit body formations of *Lentinula edodes*, even if yeast extract was added to the basal liquid media (Mohamed et al. 1992). Therefore, thiamin is not able to replace the stimulus of light; indicating that thiamin is not the inducing substance, but is the stimulating substance for the fruit body formation of *Lentinula edodes* in a more limited sense.

When the excised roots of higher plants such as pea and tomato were placed in a culture medium lacking thiamin, they grew less and less in each succeeding transfer and the growth finally ceased. Because thiamin is synthesized in the leaves and transported through the stem toward the root and acts effectively in a slight amounts, thiamin is regarded histologically as the plant hormone for the cell division in the tissue of root (Bonner et al. 1948). In this experiment, the mycelia of *Lentinula edodes* was able to grow even in the basal liquid medium lacking thiamin, but the fruit body did not form in that medium. However, by the addition of thiamin, the fruit body could

be induced at the concentration of only about 12 μg/L, which was calculated to be 4.0×10^{-8} M / L, suggesting that *Lentinula edodes* could not synthesized thiamin by itself. It could be concluded that the thiamin supplied from the culture media was the essential micronutrient for the fruit body formation of *Lentinula edodes.*

Thiamin, which is one of the water soluble vitamins, is an essential factor of the respiratory system in an organizm. Thiamin participates in the oxidation of pyruvate, and thiamin pyrophosphate is known to be the coenzyme of pyruvate decarboxylase (Bonner et al. 1948). Deficiency of thiamin would cause the problems in glucose metabolism for *Lentinula edodes,* resulting in the depression of fruit body formation. If newly occurring phenomenon in the mycelia by the incorporation of thiamin would be investigated physiologically and biochemically, it would be able to provide some information on the mechanism of fruit body formation of basidiomycetes.

Acknowledgment

We thank Mr. Naohiro Matsuo of The Nagasaki Prefectural Government for his valuable advice.

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Thiamin requirements for vegetative growth and fruit body formation of *Lentinula edodes*

Introduction

We previously reported that fruit body of a basidimycetes *Lentinula edodes* (Berk.) Sing., " Shiitake" was formed in the basal peptone-glucose liquid media by the addition of yeast extract (Matsuo et al. 1992; Mohamed et al. 1992). The activity of yeast extract was drastically lowered by heating under alkaline condition, suggesting that the fruit body inducing substance in yeast extract would be alkaline-labile thiamin. By the addition of thiamin instead of yeast extract, the fruit body was also formed in the basal peptone-glucose liquid media (Shin et al. in press). Thus it was concluded that thiamin must be the active substance in yeast extract for the fruiting of *Lentinula edodes*.

Since *Schizophyllum commune* and some other fungi were found to require thiamin for their good 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 by the use of the fungi of which fruit body was easily formed in chemically defined agar or liquid media, 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) and *Coprinus lagops* (Madelin 1956). All of these fungi required thiamin as an essential element for the fruiting.

There are few reports about thiamin requirement of edible mushroom except *Flammulina velutipes* (Yamada and Aoyama 1986). Especially, the thiamin requirement of *Lentinula edodes* for the fruiting have been still unknown because of difficulty of the fruiting in synthetic media.

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

Materials and methods

Organism

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

Media and cluture conditions

The basal liquid media (PG media) consisted of glucose; 50g, polypeptone ; 2.5g, KH_2PO_4 ; 1.0g, $MgSO_4 \cdot 7H_2O$; 0.5g, $CaCl_2 \cdot 2H_2O$; 0.5g, $FeCl_2 \cdot 6H_2O$; 10mg, $MnCl_2 \cdot 4H_2O$; 7.2mg, $ZnCl_2$; 4mg, $CuSO_4 \cdot 5H_2O$; 1mg and 1 liter of distilled water. A peptone-glucose-agar media (PGA media) which agar added at 2.5 g/L to PG media, a casamino acid-glucose-agar media (CGA media) which vitamine-free casamino acid (Difco) was added at 2.5 g/L instead of peptone in PGA media, and glutamic acid-glucose-agar media (GGA media) which glutamic acid was added at 2.5 g/L instead of peptone in PGA media were also prepared. One hundred ml of the liquid media was dispensed into a 500-ml Erlenmeyer flask and autoclaved at 121℃ for 30 min. The mycelial inoculum, an agar disk of 5 mm diameter cut from the previous colonized plate, was put on the center of the liquid media. The mycelial cultures were incubated statically for 9 weeks at 25 ℃ and 60 % relative humidity under about 200 lux of light with 12 h light and dark cycle. Thiamin was added at various concentrations in PG media before autoclaving.

