Studies on the Highly-phosphorylated Nucleotides during the Differentiation of *Aspergillus niger*

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검정곰팡이의 分化에 따르는 菌體內의
高燐酸뉴크레오티드의 消長에 관한 硏究

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Abstract: Highly phosphorylated nucleotides were investigated to assure whether the eucaryotic *Aspergillus niger* produce these substances or not during the differentation. Investigation was extended to see how organic phosphate interacts with inorganic polyphosphate during development, and high molecular weight RNA-polyphosphate complex was detected in 2.6% polyacrylamide gel by electrophoresis.

Guanosine tetraphosphate was found in vesicle and phialide forming mycelia and spore forming body by PEI cellulose TLC. It is revealed that guanosine tetraphosphate is a common substance for spore formation in eucaryotic microorganisms as well as in procaryotic.

Especially, prior to sporulation, protein bound RNA and protein bound phosphate may occur as a result of reorganization of cellular materials. The evidence was obtained by the fact of differential increase of optical density ratio between the samples from different developmental stages of this fungus.

In 2.6% polyacrylamide gel which was run to electrophoresis, high molecular weight RNA (mostly rRNA) was found to couple and to make RNA-polyphosphate complex. The complex was examined with enzymes and radioactive isotope of ^{32}P. (enzymic test was not reported here.) RNA-polyphosphate complex might be another sort of highly phosphorylated nucleotide or rRNA beside guanosine-tetraphosphate.

Introduction

Since the fruiting process is triggered by starvation, regulatory mechanisms known to respond to starvation may function in the control of fruiting. One such mechanism is mediated by increased levels of the guanosine polyphosphates, guanosine tetraphosphate (GP4) and guanosine pentaphosphate (GP5), which accumulate in response to starvation for amino acids, carbon energy sources, and other external nutrients (Cashel, M., 1975). Guanosine polyphosphates appear to mediate the "stringent" response, a broad readjustment of metabolism that includes inhibition of ribosome, peptidoglycan, and phospholipid by synthesis (Cashel, M., 1975).

Cultures of *Myxococcus xanthus* develop multicellular fruiting bodies when starved for carbon and

nitrogen sources on agar surface. Under these conditions of severe starvation, cultures rapidly accumulated a compound identified as guanosine tetraphosphate. The accumulation of guanosine tetraphosphate was reduced in the presence of tetracycline. The guanosine tetraphosphate level was also reduced in starved cultures of a mutant unable to fruit normally, although it has not been determined whether the defect in guanosine tetraphosphate accumulation is responsible for the inability to fruit (Manoil and Kaiser, 1980).

Development of multicellular fruiting bodies of *Myxococcus xanthus* can be induced by limitation of any of a number of different classes of amino acids. Gradual starvation for an essential amino acid was required for the induction of fruiting. Guanosine polyphosphate accumulation is shown to be correlated with nutritional conditions 'that induce fruiting, and therefore may serve as intracellular signal to trigger cells to end vegetative growth and initiate fruiting body development (Manoil and Kaiser, 1980).

Escherichia coli and other bacteria accumulate guanosine polyphosphate (GP4 and GP5) in response to starvation for amino acid (Cashel, M., 1969). *Caulobacter crescentus* accumulated guanosine tetra-phosphate in response to nitrogen starvation but not in response to amino acid starvation (Chiaverotti *et al.*, 1981).

In all cases with *Bacillus subtilis*, sporulation was correlated with a significant decrease of GTP, and increase of ppG pp and pppGpp (Lopez, Dromerick, and Freese, 1981).

The stringent response, usually measured by an immediate cessation of RNA accumulation, result from an increased ratio of uncharged to charged tRNA and is correlated with an increase in the concentration of the highly phosphorylated guanosine nucleotides ppGpp and pppGpp (Lopez *et al.*, 1981).

