担子菌による核酸関連物質の醗酵—I

えのきたけの液内培養における核酸関連物質の変化

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Fermentation of Nucleic Acid Related Substances by Basidiomycetes - I

Changes of nucleic acid related substances in submerged culture of *collybia velutipes* (enoki-take)

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Abstract

The mycelium of *Collybia velutipes*, one of edible mushrooms, was grown on a shaker. The change of nucleic acid and related substances, particularly 5′-mononucleotides during the culture period was investigated for the purpose of the application as a flavor substance.

Maximum growth was obtained at 15 days of culture in both medium containing ammonium tartrate or urea as nitrogen source. The pH of the medium containing ammonium tartrate was reduced to about 3.0, but that of urea medium was around 7.0 throughout the culture period. The greater part of phosphate initially existed in the medium had been incorporated into the mycelium until the time of miximum mycelial yield. More than 15% of total phosphorus of the mycelium was found to be RNA-phosphorus. Accompanied with the cessation of the increase of the mycelial yield, its RNA was degraded and mononucleotides were excreted into the

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Abbreviations used :

AMP, adenosine-5′-monophosphate
ADP, adenosine-5′-diphosphate
ATP, adenosine-5′-triphosphate
GMP, guanosine-5′-monophosphate
CMP, cytidine-5′-monophosphate
UMP, uridine-5′-monophosphate
IMP, inosine-5′-monophosphate
RNA, Ribonucleic acid
DNA, Deoxyribonucleic acid

medium. AMP and UMP were found to be the major nucleotides contained both in mycelium and in culture broth. CMP and GMP were found in a little amount. The growth phase of mycelium seems to be interpreted rather clearly with the ratio of RNA- to total phosphorus.

Introduction

Submerged cultures of mycelia of Basidiomycetes have been well established as introduced in reviews by Humfeld[1], Robinson and Davidson[2], Block[3], and Yoshida and Teramoto[4]. The process is considered to offer a promise of a large scale, low-cost production of mushroom mycelium for foods and feeds. Furthermore, some investigations on the production of useful chemicals, enzymes, and antibiotics have been carried on.

Fruiting bodies of mushrooms are valuable for food, because of their flavor. Remarkable amount of 5′-GMP, one of taste enhancing substances, is contained in some fruiting bodies[5,6], and it is supposed that 5′-GMP and other mononucleotides may contribute to the flavor of mushrooms. It is one purpose of this communication to evaluate the usefulness of mycelia produced in submerged culture by determining the amount of 5′-nucleotides.

The change of ribonucleic acid is considered to be an useful index to examine the growth phase of microbial cells[7,8]. Mononucleotides above stated are essential components of the RNA, therefore, it is interesting to investigate the metabolic change of nucleic acid related compounds in connection with the growth of mushroom mycelium. This paper reports a result of a study on the change of nucleic acid and related substances in mycelium and in medium during the shaking culture of *C. velutipes*, (Enokitake, in Japan), one of edible mushrooms.

Materials and Methods

1. Cultural methods

C. velutipes strain was maintained on agar slant composed of 2% glucose, 0.5% yeast extract, 0.1% $MgSO_4$, $7H_2O$, 0.1% K_2HPO_4, 2% agar, and the pH adjusted to 5.0, grown at 25°C for 7 days and stored cold. A pure culture of the strain was obtained by aseptically transferring pieces of the fruiting body tissue onto this agar slant in the same way as described by Block et al.[9]

The composition of medium for inoculum was that of proposed by Reusser et al.[10], with a little modification, i.e. 4% of glucose and 0.4% of ammonium tartrate were used as carbon and nitrogen sources. Inoculum was prepared stationary in 100 ml Erlenmeyer flasks containing 25 ml of the medium. Bits of mycelium planted in this medium were allowed to grow at 25°C for 7 to 10 days. The mycelium grown was washed with

sterile distilled water and broken up by homogenizing in sterile electric blendor at 15,000 rpm for 1 min containing sterile distilled water or culture medium. The resulting suspension was used as inoculum.