A 30 mL of agar media previously autoclaved were poured in 9 cm diam. Petri dishes and, after solidification, were inoculated with mycelial disks cut from a colonized PDA plats. After 11 days of incubation, mycelial disk was cut from colonized plate and transferred to fresh media. The transfer was repeated again with the same manner. Incubation was undergone 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, mycelia was extracted with 50 ml of 0.1 mol- HCl in a water bath at 100 ℃ for 30 min. 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 (15cm×4.6cm) column with acetonitrile : 0.1 mol/L $NaH_2PO_4 \cdot 2H_2O$ (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. 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. in perss).

Results

Effects of thiamin on mycelial growth in liquid media

Although the optimal concentration of yeast extract for fruit body formation of *Lentinula edodes* was 2.5 g/L, fruit body was able to be produced even at concentration as small as 25 mg/L of yeast extract (Matsuo et al. 1992). The content of thiamin in 1 g of yeast extract was 0.58 mg as a thiamin hydrochloride (Shin et al. in press). The concentration of thiamin in 25 mg/L of yeast extract was therefore calculated to be 14.5 μg/L.

Lentinula edodes was incubated for 9 weeks in basal peptone-glucose liquid media supplementing by 14.5 μg/L thiamin (PGT media). Mycelial growth in PGT media was slower than that in basal media supplementing by yeast extract, but small mycelial aggregates were formed on the surface of colony after about 4 weeks and then they grew up to primordia. Fruit body formation began after about 7 weeks. The fruiting was occurred in 60 % of cultures within 9 weeks after inoculation. In basal liquid media without thiamin, none of primordia and fruit body were produced.

The mycelial weight was significantly increased after about 4 weeks of incubation in PGT media, as shown in Fig. 1. Whereas, the effect of thiamin on mycelial weight was not found until the third week. Even in the media without thiamin, mycelia kept growing up to the 9 th week of incubation.

Effects of thiamin on mycelial growth and fruit body formation

In order to confirm the effect of thiamin for fruit body formation, *Lentinula edodes* was incubated for 9 weeks in basal liquid media supplementing by thiamin at various concentration before autoclaving. The result was shown in Fig. 2 (I). The level of fruit body formation was still maintained over 60 % at 14.5 μg/L corresponding to 1/100 of original concentration of thiamin, but decreased significantly below about 5 μg/L (1/300), and fruit body was not formed at 1.45 μg/L (1/1000). Because about 20% of thiamin added to PG media was decomposed under autoclaving at 121℃ for 30 min, the concentration of thiamin at the begining of incubation was decreased from 14.5 μg/L to 12.1 μg/L.

These results suggested that thiamin was an essential element for fruit body formation of *Lentinula edodes* and

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 included 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.

