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Polyacrylamide Gel Electrophoresis of the Cell Proteins from Differentiating *Aspergillus niger*

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검정곰팡이의 分化에 있어서 폴리아크릴아마이드 겔 電氣泳動法에 의한 단백질 패턴 硏究

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Abstract: *Aspergillus niger* van Tieghem was cultured by the method of submerged and synchronized culture for the study of differentiation. Acid-phenol soluble cell proteins of the fungus were extracted from four stages during development. Those acid-phenol soluble proteins were separated by polyacrylamide gel electrophoresis to determine the protein patterns.

A new protein band was observed from the pre-sporulation body, color density of the stained protein bands in four tubes differed according to the differentiation stages.

The number of protein bands in 10% gels varied from 18 to 16, 17, and 19 according to the course of spore germination stage, conidiophore stage, phialide maturation stage and sporulation stage.

The genetic and biochemical mechanisms which are directly involved in the regulation of differential gene expression and the biosynthesis of informational macromolecules are central to the problem of differentiation(Smith and Berry, 1974). The biochemical basis of differentiation lies in the production of specific proteins. The pattern of proteins in differentiated cells may be varied by an alteration in their rate of synthesis. In the case of enzymes, altered activity likewise leads to variation in cellular function. It is not always easy, when considering the proteins of differentiated cells, to distinguish between altered synthesis and altered activity of enzymes. Altered activity is itself generally a consequence of altered synthesis of particular subunits(Pasternak, 1970).

Differentiation of *Dicyostelium discoideum* is accompanied by the synthesis of specific enzymes, such as UDP-glucose pyrophosphorylase, UDP-galactose transferase and trehalose-6-phosphate synthetase. They appear at discrete times after commitment to aggregation(Sussman, 1967).

Numerous enzymes including TCA cycle related enzymes of cyclophorases, phosphorus metabolism related enzymes, and proteinases have been studied by many investigators (Doi *et al.*, 1969).

Protein synthesis is required for meiosis and spore formation. Cycloheximide inhibits the sporulation process up to the stage where mature ascospores in yeast are observed.

Incorporation of C^{14} amino acids into hot TCA precipitable material has shown that two periods of maximum proetin synthesis occur during sporulation of yeast(Esposito, 1969). Since proteins synthesized during sporulation of yeast have not been characte-

rized, there is no evidence at present for sporulation-specific proteins. On the other hand, ascospores contain an immunologically distinct antigen absent in vegetative cells, which has been tentatively identified as a protein structure component(Snider and Miller, 1966).

There is little information available on the profiles of specific proteins in sporulating *Aspergillus.*

The electrophoretic patterns of cell proteins in polyacrylamide gels were used for the study of several taxonomic problems in the fungi(Razin, 1968: Chessen, Morgan and Codner 1978).

Schnaitman(1970) examined the protein composition of the cell envelope of *Escherichia coli* by polyacrylamide gel electrophoresis. Electrophoresis on sodium dodecyl sulfate-containing gels yielded from 20 to 30 well-resolved bands of protein.

The proteins of *Mycoplasma* cells of various species produce highly reproducible and species-specific electrophoretic patterns in polyacrylamide gels containing 5M urea and 35% acetic acid. These electrophoretic patterns can be used for the rapid identification and classification of *Mycoplasma*(Razin and Rottem, 1967).

Comparative electrophoretic study of proteins of *Acremonium* like *Hyphomycetes* with use of phenol-acetic acid-water(2 : 1 : 0.5 w/v/v) was reported by Chesson, Morgan and Codner(1978). The acid-phenol protein patterns were proved to be characteristic for total protein.

Polyacrylamide gel electrophoresis of soluble protein extracts stained for both protein and specific enzyme activity has been applied to fungal taxonomy (Chang, Srb, and Steward, 1962). Gels stained for enzyme activity also have been of value although the greater variation in the patterns produced indicate that their value may be below species level (Hall, 1969). While electrophoresis in the fungi has been exclusively concerned with the separation of soluble proteins, electrophoresis of solubilized water insoluble proteins have proved of value in bacterial taxonomy.

