韓 國 菌 學 會 誌 第6卷 第2號
Kor. J. Mycol. Vol 6, No. 2, p1~10. 1978

Biosynthesis of the extracellular enzymes *in de novo* during the differentiation of *Aspergillus niger*

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검정곰팡이의 형태분화에 따른 세포외성효소의 신생적생합성에 관한 연구

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Abstract : In *de novo* biosynthesis of the extracellulor enzymes-proteinsaes, alpha and gluc-amylases during the synchronized differentiation of *Aspergillus niger* in submerged culture and surface liquid culture were investigated.

Gluc-amylase was synthesized in the stage of presporulation in which phialide formation is involved. Proteinase was synthesized both in the stages of conidiophore formation and presporulation.

Alpha-amylase was synthesized during presporulation and sporulation stages, the activity of enzyme lasted for seven days on surface liquid culture. It seemed that the synthesis was occured in *de novo* partly repressed by the catabolite, and its nature was found to be constitutive since it is produced in non-starch medium.

Polyacrylamide gel electrophoresis have shown that presporulating and sporulating body produced diverse types of the proteins whereas the earlier stages of vegetative body showed simpler profiles. The uptake of C-14 uracil into RNA and C-14 glutamate into protein were shown to be vigorous in presporulating body rather than those in sporulating body.

Coincidence of alpha-amylase biosynthesis in *de novo* and sporulation may be significant in the study of differentiation in which gene expression is involved.

Introduction

Asexual reproduction of *Aspergillus niger* in synchronized and submerged culture in which replacement technique was applied have been intensively studied by Smith et al (1974) and Anderson(1971).

The biochemistry of development in *Aspergillus niger* was well reviewed recently by Smith and Berry(1974), and Ashworth and Smith (1973).

Asexual sporulation of *Aspergillus miger* occured in submerged culture in a liquid minimal medium without added nitrogen, in low ammonium-N concentrations, and in a wide range of nitrate-N concentrations. Glyoxylate and several intermediate of the tricarboxylic acid (TCA) cycle also promoted conidiation in the presence of ammonium. There was no clear correlation between the activities of these enzymes and conidiation of this fungus(Galbraith, J.C. and Smith, J.E. 1969).

The specific activities of certain enzymes of the Embden-Meyerhof-Parnas (EMP) and pentose phosphate pathways varied in cultures of *Aspergillus niger* in relation to the stage of the growth cycle and the nature of the growth medium. The levels of most EMP pathway enzymes were higher in extracts from sporula ting than from non-sporulating medium (Smith, J.E., Valenzuela-Perez, J. and Ng, W.S. 1971).

The concentrations of the EMP intermediates were considerably higher in extracts of mycelium from sporulation medium than from non-sporulation medium. Major changes in metabolism are taking place in mycelium which will ultimately differentiate into sporing mycelium, long before nutrient exaustion has occured in the growing medium(Smith, J.E., and Valenzuela-Perez, J. 1971).

Sporulation depends on the functioning of the complete glycolytic pathway and the tricarboxylic acid cycle. Addition of glycolytic intermediates to cultures on non-sporulation medium induces sporulation (Valenzuela-Perez, J. and Smith, J.E. 1971).

For fungal conidiation as for other differentiating systems the normal sequential patern of the expression of the genetic potential will depend on the environmental conditions (Turian, G. 1966). It is accepted that differfntiation is the result of differential gene action (Gross, P.R. 1968).

The requirements for sexual and asexual reproduction in a given species are usually not alike, so that in a sense the two types complete. Perhaps the most promising suggestion in that injured or moribund cells release substances which act on surviving cells and divert them into a new developmental path (Cochrane, V.W. 1958).

The transformation of a vegetive bacterial cell into a spore is initiated by lack of nutrients. Some enzymes, and proteins in general, have altered properties compatible with heat and radiation resistance. In slime moulds differentiation is accompanied by the synthesis of specific enzymes, such as UDP-glucose pyrophosphorylase, UDP-galactose transferase and trehalose-6-phosphate synthetase(Pasternak, C. A. 1970, Sussman, M. 1967).