Fig.1

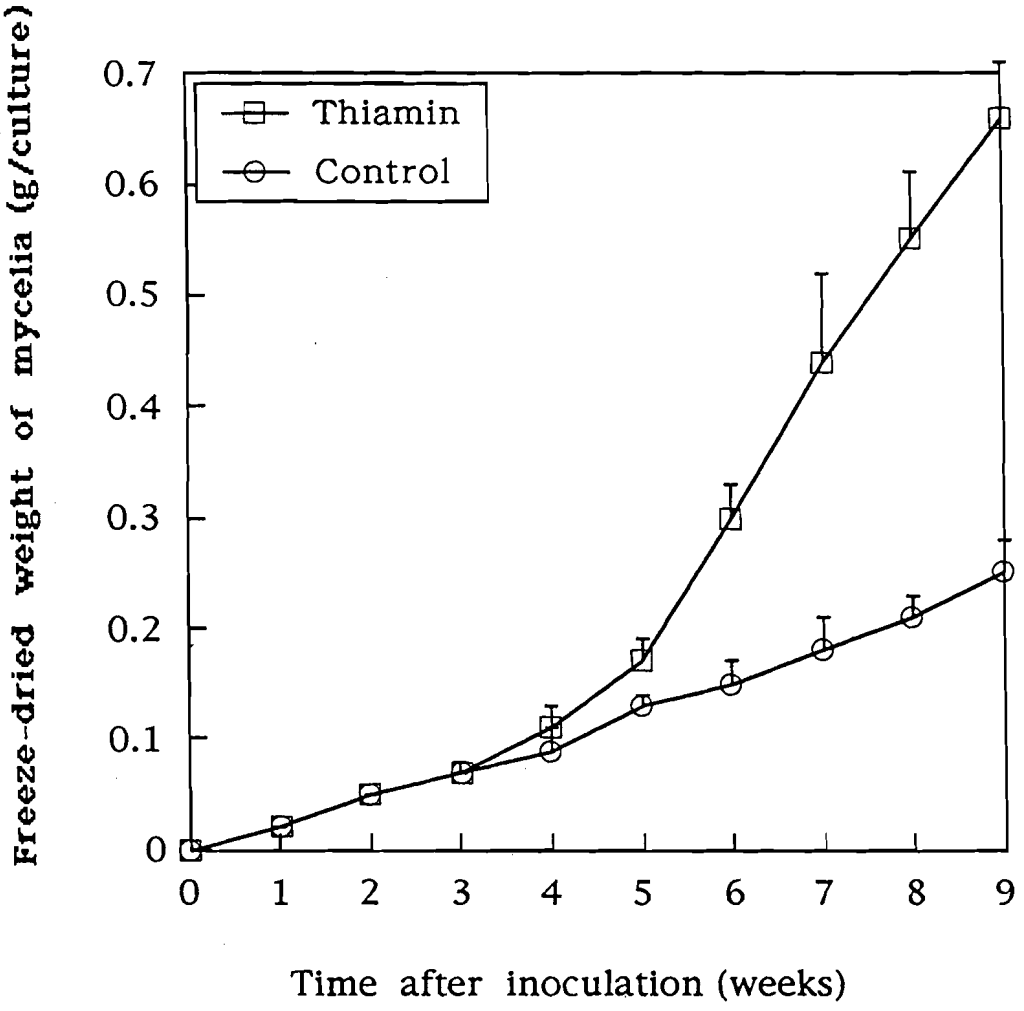

Freeze-dried weight of mycelia (g/culture)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Thiamin
Control
0
1
2
3
4
5
6
7
8
9
Time after inoculation (weeks)

Fig.2. Effect of thiamin concentration in the region of 1.45 mg-1.45 μg/L on : (Ⅰ) fruit body formation, (Ⅱ) mycelial growth. The symbol A in the figure means 1.45 mg/L which is the thiamin concentration contained in 2.5 g/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 1.45 mg/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.