Guanosine5' diphosphate-3' diphosphate (ppGpp) controls a remarkably broad range of processes in transcription, translation, and metabolism in *E. coli* (Cashel, M, 1975). The possible occurence of this regulatory nucleotide in eucaryotic cells has

therefore received considerable attention. The results of these nucleotide hunting expeditions have been generally negative. Evidence of ppGpp in *Saccharomyces cerevisiae* subjected to heat shock was presented by Pao, Paietta, and Gallant in 1971, and they suggested that it is produced on the mitochondrial ribosome. The aim of the present study is to investigate the presence of such highly phosphorylated nucleotides in the spore forming mycelia of *Aspergillus niger* of eucaryotic.

Materials and Methods

Strains and medium. *Aspergillus niger* van Tiegham (IMI 41873) was used in this study. The synthetic media of four replacement cultures for shaking incubation and submerged culture were those of Andersons and Smith's formula (1971) desinged for synchronous initiation and sporulation of *Aspergillus niger*. With these four media, conidiophore maturation and sporulation was gained as the synchronously differentiating state in the jar fermenter. The details of the formula of media are described by Anderson & Smith (1971), and Kim, J.H. (1980).

Germination medium (A-medium) from the spores is composed of basal minerals $(NH_4)_2SO_4$ 3. 968g, glucose 20g, distilled water 1L. initial pH 2.3. Basal minerals of KH_2PO_4 1g, $MgSO_47H_2O$ 0. 25g, $CuSO_4$ $8H_2O$ 0. 234mg, $FeSO_47H_2O$ 6. 32mg, $ZnSO_47H_2O$ 1. 1mg, $MnCl_2$ 46. 7mg were added in order into 1L of distilled water. A-medium is for shaking culture to support germination of the spore within 48 hours.

Low-nitrogen medium (B-medium) contains basal minerals (same as above), $(NH_4)_2SO_4$ 0. 66g, glucose 10g, antifoam agent 1ml, and distilled water 1L. Initial pH is 4.6. This B-medium allow submerged culture and the growth of foot cell and conidiophore in the jar-fermenter.

Citrate-ammonium medium (C-medium) is prepared with basal minerals (same as above), $(NH_4)_2SO_4$ 1. 98g, citric acid 12. 6g, antifoam agent 1ml, and distilled water 1*l*. Initial pH was adjusted to 4. 6.

C-medium can support conidiophore to produce

vesicle and phialides in submerged cultture with jar-fermenter.

Nitrate-glucose medium (D-medium) is made up with basal minerals except KH_2PO_4, KH_2PO_4 13g, K_2HPO_4 1.13g, $NaNO_3$ 5.1g, glucose 20g, antifoam agent 1ml, and distilled water 1l, by adjusting pH to 5.5 at first. In D-medium this fungus formed spores in submerged culture with jar-fermenter.

Determination of total phosphate, inorganic phosphate, and polyphosphate levels. Harvested mycelia at developmental stages were washed and the lipids were extracted with acetone twice. Residues were treated with 0.5N cold PCA (perchloric acid) at 4°C for 14minutes twice (Harold, 1960). Total phosphate level of cold PCA soluble fraction was determined by the method of Fiske and Subbarow's (1925) with the spectrophotometer. Inorganic phosphate level of it was assayed by the method of Martin-Dorty's (1949) employing spectrophotometer.

The level of the organic phosphate (cold PCA soluble fraction) was determined by its UV absorption with DU-2 Spectrophotometer at 260nm. The level of inorganic polyphosphate was determined as follows.

Inorg. poly p. = Total phosphate − (Inorg. p. + Org. p.)

Hot PCA (0.5N) soluble phosphate fractions were also collected twice, and then assayed by the same methods.

Determination of total RNA. Crude mycelial RNA was extracted by the method of Crestfield (1955), and the levels in the developmental stages were assayed with ultra-violet absorption spectrometry by contrasting with orcinol reagent method. The ribonucleate is extracted from washed mycelia by short heating with an aqueous solution of sodium dodecyl sulfate the crude ribonucleate is precipitated by alcohol (Crestfield, 1955).

Extraction of phosphorylated nucleotide from the differentiating mycelia. The mycelia of conidiophore maturation stage and of spore forming body were treated separately with an equal volumes of 2M formic acid. Potter type homogenizer was employed for the extraction. After incubating in an

ice bath for at least 15 min., the suspension was transferred to a centrifugal tube, and the mycelia were sedimented 1 min., at 12,000×g. The cell free supernatants were spotted directly on polyethyleneimine cellulose chromatograms of were frozen and chromatographed later (Cashel, 1968).