The cultures were carried out in two kinds of media. The basal composition of two media was that of Reusser et al.[10] The medium which contained 0.4% of ammonium tartrate as nitrogen source was referred to as medium T. Medium T allowed the reduction of pH from 5 to 3, during the culture period, because of the formation of organic acids. The medium which contained 0.132% of urea as nitrogen source (it corresponds to 0.061% of total nitrogen) was referred to as medium U (Urea). Medium U allowed to keep pH around 7, because urea neutralized the formed acid. In medium U, another 0.0264% (1/5 to initial amount) of urea was added after 12 days if the pH of culture broth became below 7. Each 500 ml shaking flask containing 100 ml of sterile medium was inoculated with 2 to 5 ml of mycelial suspension. The cultures were shaken at 120 rpm on a reciprocating shaker having a path of 6 cm at 25°C.

The mycelium was harvested at the ages of the maximum growth rate (about 7 days' incubation), the maximum mycelium yield (about 15 days' growth), and the autolyzation of mycelium (about 30 days after).

2. Analytical methods Duplicate samples were removed from shaker, and the mycelial pad was separated from the fermented broth with centrifugation. The moist mycelium was analysed for nucleic acid related substances. The residual broth was analysed for the pH, the absorption at 260 mμ, reducing sugar and nucleic acid related substances. Mycelium weight was obtained by washing the mycelium with distilled water and drying below 90°C with infrared rays. Absorbancy at 260mμ of the broth was measured spectrophotometrically at pH 2.0 after diluted adequately. Reducing sugar was determined colorimetrically with the alkaline copper reagent of Somogyi[11].

Nucleic acid and related substances contained in mycelium and in fermented broth were isolated and divided into acid soluble-, RNA-, and DNA- fractions according to the procedure of Schmidt-Thannhauser-Schneider. Cold perchloric acid (5%) was used for the extraction of acid-soluble fraction. The amounts of phosphorus of above fractions were determined by the method of Fiske and Subbarow. The individual nucleotide in acid-soluble fraction was determined according to the procedure proposed by Bergkvist et al.[12] and modified by Nakajima et al.[5]

Results

1. Effect of some factors on the growth of the mycelium Preliminary experiments were carried out to clarify the factors, for example, nitrogen source, required amount of inoculum, and extent of aeration, for the growth of *C. velutipes*.

The nitrogen source, namely, ammonium tartrate, ammonium sulfate, ammonium chloride, ammonium nitrate, potassium nitrate, urea, or Casamino acid (Difco) was examined individually at a concentration of 0.0608% as nitrogen (it corresponds to

Table 1 Effect of nitrogen sources on the growth* of mycelium.

Nitrogen sources**	pH of finished broth	Mycelium (dry weight, mg/100ml)	Reducing sugar, consumed (mg/ml)
Ammonium tartrate	2.90	663	32.0
Ammonium sulfate	3.00	67	5.0
Ammonium chloride	2.90	40	3.0
Ammonium nitrate	2.91	65	4.4
Potassium nitrate	4.10	54	5.0
Urea	5.20	889	42.2
Casamino acid	4.88	749	45.0

* cultured at 25°C for 16 days

** Each nitrogen source was added in a concentration of 0.0608% as nitrogen.

0.4% of ammonium tartrate). The amount of mycelial growth was found to depend markedly upon the kinds of nitrogen sources, as shown in Table 1.

Ammonium tartrate, urea, and Casamino acid supported the mycelial growth evidently and also the utilization of sugar. The growth on the following nitrogen sources were very poor: ammonium sulfate, ammonium chloride, ammonium nitrate, and potassium nitrate. The pH of growing culture became lower when ammonium tartrate was used without pH adjustment.

Table 2 Relation of inoculum size and the growth* of mycelium.

Inoculum, mycelium (dry wt. mg/100 ml)	pH of finished broth	Mycelium (dry wt.) (mg/100 ml)	Reducing sugar, consumed (mg/ml)
2	3.5	545	17.8
4	3.5	650	18.4
13	3.5	605	18.7
21	3.4	710	21.0
42	3.5	585	21.3

* cultured at 25°C for 12 days

Table 2. shows the relation of the size of inoculumn and the growth of mycelium. The mycelial growth over 585 mg/100ml was obtained under the condition that 4 to 42 mg/100 ml of mycelium was inoculated and cultured for 12 days. The inoculum size in a range of 4 to 42 mg/100 ml was used in succesive experiments.