Fig.3

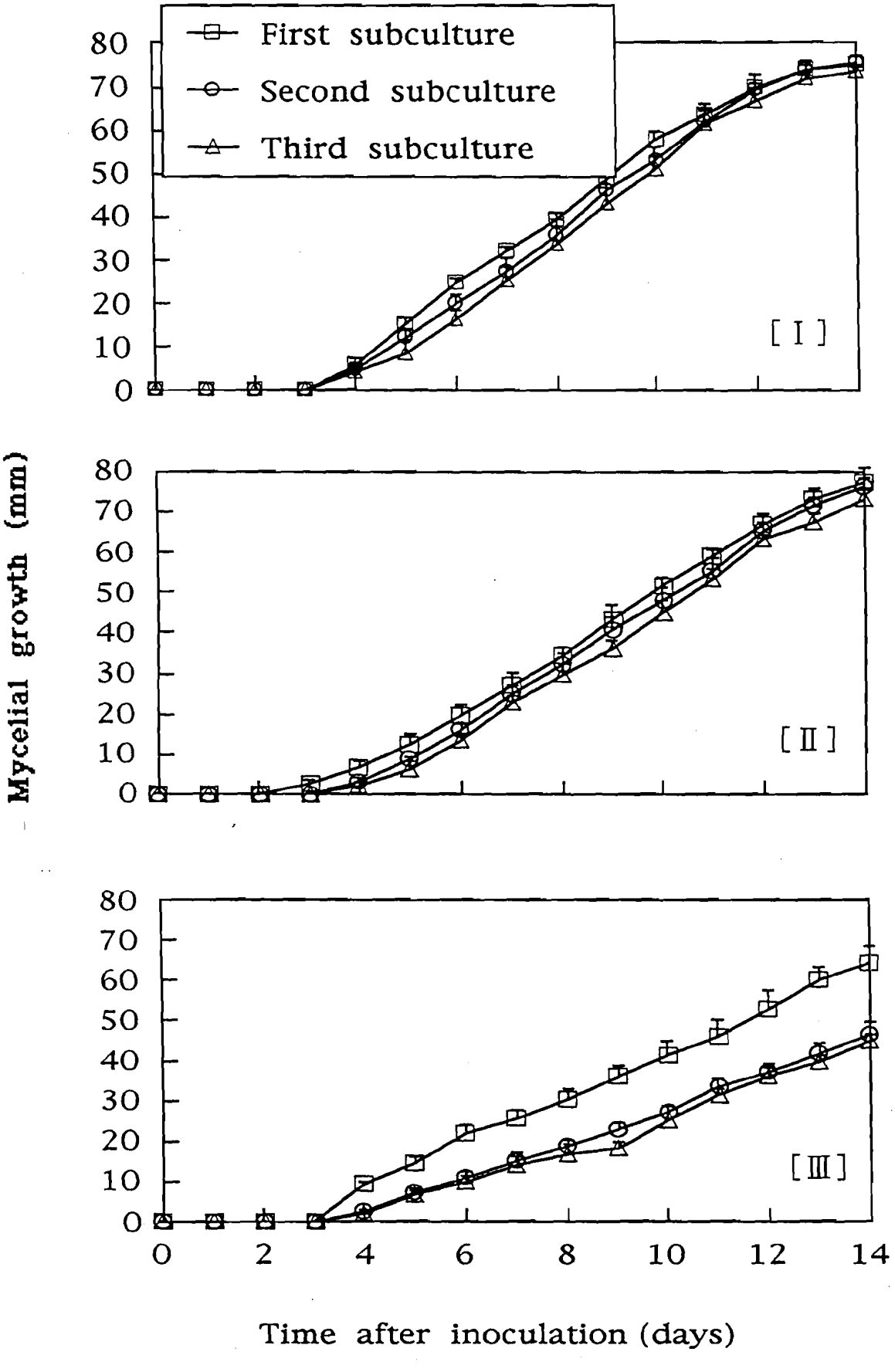
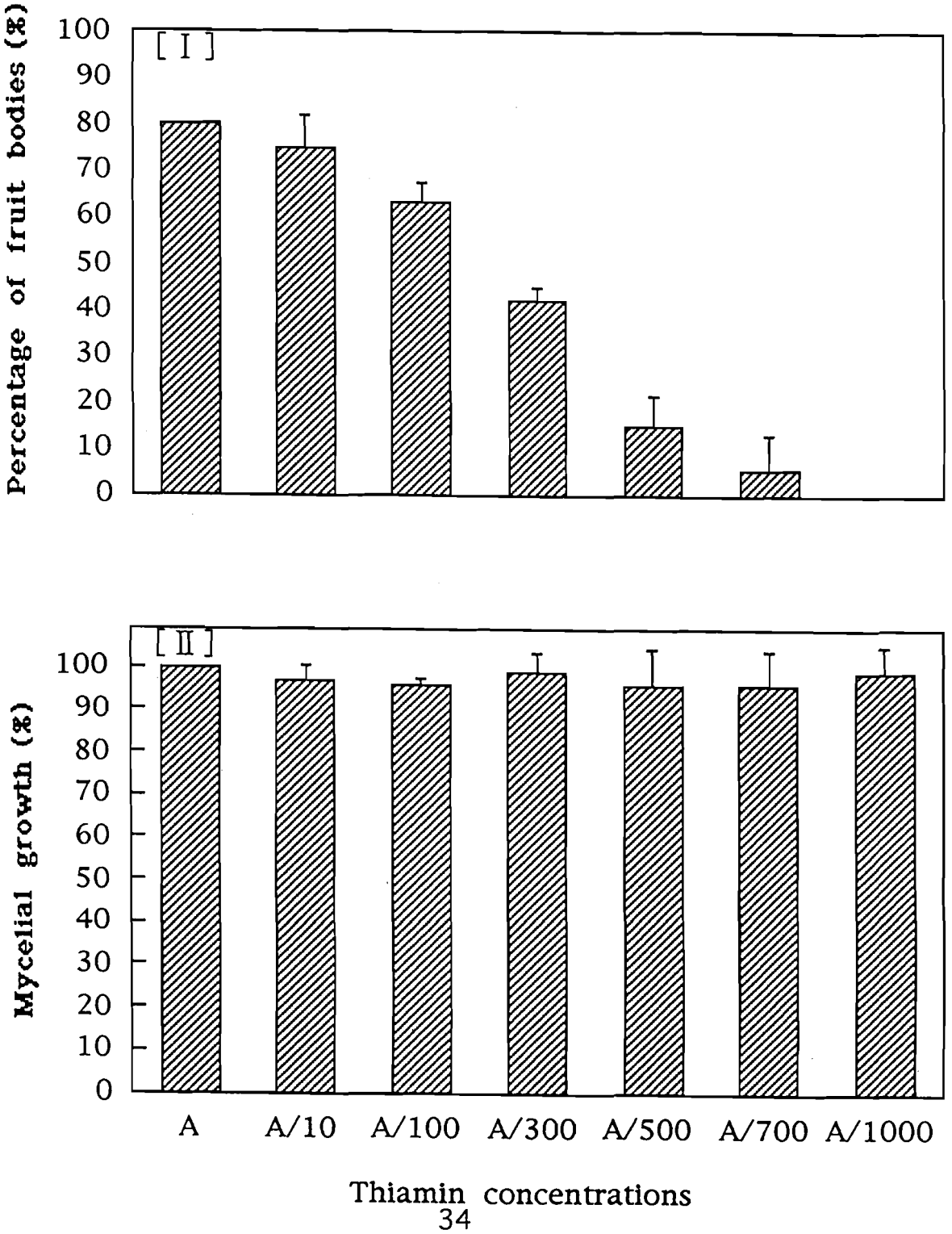