Since ontogeny is a shrinked diagram of evolution, so the phase specificity in the developing body may exhibit specific protein patterns which characterize the differentiation.

If the differentiation occurs in the defined environment of culture and in the synchronized phase, the specific protein pattern may be detectable.

The aims of the present study is to investigate whether specific protein be synthesized during the differentiation of this fungus. In this context, acid-phenol extractable proteins were investigated.

Materials and Methods

Aspergillus niger van Tieghem (IMI 41873) was used in these experiments. Stock cultures were maintained on potato-glucose agar slopes in the culture tube at 27°C. The basal mineral(M) medium contained in 1*l* distilled water: KH_2PO_4 1.8g, $MgSO_4$ $7H_2O$ 0.25g, $CuSO_4 \cdot 8H_2O$ 0.234mg, $FeSO_4 \cdot 7H_2O$ 6.32mg, $ZnSO_4 \cdot 7H_2O$ 1.1mg, $MnCl_2 \cdot 4H_2O$ 3.5mg, $CaCl_2$ 46.7mg.

Four liquid synthetic media were used as replacement culture media(Anderson and Smith, 1971). Medium A is a shake culture medium for the germination of spores. The constituents of its are as following. Basal mineral(M) medium: $(NH_4)_2SO_4$ 3.96g, glucose 20.0g, distilled water 1*l*; pH was adjusted to 2.2. Medium B contains basal mineral(M) medium: $(NH_4)_2SO_4$ 0.66g, glucose 10.0g, polypropyleneglycol P 200 1.0ml, distilled water 1*l*; pH was adjused to 4.6. Medium C contains basal minerals (M) medium: $(NH_4)_2SO_4$ 1.98g, citric acid 12.6g, polypropyleneglycol P 200 1.0ml, distilled water 1*l*; pH was adjusted at first 4.6. Medium D contains basal minerals except KH_2PO_4: KH_2PO_4 13.0g, K_2HPO_4 1.13g, $NaNO_3$ 5.1g, glucose 20g, polypropyleneglycol P 200 1ml, distilled water 1*l*; pH was adjusted to 5.5 at first.

The homogenate was left for 24 hours at 30°C, after then it was spun 40 minutes with the centrifuge at 18,800rpm(43,544g) by cooling at 4°C. The supernatant (the acid-phenol extract) was stored at −20°C(Chessen A, Morgan J.J. and Codner R.C. 1978)

The assay of total protein was performed by the method of Lowry using Folin-phenol reagent. The

above acid-phenol soluble protein was assayed by the method of Bramhall *et al.* (1969).

The filter papers of 2×2cm squares, in which 50~200μg protein were spotted, and dried in warm air, and then these were put into 2ml of cold 7.5% trichloroacetic acid solution to fix the proteins. The acid solution was then heated and maintained at 80°C for half an hour to hydrolyze and remove nonproteinaceous material. The acid solution was then discarded; the papers washed with ether/ethanol(1/1, v/v) and then ether was applied to remove the trichloroacetic acid and lipids and to dry the papers. Samples containing protein were stained with 2ml of naphthalene blue black solution(10mg/ml) in acetic acid/methanol/water; 1/4/5, v/v/v at 50°C for 15 minutes.

Medium A was used for germination of the spores in the shaker for 48 hours. in which the spores became young hyphae. Medium B was used for vegetative growth in the jar fermenter for 36 hours varying the aeration from 0.33*l* to 0.51*l* against 1*l* volume of the medium. Medium C was used for conidiophore maturation for 24 hours, and then the washed mycelia were transfered to Medium D for sporulation in the jar fermenter, culture period 20 hours. The time table of replacement culture was slightly varied according to the progress in differentiation. All submerged cultures were held at 30°C. Fermenter culture was held in 1*l* jar, aeration volume was 0.5*l* per minutes except in Medium B in which first 12 hours aeration was 0.33*l*. Agitation rate was kept to 600 rev/min throughout the culture.

Mycelia or spore bearing body from each developmental stage were washed with distilled water three times after harvest, and then these were ground for 15 minutes at 3,000 rpm in a glass homogenizer which stood in an ice bed. Ground mixture consisted of 1.5ml of 4% NaCl solution, 100mg of aluminum oxide, and 3ml of acid-phenol solution(acid: phenol: water=1:2:0.5).