The effect of agitation was examined with increasing volumes of medium in 500 ml shaking flasks as shown in Table 3. Mycelial growth of over 630 mg/100 ml was obtained in media of volumes in a range of 30 to 300 ml. Usually, the mycelium was cultured in media of 100 ml.

2. Culture in medium T Table 4. shows the growth of mycelium in medium T., in which ammonium tartrate was used. The pH of medium was reduced accompanied with the growth of mycelium, and when it was not adjusted it reached to a

Table 3 The growth* of mycelium in various volumes of medium under shaking culture.

Volumes of medium in 500 ml-flask (ml)	pH of finished broth	Mycelium (dry wt., mg/100 ml)	Reducing sugar, consumed (mg/ml)
300	3.4	660	23.2
200	3.4	705	24.2
100	3.4	630	25.9
50	3.5	705	27.1
30	3.6	630	22.3

* 25.1 mg (dry wt.)/100 ml medium of mycelium was inoculated in each flask and cultured at 25°C for 15 days.

Table 4 Growth of mycelium and changes of pH, reducing sugar, and absorbancy at 260 mμ in medium T.

	Days, passed						
	0	3	5	8	11	15	30
Mycelium(dry wt. mg/100 ml)	18	23	221	437	570	681	598
pH of medium	5.12	4.90	4.20	3.80	3.64	3.24	3.14
Reducing sugar (mg/ml)	44.01	43.09	41.02	34.00	29.07	17.07	9.04
Absorbancy* at 260 mμ	0.03			0.15		0.37	0.62

* Cell-free broth was diluted 25-fold with N/100 HCl.

value of 3.1. The maximum yield of mycelium (as dry matter) was obtained after 15 days. Reducing sugar decreased day by day. It was remarkable that the absorbancy at 260 mμ of culture broth increased after 15 days. The broth had a peak of absorption around 260 mμ. It was recognized that the nucleic acid related compounds were excreted and accumulated in a period associated with the cessation of growth.

The change of phosphorus in various fractions of nucleic acid and related compounds is shown in Table 5. The total-, acid soluble-, RNA- and DNA- phosphorus in moist mycelium were compared with the remained total phosphorus in the broth. The initial amount of phosphorus was 370 μmol/100 ml, originated in KH_2PO_4 of medium component. The mycelium containing 7 μmol of phosphorus was inoculated. According to the growth, the phosphorus of broth had been transferred to cell constituent until 15 days. The phosphorus in mycelium decreased with increasing in broth after 30 days. Remarkable decrease in the ratio of RNA-phosphorus to a total phosphorus was observed in the mycelium. The excretion of nucleic acid related compounds into the broth coincided with the decrease of RNA in mycelium.

Grown mycelium was collected by centrifugation from the medium after 8, 15, and 30 days' culture. As shown in Table 5, acid soluble fraction formed about 50% of a total phosphorus in mycelium. Extracts of the mycelium and the broth were loaded on a Dowex 1×8 column for the assay of nucleotides. Typical chromatograms of nucleotides in mycelium and the broth are shown in Fig. 1 and Fig. 2. The peak of

Table 5 Changes of nucleic acid related substances during the culture in medium T.