First subculture
Second subculture
Third subculture
[I]
[II]
[III]
Mycelial growth (mm)
Time after inoculation (days)
0
10
20
30
40
50
60
70
80
0
2
4
6
8
10
12
14

Fig.2


[I]
Percentage of fruit bodies (%)
100
90
80
70
60
50
40
30
20
10
0
[II]
Mycelial growth (%)
100
90
80
70
60
50
40
30
20
10
0
A
A/10
A/100
A/300
A/500
A/700
A/1000
Thiamin concentrations

the minimum thiamin requirement was estimated to be around 10μg/L.

The effects of thiamin on mycelial weight during the vegetative growth stage until the third week of incubation were investigated. The vegetative mycelial growth was litter influenced by the addition of thiamin in the range of 0-1.5 mg/L, as shown in Fig. 2(Ⅱ).

Effects of subculture on mycelial growth

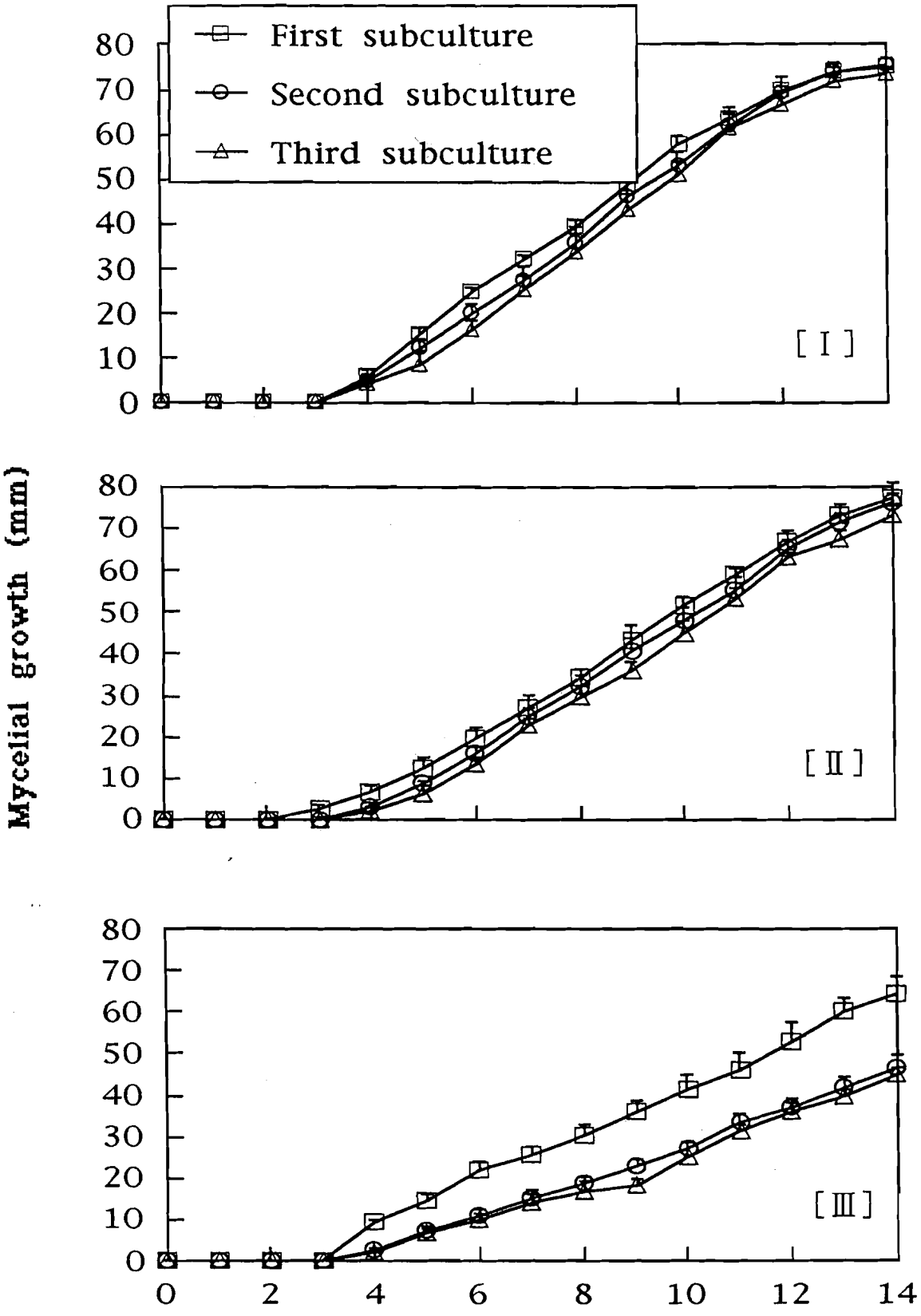
The mycelial growth rates were also investigated by the usual technique of subculturing in peptone-glucose-agar (PGA) media. *Lentinula edodes* which has been maintained on potato-glucose-agar (PDA) media, was subcultured three times in PGA media, the decrease of mycelial growth was however insignificant in each succeeding transfer, as shown in Fig. 3 (Ⅰ). While PGA media supplemented by thiamin were used for the second or third transfer, the mycelial growth rate was not influenced as compared with that in the media free from thiamin.