Thin layer chromatography of the nucleotides. Randerath (1964) and Cashel's (1968) methods were basically adopted. Polyethyleneiminecellulose thin layer sheets were obtained commercially (Merck, West Germany, PEI cellulose F) and prior to use were soaked in distilled water for at least 20min, then dried at room temperature with a fan.

For two-dimensional chromatography, 2M formic acid-1.5M, LiCl (1 : 1) solvent was used for the first dimension, and 1.5M KH_2PO_4 (PH 3.5) solution was for the second dimensional development. Chromatograms were developed in the first dimension until the solvent front reached the end of the sheet; then sheets were dried and washed for 15 min. with occasional agitation in absolute methanol. Each chromatogram was dried and then developed in the second dimension, again to the end of the sheet. The chromatograms were again allowed to dry. The positions of standard nucleotides, which had been obtained from Sigma Co., U.S.A., were determined using short-wavelength UV light (Mineralight UV lamp, San Gabriel, Calf. U.S.A.).

Preparation of RNA. Kirby's (1965) method was applied. Mycelia which was grown in low-nitrogen (B-medium), citrate (C-medium), and nitrate glucose (C-medium) were washed with chilled distilled water, and then were homogenized in the glinding solution which was composed of 1M. tris 0.3M NaCl, 1N HCL, pure phenol, tri-isopropyl-naphthalen sulphonic acid, and para-amino salicylic acid. After glinding it was deproteinized by extracting three times with phenol solution which was a mixture of phenol, m-cresol, 8-hydroxyquinoline, and distilled water. Finaly sodium acetate (0.5M) and absolute ethyl alcohol were added to precipitate ribonucleic acid. Precipitated RNA was spun down and washed with 80% ethyl alcohol, final precipitate was usually dissolved in running buffer for acryl-

amide gel electrophoresis.

Fractionation of high molecular weight RNA (mostly ribosomal RNA) by polyacrylamide gel electrophoresis. Loening's method (1967) has been basically applied. Gel concentration was 2.6%, which was prepared with acrylamide (Eastman-Kodak, Organic Chemicals), bis-acrylamide, tetramethylethylene-diamine (TEMED), ammonium persulphate, and tris (Sigma Co.,) buffer solution. This buffer was made up with tris, sodium phosphate, ECTA sodium salt, and water to adjust pH 7.8. Perspex tube ($\frac{1}{4}$ in. internal dia. ×5in. long) with plastic ring was used. The current of 60 volts and 30 mA. per 6 tubes was employed for up to 1 hour to remove the imputities. The RNA sample (from 20 to 200 μg. dissolved in from 10 to 100 μl. of the buffer containing 5% (W/V) of sucrose) was then layered over the gel top and electrophoresis was taken for 0.5~3 hours.

Scanning UV absorption and radioactivity of the electrophoresis gel. The gels were scanned with Joyce-Loebl Scanner U.K. at 265 nm. The absorption of UV ray was recorded by the instrument. The radioactivity of ^{32}P in the gel after electrophoresis was detected with the aid of solid scintillation counter (Panax Scintillation counter GSA 3-Panax Equipment Ltd. England). Before counting gels were sliced to 1mm thick, and then those were dried on adhesive labels and were stuck to counting planchets.

Incorporation of ^{32}P tracer into fungus. ^{32}P isotope tracer (Radiochemical Centre, Amersham, England) which specific activity was 1 mCi 1ml as orthophosphate form in HCl solution contained in the vial. The tracer was added into 50ml. of culture in the flask which was being differentiated, after then the culture broth was shaken for 3 hours to obtain uniformly labelled RNA. The extraction of RNA from the mycelia and method of gel electrop-horesis are described above.