	Days, passed			
	0	8	15	30
Mycelium (dry wt. mg/flask)	18	437	681	598
Total phosphorus (μmol-P/flask)				
in filtered broth	370	195	115	165
in mycelium	7	175	251	204
In mycelium(per mg dry mycelium)				
total phosphorus, μmol-P		0.401	0.368	0.340
acid soluble, 〃		0.193	0.192	0.169
RNA, 〃		0.073	0.055	0.034
DNA, 〃		0.018		0.012
Ratio of phosphorus				
acid soluble/total (%)		48.2	52.1	49.6
RNA/total (%)		18.2	15.0	10.0
RNA/DNA		4.1		2.8

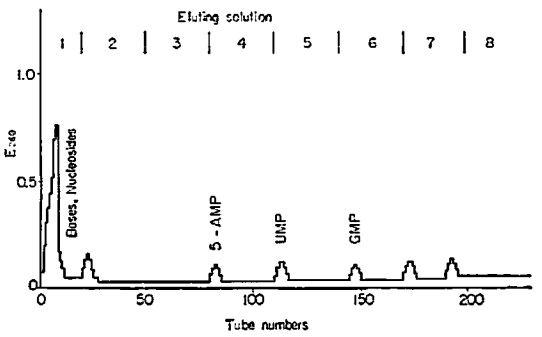

Fig. 1 Chromatogram of the extract of mycelium grown in medium T for 15 days.

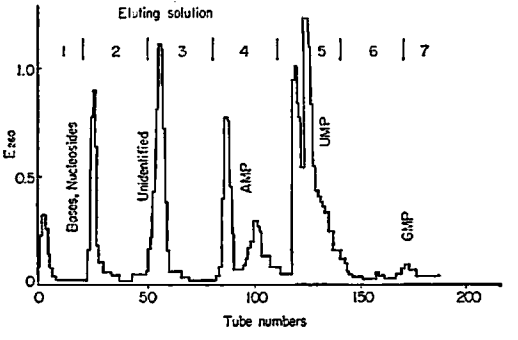

Fig. 2 Chromatogram of the mycelium-free broth of medium T, after 30 days.

a mixture of bases and nucleosides was found to be rather large. The peaks of mono nucleotides were identified by usual methods.

The amount of nucleotides in mycelium and in culture broth is shown in Table 6. Among five kinds of nucleotides generally found in nature, 5'-AMP (including 3'-AMP), 5'-UMP (including 3'-UMP) were found to be in fairly large amount. GMP and CMP were found in a trace amount. No IMP was contained either in mycelium or in culture broth.

3. Culture in medium U The outline of the culture in U medium, in which urea was used is shown in Table 7. The pH of the broth was 6.62 at initial and became a slight alkaline after 7 days. Accompanied with the supplement of urea after 12 days, the pH was kept over 7.0. The mycelium increased day by day until 14 days, afterwards it decreased gradually. The greater part of reducing sugar was found to

Table 6 Changes of mononucleotides in mycelium and filtered broth during the culture in medium T.

Nucleotides	Days, passed		
	8	15	30
In mycelium, (μmol/g dry wt.)			
CMP	0	trace	trace
AMP (5′-and 3′-)	trace	0.202	0.424
UMP (5′- and 3′-)	0.138	0.248	0.308
GMP	trace	trace	trace
IMP	0	0	0
In filtered broth, (μmol/100ml)			
CMP	trace	trace	trace
AMP (5′- and 3′-)	1.25	1.81	0.99
UMP (5′- and 3′-)	0.33	1.36	1.83
GMP	trace	trace	trace
IMP	0	0	0

Table 7 Growth of mycelium and changes of pH, reducing sugar, and absorbancy at 260 mμ in medium U.

	Days, passed					
	0	5	7	9	14	28
Mycelium (dry wt. mg/100ml)	16	257	374	775	959	790
pH of medium	6.62	7.03	7.72	6.63	7.50	8.50
Reducing sugar (mg/ml)	44. 3	36. 6		26. 0	18. 2	0. 1
Absorbancy* at 260 mμ	0.03	0.07	0.11	0.21	0.25	0.44

* Cell-free broth was diluted 25-fold with N/100 HCl.

be consumed after 28 days. The absorbancy at 260 mμ of the residual broth became higher as time passed by.

Table 8 shows the change of phosphorus contained in acid soluble-, RNA-, and DNA- fractions and also a total amount of phosphorus, during the culture in U medium. The exogenous phosphorus of the medium decreased remarkably accompanied with the growth of the mycelium. The amount of phosphorus in mycelium was maximum after 14 days, afterwards it decreased gradually corresponding to the decrease of mycelium weight. A ratio of RNA- to a total-phosphorus was 15.8% after 14 days and it decreased to 9.0% after 28 days. The fact may be accounted for the autolyzation of RNA.