The papers were rinsed with hot 7% acetic acid several times to remove excess dye. The liquid was dsicarded and the papers put into individual tubes. Five ml of 0.05N NaOH were added to each tube and the dye released was measured at 620nm in spectrophotometer. Bovin serum albumin(Sigma Co.) was used as the standard, and was treated by the same process.

Acid-phenol soluble(APS) proteins containing 200~250g/100μl were layered on the top of 10% polyacrylamide gel in the tube (Razin and Rottem, 1967). The buffer tanks were filled with 10% acetic acid and the upper tank was connected to the cathode of power supply. Those tanks were kept at 4°C to prevent from warming. 5mA current per tube was applied for each 150, 180, 210 minutes. Dichloromethylsilane was coated inside of the tube as a lubricant.

Amidoblack dye solution(0.3%) was applied for staining the protein bands in the gels. The stained bands were washed with running water, and then destaining solution(acetic acid/methanol/distilled water; 70ml/50ml/880ml.) was employed until clear resolution appeared.

The catalase, alpha-amylase, alcohol dehydrogenase, ribonuclease, and trypsin that are Sigma products were used as the standard reference molecules. And these were dissolved in acidic phenol saline solution (acetic acid/phenol/water/saline water; 2:4:1:3.5) in order to match the acid-phenol solution which is extracting solution. The methylgreen dye was used as a marker. Electrophoretic mobility of the protein (Ef value) was calculated as

$$Ef = \frac{\text{distance of protein migration}}{\text{length after destaining}} \times \frac{\text{length before staining}}{\text{distance of dye migration}}$$

The mobilities were plotted against the known molecular weights expressed on a semi-logarithmic scale (Weber and Osborn, 1969).

Results

Differentiation of *Aspergillns niger* including germination, vegetative growth, and sporulation synchronously occurred through replacement cultures in the jar fementer as Anderson and Smith had described(1971). Plates I, II, III, IV and V show the sequencial development of the fungus.

According to the differentiation the acid-phenol soluble protein bands differed each other in their numbers and mobilities(Fig. 1, Pl. VI and VII).

In vegetative growth stage and conidiophore stage, 16 and 17 bands were observed, although 19 bands were checked from the sporulating body. Those APS bands were separated in 10% acrylamide gels and 3 hour's electrophoresis with 5mA/gel current.

The fourth band of A tube which involve APS protein of vegetative hyphae disappeared at the same loci in B and C tubes, but it was appeared again in D tube which contains APS protein of sporulating body. The molecular weight of the fourth band was revealed as 48,000 by comparison of mobility of standard proteins.

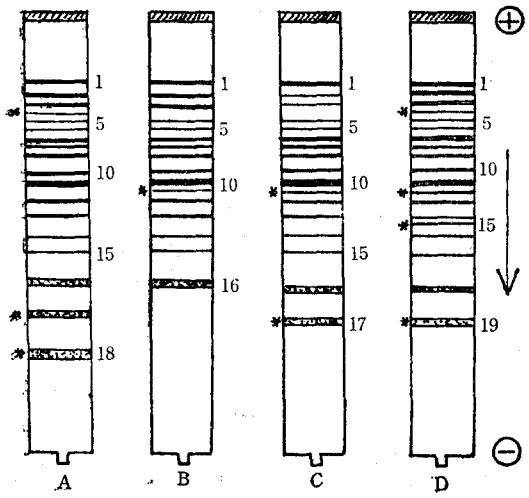

Fig. 1. Schematic drawing of acid-phenol soluble protein of *Aspergillns niger* polyacrylamide gel electrophoresis during the differentiation. A: Vegetative stage, B: conidiophore stage, C: Vesicle and phialide stage, D: Sporulation stage, Running condition; 10% gel, 3 hours, 4°C, 10% acetic acid buffer solution.