Results

Levels of total phosphate in each germinating,

(stage I) conidiophore elongating (stage II), vesicle and phialide forming (stage III) and spore forming body (stage IV) through replacement cultures were shown in Figure 1. The amounts of nucleotide and organic phosphate which are acid soluble increased at the time of conidiophore maturation, vesicle and phia-lides forming, and sporulation stages, whereas levels of inorganic poly-phosphate fell down with inverse ratio. The levels of total phosphate dropped at the end of hyphal growth, and then the level did not change until sporulation occurs. The inorganic phosphate was kept constant in its initial level
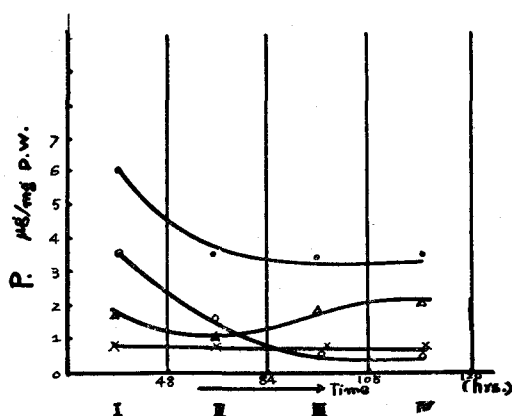

Fig. 1. Levels of various type of phosphates from differentiating *Aspergillus niger* by synch-ronous culture. ●···Total P., ×···Inorganic P., and acid labile P., △···Organic P., (nucleotides) ○···Inorganic poly P.
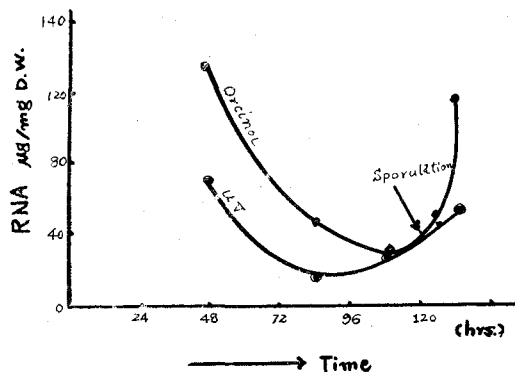

Fig. 2. Levels of extracted RNA from differentiating *Aspergillus niger* by synchronous culture. ●··········Orcinol reaction ◉··········260nm UV absorption

Table I. Ratio of optical density(280/260nm) among cold PCA soluble fractions, and those among RNA extracts from *Asp. niger* at different developting stages.

Developmental stages	Cold PCA sol. fractions	RNA extracts*
Stage I	0.55	0.53
Stage II	0.62	0.58
Stage III	0.82	0.60
Stage IV	0.55	0.52

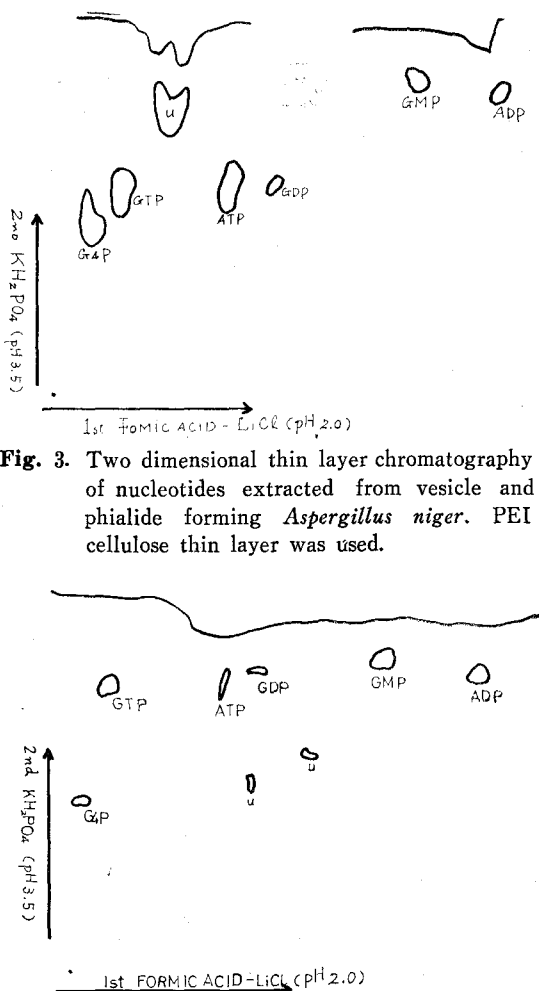* Yeast RNA (Sigma Co.) was used as a reference reagent.