A typical chromatogram of a sample of the broth obtained from 28 days' culture is shown in Fig. 3. Noticeable amounts of 5′-AMP, 3′-AMP, UMP and a trace amount of CMP and GMP were found to be in the broth. Some fractions including nucleoside polyphosphates were eluted succeeding to mononucleotide fractions. These were assumed to be the fractions of adenosine triphosphate, adenosine diphosphate, and uridine

Table 8 Changes of nucleic acid related substances during the culture in medium U.

	Days, passed			
	0	7	14	28
Mycelium (dry wt. mg/flask)	16	374	959	790
Total phosphorus (μmol-P/flask)				
in filtered broth	364	242	32	83
in mycelium	6	121	310	281
In mycelium (per mg dry mycelium)				
total phosphorus, μmol-P		0.323	0.323	0.356
acid soluble, 〃		0.182	0.156	
RNA, 〃			0.051	0.032
DNA, 〃		0.011	0.014	0.015
Ratio of phosphorus				
acid soluble/total (%)		56.4	48.4	
RNA/total (%)			15.8	9.0
RNA/DNA			3.6	2.1

diphosphate sugars, however, further detailed identification was not done.

As shown in Table 9, 5′-AMP (including 3′-AMP) and 5′-UMP (including 3′-UMP) were found to be the major nucleotides, GMP and CMP were in a trace amount. No IMP was contained in either mycelium or broth.

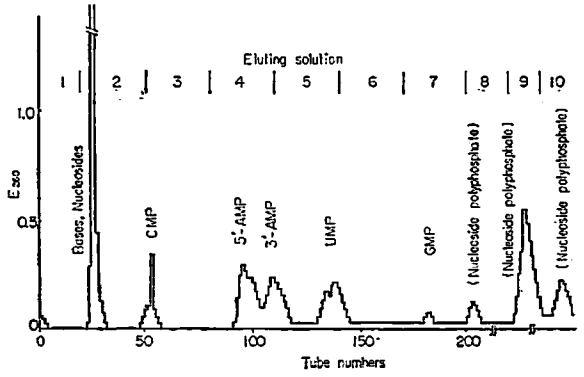

Fig. 3 Chromatogram of the mycelium-free broth of medium U, after 28 days.

As the results, the maximum mycelial growth in medium U (Table 7) was more than that in medium T (Table 4). The comsumption of sugar in medium U was more than that in medium T. To compare the metabolic change related to nucleic acid in medium T with medium U, the amounts of a total phosphorus, RNA, and nucleotides were calculated on a same volume of the medium. There was no difference between two media, referring to the ratio of RNA-phosphorus to the dry weight of mycelium (Table 5, 8). The amounts of exogenous 5′-AMP and 5′-UMP of the medium T was more than those of medium U. Thus, it was found that the excretion of nucleotide into medium T was more than that into medium U.

Table 9 Changes of mononucleotides in mycelium and filtered broth during the culture in medium U.

Nucleotides	Days, passed		
	7	14	28
In mycelium, (μmol/g dry wt.)			
CMP	0	trace	trace
AMP (5′- and 3′-)	0.166	0.093	0.236
UMP (5′- and 3′-)	0	0.160	0.275
GMP	trace	trace	trace
IMP	0	0	0
In filtered broth, (μmol/100 ml)			
CMP	0	trace	trace
AMP (5′- and 3′-)	0.05	0.10	0.72
UMP (5′- and 3′-)	0.02	0.04	0.83
GMP	trace	trace	trace
IMP	0	0	0

Discussion

As shown in Table 5 and 8, 68% and 85% of inorganic phosphate initially existed in culture broth had been incorporated into grown mycelium in medium T and U, respectively, until the time of maximum growth. The ratios of RNA-phosphorus to a total phosphorus and RNA/DNA were more than 15% and 3.6~4.0 during the growth in good condition, however, they decreased to about 10% and 2.1~2.8 after the cessation of the growth, respectively. These ratios may be considered as an index of growth phase, agreed with the cases of bacteria as described by Mimura et al[7] and Aiba et al[8].