Lentinula edodes was also subcultured three times in a CGA media containing a casamino acid which was specially purified for vitamin assay, insted of peptone in PGA media. The inhibition of mycelial growth was also little in each subculture, as shown in Fig. 3 (Ⅱ).

When investigated in the chemical-defined media (GGA

Fig.3. 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 culture.

Fig.3


First subculture
Second subculture
Third subculture
[I]
[II]
[III]
Mycelial growth (mm)
Time after inoculation (days)
0
10
20
30
40
50
60
70
80
0
2
4
6
8
10
12
14

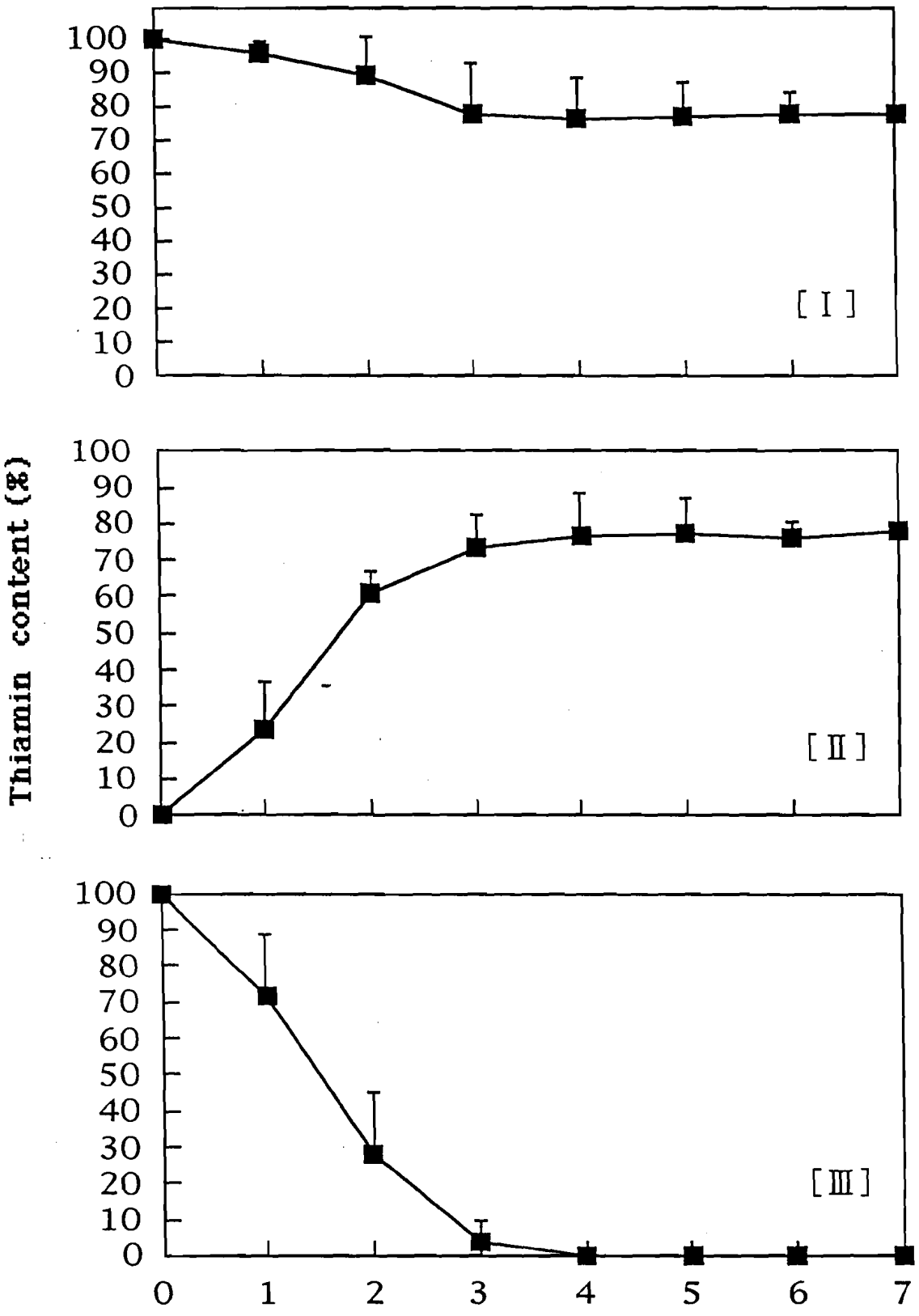
media) containing glutamic acid instead of peptone in PGA media, the mycelial growth was significantly inhibited in the second subculture, as shown in Fig. 3 (Ⅲ) . However, the further inhibition of mycelial growth did not suffered by a successive transfer. Because the mycelial growth was not recovered even when GGA media supplemented with thiamin were used for the second transfer, the deficiency of thiamin was not responsible for the inhibition of mycelial growth by subculturing.