Fig. 3. Two dimensional thin layer chromatography of nucleotides extracted from vesicle and phialide forming *Aspergillus niger*. PEI cellulose thin layer was used.

Fig. 4. Two dimensional thin layer chromatography of nucleotides extracted from spore forming *Asp. niger*. PEI cellulose thin layer was used.
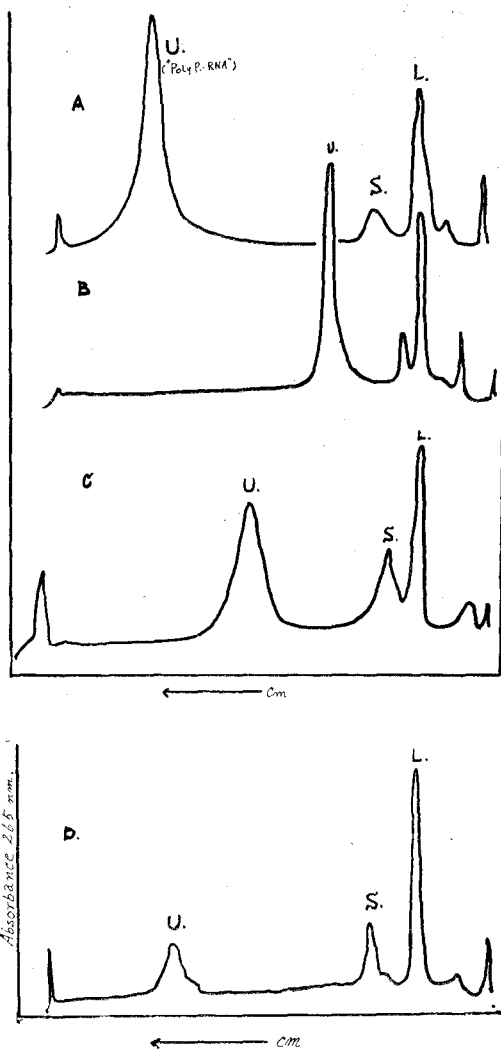

Fig. 5. Electrophoretic profiles of high molecular weight RNA in 2.6% polyacrylamide gel.

A. 107μg of RNA sample from hyphae of *Aspergillus niger* was run for 2 hours.

B. 85μg of RNA sample from grown hyphae of *Aspergillus niger* was run for 1.5 hours.

C. 85μg of RNA sample from conidiophore forming mycelia of *Aspergillus niger* was run for 1.5 hours.

D. 30μg of RNA sample from spore forming body of *Aspergillus niger* was run for 2 hours.

※ L: Large subunit r-RNA, S: Small subunit r-RNA

U: Unknown substance, probably polyphosphorylated RNA

through the whole stages of development.

The transition of ribonucleic acid level through developmental stages were shown in Figure 2. The levels of RNA dropped according to the course of growth and conidiophore maturation, althouth it has increased at the threshold of sporulation. RNA level calculated by orcinol reaction and spectrophotometry was more higher than those of UV absorbance.

Detection of phosphorylated nucleotides were arranged by thin layer chromatography after acid extraction of *Aspergillus niger* cultures. Standard nucleotides (Sigma Co., products) had been developed on PEI cellulose plate as preliminary test to determine the R*f* value on chromatogram. Two dimensional thin layer chromatography of nucleotides and phosphorylated nucleotides are shown in Figures 3 and 4.

Nucleotides extracted from vesicles and phialides producting mycelia are shown on figure 3, those from spore forming body are in Figure 4.