The periodical change of a pattern of phosphorus compounds in shaking culture shown in our results progressed more rapidly than that in static culture observed by Wakita[13]. He indicated that the RNA of *C. velutipes* No. 67 var. had the same nitrogen- and phosphorus- contents as the yeast RNA. Then, it may be reasonable to calculate the RNA-content from the RNA-phosphorus content of mycelium, in the same way as in yeast RNA. The RNA-phosphorus (RNA-P) of mycelium after 8 days in medium T is 0.073 μmol-P/mg dry cell. The value, 0.073 μmol corresponds to 2.26 μg of phosphorus. 2.26 μg is multiplied by 1/0.095 (0.095 is the mean value of RNA-P/yeast RNA) and then the amount of the RNA is calculated as 23.8 μg/mg dry cell (=2.38%). This value resembles to those of *Saccharomyces* sp., *Penicillium* sp., *Aspergillus* sp. described in a paper of Kuroiwa and Horie[14].

The major components of nucleotides both in mycelium and in culture broth were AMP and UMP. CMP and GMP were found in many cases, but in a little amount. The distribution pattern of nucleotide may belong under the plant type as proposed by

Shimazono[15].

Comparing the total amount of mononucleotides of the culture broth with that of the mycelium, it was fiound that the mononucleotide in mycelium was one fifth to one tenth of that in cell-free medium, both in cases of T and U medium. The total amount of free mononucleotides was no more than one twentieth of the amount of mycelium RNA. As like as our observation[6] in fruiting body of *Lentinus edodes* and *Psalliota bisporus*, the free mononucleotides were contained in a slight amount compared with its own RNA, in the case that the mycelium was grown in a satisfactory condition.

Nakao et al[16] reported the formation of 3'-and/or 5'-nucleotides by the degradation of endogenous RNA in yeasts. The pattern of nucleotides formed was differed in species and also in the pH of medium used. It is reasonable to consider that the enzymes which degrades nucleic acid may attack the mycelium RNA in a certain optimal pH range, after the time attained at the maximum growth. From the point, one culture was carried out under the pH range below 5 (in medium T), and the other around 7 (in medium U). As a result, the excretion of 260mμ absorbing substances and of nucleotides was larger in medium T than in medium U. The fact may be interpreted by the action of nucleic acid degrading enzyme in a region of acid side. The enzymes which decompose yeast RNA in acid side were found in fruiting body of *Lentinus edodes*[17] and *Psalliota bisporus*.[18] The crude enzymes from the mycelium of *Collybia velutipes* grown in static culture[19] degraded its own RNA.

For the purpose of application of grown mycelium to foodstuffs, we are now carrying on some experiments referring to the formation of 5'-GMP and other nucleotides by the degradation of mycelial RNA with its own enzymes. The results will be published in a successive paper.

要　　旨

シイタケ，シメジなどきのこ類には 5'-グアニル酸（5'-GMP）が見出されるが，これは旨味の強い呈味成分である．きのこ類の液内培養は菌糸体を食用，飼料用に利用する目的で開発されているが，この菌糸体や培養液に 5'-GMP などヌクレオチド類が蓄積されるか否かは興味深いことである．

本報では食用きのことえのきたけの菌糸体を液内振盪培養したときの 5'- ヌクレオチドなど核酸成分の変化をしらべた．

窒素源として酒石酸アンモンあるいは尿素が使用されたが，前者では pH が 3.0 迄低下し，後者では pH は 7.0 付近を上下した．はじめ培地成分として加えられた無機燐酸塩の大部分が菌糸体内に取り込まれ，菌糸体内の総燐の 15% 以上が RNA-燐で占められた．菌糸体の生育が止った後にそのRNAが分解され，ヌクレオチドが培地内に分泌された．全燐に対するRNAの比率は菌糸体の生育相に相関していた．菌糸体及び培地内の主要なヌクレオチドはAMPとUMPであった．

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