Uptake of thiamin

Lentinula edodes was incubated in a 100 ml of PG basal media containing 1.45 μg/L of thiamin, and the contents of thiamin were determined both in mycelia and culture filtrate every week after inoculation. As shown in Fig. 4, about 60 % of thiamin in the media have already been taken into mycelia only after 2 weeks incubation. Almost all thiamin existed in the media was found in the mycelia at the third week, the content of thiamin in the mycelia stayed fairly constant after 3 weeks. We could not detected thiamin in the culture filtrate after 4 weeks under the condition of this experiment. These results suggested that almost all thiamin contained in the media was taken and accumulated in the mycelia during only 3 weeks on the early stage of totally 9 weeks incubation.

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.

Fig.4

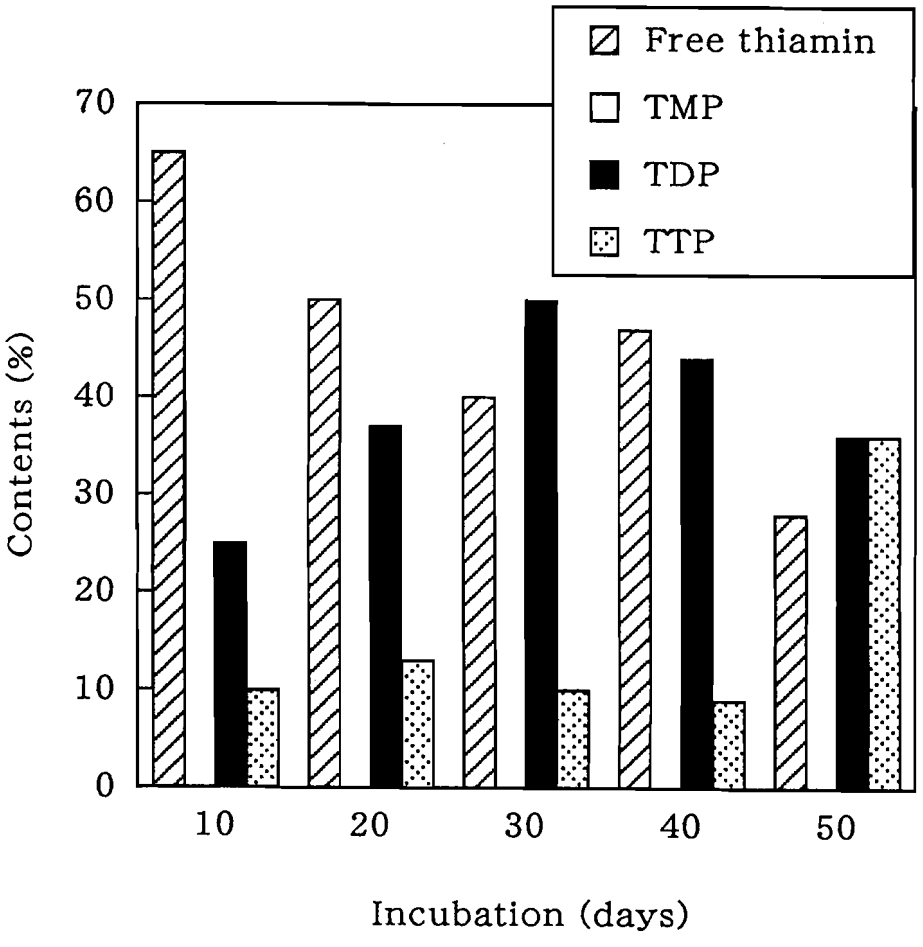

Thiamin content (%)
100
90
80
70
60
50
40
30
20
10
0
[I]
[II]
[III]
0
1
2
3
4
5
6
7
Time after inoculation (weeks)

The contents of thiamin, thiamin monophosphate (TMP), diphosphate (TDP) and triphosphate (TTP) in mycelia were determined separately during incubation. The result was shown in Fig. 5. Over 60% of thiamin taken in mycelia was existed as free-form and the content of phosphate esters was significantly low in the first 10 days of incubation. The ratio of free thiamin was gradually decreased during the course of incubation, and TDP content was contrarily increased.The level of TDP reached to the maximum at the fourth week from which the primordia formation started to occure, and then decreased. The content of TTP increased to the same level as compared with that of TDP at the seventh week of incubation. None of TMP could be detected in the mycelia.