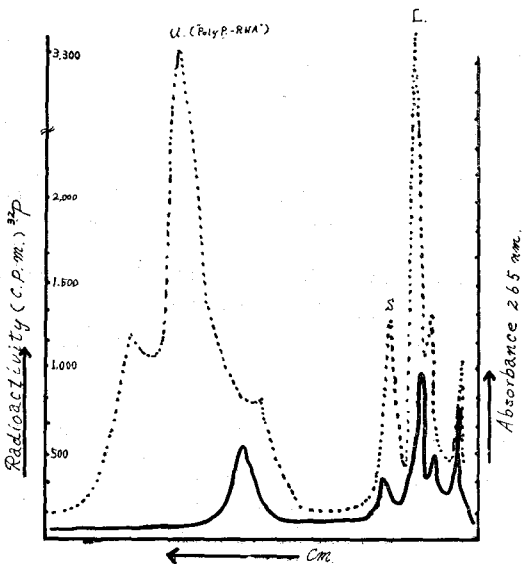**Electrophoresis of ribosomal ribonucleic acid.**

Fig. 6. Electrophoretic profile of high molecular weight RNA in 2.6% polyacrylamide gel. Radioactive tracer of ^{32}P was incorporated into the culture for 3 hours. UV (265nm) absorption was scanned together.

Ribosomal ribonucleic acid and phosphorylated RNA fractions obtained by polyacrylamide gel electrophoresis were well shown in Figurs 5 and 6. Evidence that "poly p.-RNA" band might be phosphorylated RNA was supported by the use of radioactive phosphorus (^{32}P) for the culture, ultra-violet ray absorption with UV gel scanner (Joyce-Loeble Co. U.K.) for unstained gel, and application of various enzymes-protease, alpha-amylase, ribonuclease for the gel to identify the substrate. Details of enzymic tests are not reported in this paper.

Table II. Relative amount of labelled phosphorous in rRNA during the vegetative stage of growth.

Units	Radioactivity (c.p.m.)	Optical density (265nm absorbance)
Amount of total RNA	115,750	
Large unit r-RNA(L)	4,190	1.46(cm²)
Small unit r-RNA (S)	2,171	0.32
Unknown band (U)	30,060	2.02

Table III. Relative abundance of ribosomal r-RNA at different stages of differentiation.

Unit or Band	Initial vegetative growth stage	Conidiophore forming stage	Vesicle and Phialide stage	Sporulation stage
Large unit r-RNA (L)	1.6(cm²)	3.06(cm²)	3.95(cm²)	2.66(cm²)
Small unit r-RNA (S)	0.4	1.3	1.32	0.68
Unknown substance band (U)	4.7	4.8	not detected	1.58

Discussion

The first morphological event of conidiation, foot cell formation (stage I), is induced by growth in a medium in which N is the limiting nutrient (LN medium). Conidiophore elogation (stage II) occurs in LN medium after the exhaustion of exogenous N.

Replacement of the culture to a new medium containing a N source and a TCA cycle intermediate

(citrate) as the carbon source induced vesicle and phialides formation (stage III). Conidia formation (stage IV) is most effectively induced by transfer into a medium with glucose as the C source and nitrate as the N source. Thus by means of nutrient replacement in the controlled environment of the fermenter it has been possible to follow the synchronous maturation of conidiophores (Anderson and Smith. 1971) (Figure 7).
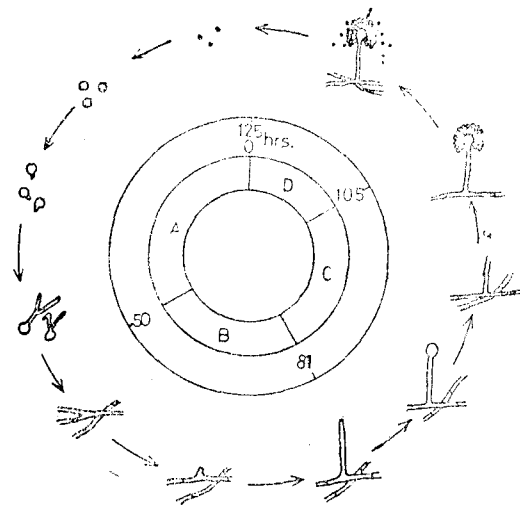

Fig. 7. Differentiation of *Aspergillus niger* throughout life cycle.