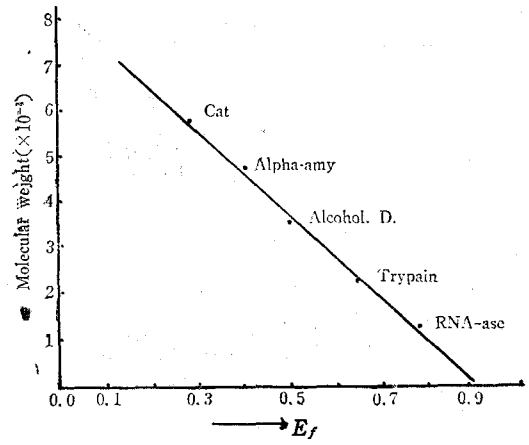

Fig. 2. Relative curve of molecular weight against their mobilities(E*f*) on polyacrylamide gel electrophoresis.(10% gel, 3 hours, acetic acid buffer solution).

The molecular weight of eleventh and tweleveth bands in B, C and D tubes were about 34,000. The fifteenth band seemed like a newly occurred protein band, its molecular weight was revealed as 27,000. The seventeenth and eighteenth bands were estimated 10,000 and 3,000 in molecular weight.

Relative curves of molecular weight of standard proteins against mobilities are shown in Figure 2 and Plate II. Density of color in amido black stained bands differed each other. Seventh, eighth, nineth, tenth and eleventh band in all gel tubes were distinct and darker, especially eleventh band was larger and thicker.

Discussion

In the sporulation of bacteria, some enzymes and proteins in general have altered properties compatible with heat and radiation resistance(Pasternak, 1970). Some enzymes that are correlated with differentiation of microorganisms are generally functional protein but not structural one.

The electrophoretic patterns of cell proteins in polyacrylamide gels were used for the study of several taxonomic problems in *Mycoplasmatales* by Razin (1968). As a taxonomical criterion, acid-phenol electrophoresis of whole cells solubilized with phenolacetic acid water provided characteristic total protein patterns(Chesson, Morgan and Codner, 1978).

The results of the present investigation are concerned with the protein changes during the differentiation through the replacement cultures.

In this experiment, 10% gel of polyacrylamide was employed, because of reproducibility, and therefore relatively smaller molecules of the proteins were retained in the gel. Larger size molecules of the proteins were hoped to be elucidated. The phase specificity, which was expressed as the difference of size in the macromolecules or the proteins patterns in the tissue or organ of the body, may be identified by the gel

electrophoresis method, and this specificity might define the differential gene actions in the differentiation.

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要 約

검정곰팡이(*Aspergillns niger*)의 同調的 分化를 施行하면서 acid-phenol溶性인 細胞蛋白質을 抽出하여 胞子形成 前後의 蛋白像의 變化를 polyacrylamide gel 電氣泳動法으로 追究하였다. 結果에서 1種의 蛋白質(分子量 約 27,000程度)이 胞子形成期에 新生함을 알았다. 胞子의 發芽時에는 18개의 蛋白質밴드가 있으며 胞子形成時에는 19개가 있었다. Amido black 色素의 染色度는 分化時期에 따라서 各 밴드 마다 각각 많이 달랐다.

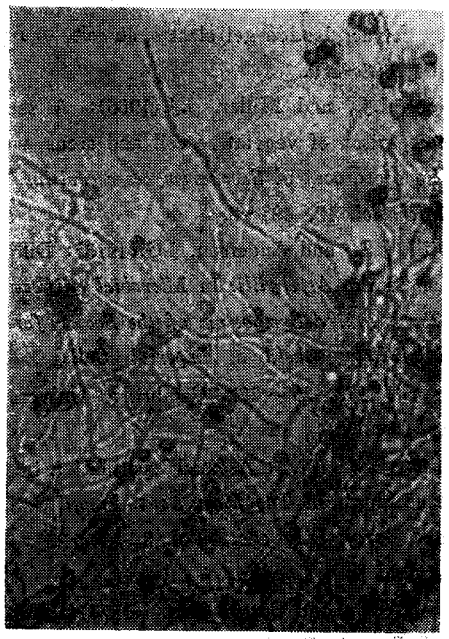


Plate I.