Discussion

Raper and Krongelbha investigated the effect of thiamin on mycelial growth and fruit body formation of *Schizophyllum commune* in glucose-asparagine media (Raper and Krongelb 1958). They showed that thiamin was a necessary factor for fruiting in *Schizophyllum* and the thiamin requirement was variable for different strains in the region of 7.5-30 μg/L. It was also reported by Oyama and others that *Schizophyllum commune* required 30-100 μg/L thiamin for fruiting in glucose-asparagine media (Oyama et al. 1976).

Fig.5. The contents of thiamin, thiamin monophosphate(TMP), diphosphate(TDP) and triphosphate(TTP) in mycelia of *Lentinula deodes*.



The thiamin requirements for fruiting of *Favolus arcularius* in maltose-casamino acid-agar media, and that of *Coprinus lagops* in alanine-glucose-agar media were found to be 3 and 10 μg/L, respectively (Kitamoto and Kasai 1968a, 1968b; Kitamoto et al. 1980).

Flammulina velutipes, which is one of economical important edible mushroom in Japan, required over 10 μg/L of thiamin for fruiting in asparagine-glucose-agar media (Yamada and Aoyama 1986). We previously reported that *Lentinula edodes* formed fruit body in the basal peptone-glucose liquid media by adding 12.3 μg/L thiamin instead of fraction Ⅱ of yeast extract. In this experiment, *Lentinula edodes* gave 60 % of the fruiting by adding thiamin at 14.5 μg/L (The concentration after autoclaving was 12.1 μg/L). Therefore, it seemed that thiamin requirement of *Lentinula edodes* for the fruiting was almost the same level as those of fungi described above.

Though the mycelial growth of *Schizophyllum commune* was also promoted by thiamin, the requirement of thiamin for mycelial growth was lower than that for the fruiting (Raper and Krongelb 1958). Whereas, Oyama and others reported that thiamin had little effect on the vegetative growth of *Schizophyllum commune* (Oyama et al. 1976). Ishikawa investigated the effect of thiamin on the mycelial growth of *Lentinula edodes* by using ammonium

tartarate-glucose liquid media. He reported that the dry weight of mycelia was increased about three times by adding thiamin at 100 μg/L (Ishikawa 1967). In this experiment, there was not a significant difference in 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. The thiamin requirement of *Lentinula edodes* was seemed to be variable for different strains.

It was assumed that the relative abundant vegetative growth in the media without thiamin was due to thiamin contained in the agar itself for *Coprinus lagopus*, and in inoculum for *Flammulina velutipes*, respectively (Kitamoto and Kasai 1968a; Madelin 1956). If the inoculum contained thiamin, thiamin took into the culture would be decreased and extinguished at last by successive transferred 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 was however insignificant. Peptone is made from natural substance so that it may possibly contains thiamin as impurity. However, even in the case of using vitamin-free casamino acid or glutamic acid instead of peptone, the deficiency of thiamin for the mycelial growth did not occur

by transferring.

Because the uptake of thiamin into the mycelia from the media was very slow and /or it started in the latter half of incubation, thiamin should not effectively work for promoting the mycelial growth of *Lentinula edodes* until 3 weeks after incubation. From the determination of thiamin contents both in mycelia and culture filtrate, it was found that almost all thiamin contained in the media was taken and accumulated in the mycelia during the vegetative mycelial growth. These results showed that the amounts of thiamin which *Lentinula edodes* required for the vegetative mycelial growth, should be trace if thiamin was essential for it. Because *Lentinula edodes* required around 10 μg/L thiamin for the fruiting, almost all thiamin added to basal media must be used for fruit body formation.

It has been well known that thiamin is one of water soluble-vitamins and it's diphosphate is an essential factor for carbohydrate metabolism of organism. Thiamin is the coenzyme of the enzyme pyruvate carboxylase and it takes part in the oxidation of pyruvic acid (Bonner, J., and Bonner, H. 1948). From this experiment, it was found that free thiamin accumulated in mycelia was gradually decreased during the course of incubation and the ratio of TDP was contrarily increased. It should be very interesting phenomenon for investigating the roll of thiamin on fruit body formation of *Lentinula edodes* that TDP content reached the

maximum level at that time when the primordia formation startet.

Acknowledgements

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

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