    A: Shaking culture medium
    B: Low nitrogen medium
    C: Citrate-ammounium medium
    D: Nitrate-glucose medium

During the conidiophore maturation and vesicle formation, the levels of organic phosphate, in which nucleotide and nucleic acid are involved, increased rapidly until sporulation begun to takae place (as shown in Figure 1). Reduction of the level of inorganic polyphosphate is considered as it had been consumed for the synthesis of organic P.

Level of total RNA extracted from mycelia increased at the onset of sporulation is rather than those of UV (260nm) absorption. It may have so much pentose polymer, it might be associated with cell wall component as a RNA-pentose polymer complex.

Optical density ratios of 280nm per 260nm wave-

length in cold PCA soluble phosphate fraction RNA extract from vesicle and phialides bearing mycelia were a little bit higher than those from other mycelia.

It can be interpreted that presporulating mycelia may reorganize cellular component, just before sporulation. Protein might combine with phosphate or RNA to make a kind of phospho-protein complex or ribonucleo-protein as the stringent response.

Thin layer chromatogram of phosphorylated nucleotides are shown in figure 3 and 4. Thus Guanosine-tetraphosphate can be detected in vesicle and phialide forming mycelia and spore forming mycelia. The presence of this compound in the fruiting body of the procaryote has been reported by Manoil (1980 a, b), Maeba and Shipman (1978), Lazzarini and Cashel (1971), Lopez, Dromerick, and Freese (1981), Chiaverotti *et al.* (1981), and in yeast by Pao, Paietta, and Gallant (1977).

Guanosine-tetraphosphate in *Aspergillus niger* was well detected with PEI cellulose TLC in this study.

From the polyacrylamide gel electrophoresis, electrophoretic profiles of the high molecular weight ribonucleic acid (mostly rRNA) were obtained (figure 5 and 6). In this experiment, r RNA was separated into two bands such as *L* and *S*, although a large band ("poly P.-RNA"), which has radioactivity of ^{32}P and UV absorption (265nm) appeared in every gel. The band occurs throughout whole life cycle from foot cell stage to sporulation stage. Alpha-amylase, protease, and ribonuclease were treated if it could be removed or digested away. However, it was not affected by these enzymes. Therefore, it was concluded that the band might be polyphosphate-RNA complex. The size of band at different developmental stages varied and was becoming smaller, at last that of spore forming stage reduced to a quarter of initial band of vegetative growth stage in size. It could be considered that this polyphosphate-RNA complex might be consisted of various kinds of polyphosphorylated nucleotide such as that of adenosine and guanosine. However, the characteristics of the substance in the band from conidiation stage were not yet detected.

Sequential mobilization of polyphosphorylated RNA for stringent response as energy source or shift-down initiator may occur according to the development (Cashel, 1975).

Acknowledgment

要 約

검정곰팡이(*Aspergillus niger*)의 胞子形成을 液浸培養法과 同調的方法으로 施行하면서 燐酸化合物, 高燐酸化뉴크레오티드, 및 RNA(核酸)의 動態를 研究하였다. 結果는 다음과 같이 要約된다.

1. 곰팡이의 胞子形成時에 有機燐酸化合物의 量이 增加하였으며, 無機 polyphosphate의 量은 反對로 減少하였다.

2. 無機燐酸量은 變動하지 않았으며, 總燐酸化合物의 量은 初期菌絲生長時에는 減少하고 胞子囊柄時期부터는 變動하지 않았다.

3. RNA核酸區劃에 있어서 orcinol 反應陽性區의 吸光度가 急激히 增加하였다. 그러나 UV吸光度는 완만하게 增加하였다.

4. 胞子囊柄및 胞子形成時期에 guanosinetetraphosphate가 檢出現되었다.

5. 高分子量性 RNA (rRNA 分劃)의 polyacrylamide gel 電氣泳動을 2.6% gel로서 施行한 바 polyphosphate와 RNA의 結合物이 相當量 存在함을 알았다.

以上의 結果로 보아 검정곰팡이의 分化過程에 있어서 高燐酸化 RNA 化合物의 存在가 確認되었으며, 이 化合物 가운데 guanosine tetraphosphate (G4P)의 存在가 眞核微生物인 곰팡이에서도 檢出되었음을 指摘한다.

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