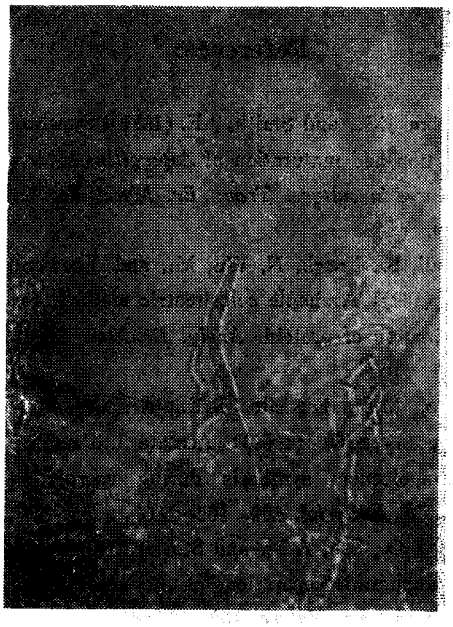
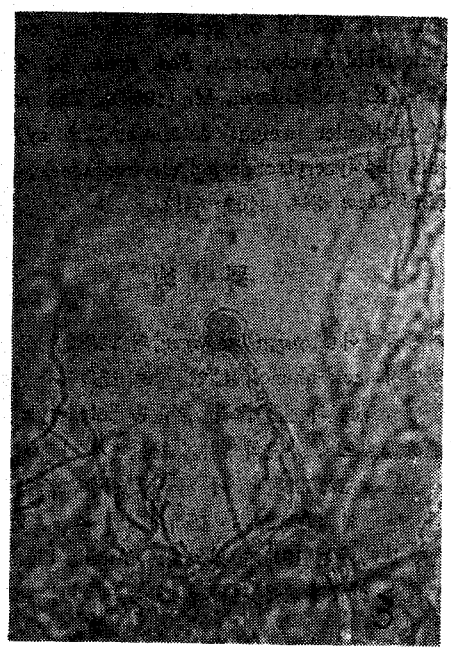


Plate II.



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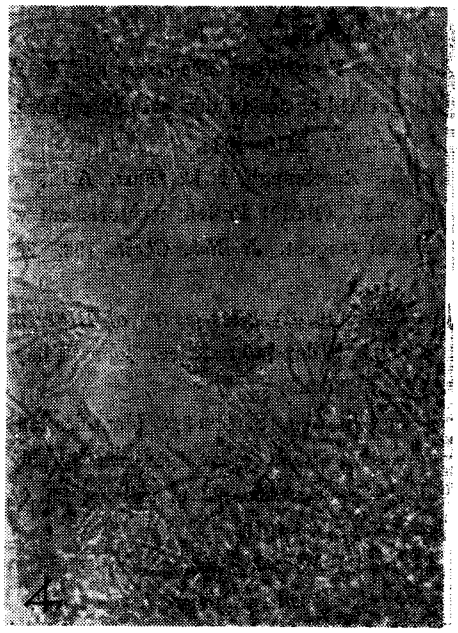

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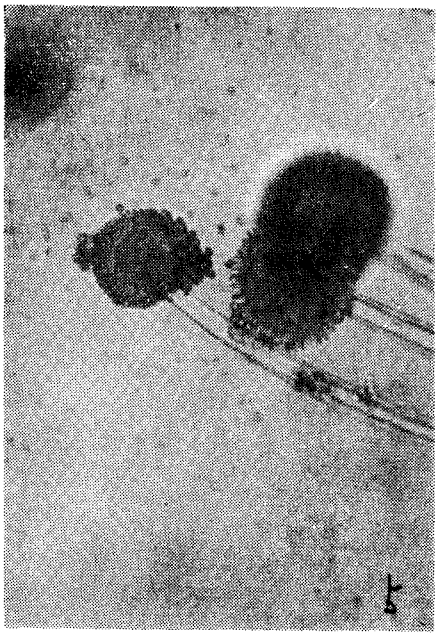

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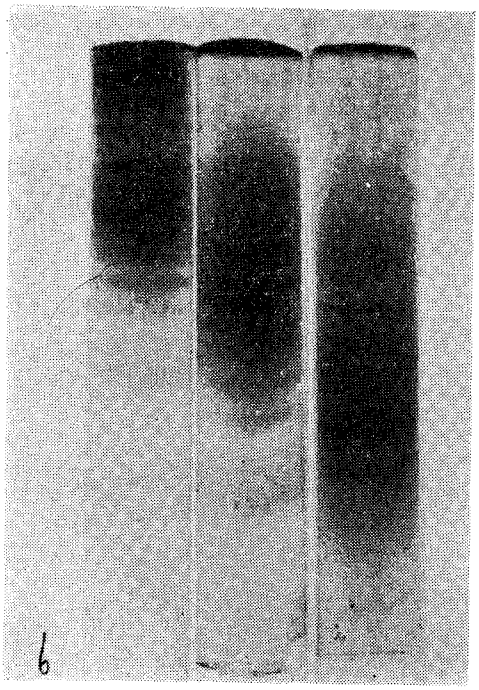

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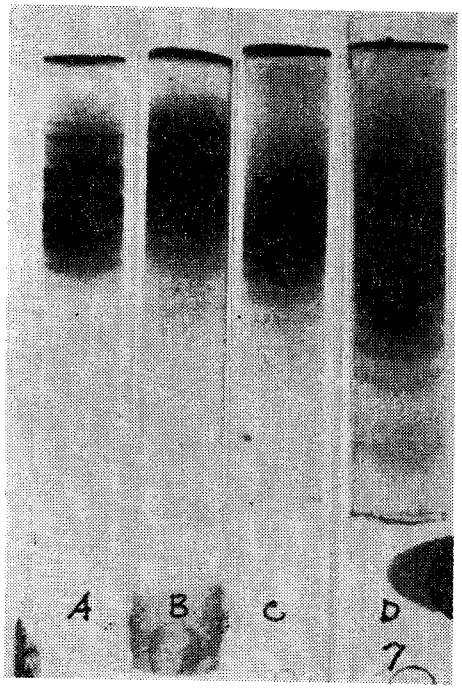

Plate VII.

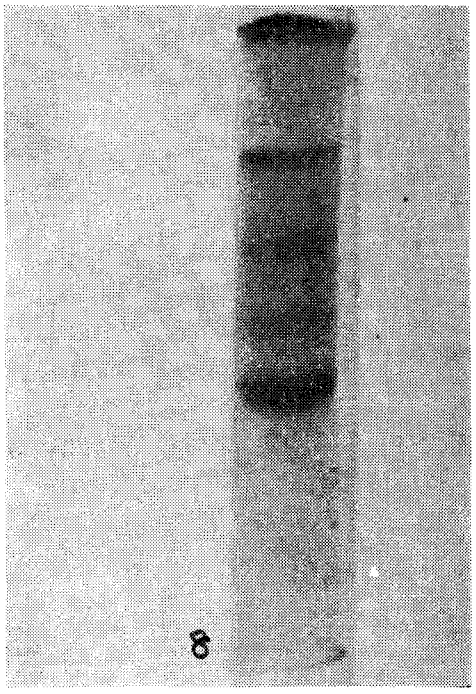

Plate VIII.

Explanation of Plates

Plate I. Geminating mycelia in shaken culture.

Plate II. Conidiophore development(early stage) in B medium.

Plate III. Conidiophore maturation in B medium.

Plate IV. Phialide development in C medium.

Plate V. Asexual spores are formed in D medium.

Plate VI. Polyacrylamide gel electorphorsis of acid-phenol soluble proteins on 10% gel, 4°C, 2.5 hours (left), 3 hours(middle), and 3.5 hours(right) running, 5mA was employed.

Plate VII. Polyacrylamide gel electrophoresis of acid-phenol soluble proteins from mycelia at four developmental stages. D tube contains sporulating stage proteins. 3 hour's running was held with the same condition stated above.

Plate VIII. Polyacrylamide gel electrophoresis of catalase, alpha-amylase, alcohol dehydrogenase, trypsin, and ribonuclease(all Sigma Co. products) on 10% gels for 3 hour's run with 5mA.