The stimulus for differentiation is sometimes environmental (Cantino, E.C. and Lovett, J.S., 1964). 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, overall function depends not only on the kind and amount of enzyme present but also on its actiyity. Activity is determined by many factors, including the presence of specific effectors such as a substrate, product, hormone or some other molecule (Pasternak, C.A., 1970).

Protein turnover during sporulation of *Bacillus subtilis*, in that protein newly synthesized is degraded at the same rate as pre-existing vegetative cell protein. The percentage of protein newly synthesized in the forespore compartment during sporulation is significantly higher than that in the surrounding mother cell (Spudich, J.A. and Kornberg, A. 1968).

Incorporation of radioactive precursors into RNA continued at high rate throughout the period of diffenentiation of water mold Achlya, and DNA-deperdent RNA synthesis was required for the differentiation of sporangia (Griffin, D.H. and Breuker, C. 1969). The change in enzyme activity appeared to be related to a morphological change in the cells and indicated an altered metabolic pattern for sporulating cells of *Clostridium thermosaccharolyticus* (Hsu, F.J. and ordal, Z.J. 1970). In the sporulation of yeast the specific activities of protease A and B were shown to be highly increased (Tsuboi, M. 1977). Molecular hybridization experiments with pulse labeled RNA of *Neurospora crassa* showed differential transcription during growth periods Bhagwat, A.S. and Mahadevan, P.R. 1973). Such a

regulation has been suggested as a basic molecular event behind the processes of differentiation and morphogenesis. Conditions that are optimal for cellulase production by *Myrothecium verucaria* grown on non-cellulosic substrates are similar to those required foresporulation and often for antibiotic production, *viz* an environment which no longer favours balanced vegetative growth (Hulme, M.A. and Stranks, D.W. 1971). The multiplicity of the biologically active substances excreted by spore-fo rmers into their culture medium is well known, but remains unexplained. In bacilli, which will be considered first, these are mainly antibiotics and exo-enzymes, in Clostridia, they include toxins.

The purposes of this investigation were to determine (a) the uptake rate of C-14 L-glutamate into protein and C-14 uracil into RNA during sporulation of *Asergillus niger*, (b) the profiles of exo-enzymes from the sporulation medium in the disc electrophoretic gels, (c) sequencial emergence of gluc-amylase and alpha-amylase during the sporulation of this fungus, (d) Pattern of genetic expression of alpha-amylase synthesis in the non-starch medium.

Materials and Methods

Aspergillus niger van Tiegham (IMI 41873) was used in these experiments. This strain was cultured on potato-glucose-agar slopes at 29°C. Submerged culture was done with four types of replacement media (A,B,C, and D medium) those have been indicated by Smith, J.E. et al. (Anderson, J. G.and Smith, J.E. 1971, Gaibrith, J.C. and Smith, J.E., and Kim, J.H. 1971).

Shake culture for the germination of spores was performed with A medium. In order to grow the vegetative cells and to get spores (ssexual spores) B,C, and D replacement media were used, then synchronous differentiation was acquired in each medium (Smith, J.E. and Anderson, J.E. 1973).

Proteins from the culture medium were obtained by precipitating the culture filtrate with two holds volume of cold acetone (Keller, S. and Block, R.J. 1966). The precipitation was held in the refrigerator for 24 hours then centrifuged out.

To determine the activities of three kinds of proteases such as acidic, neutral, alkaline Hagihara's (1953) method that is a modification of Anson's was applied in corresponding pH ranges and with Hammersten milk casein (E. Merck Co.). The method is to assay the level of TCA soluble fraction which is enzymic hydrolysate with Folin-phenol reagent (Lowry, O.H. 1951). The proteinase unit was expressed as the activity to produce the TCA soluble fraction, and the equivalence to 1mg. equivalent of tyrosine (in optical densiy) per minute was computed.

The above cold acetone treated precipitate was used for the determination of gluc-amylase actity and alpha-amylase activity respectively.

Saccharogenic activity of the gluc-amylase was expressed as the level of glucose which is produced by hydrolysis of soluble starch with the enzyme per hour, the assay of the glucose was done by the method of Somogyi et al. (1954). Liquifying acti vity of the alpha-amylase was determined by the method of Wohlgemuth(1908) with use of highly diluted (0.01 N.) KI and iodine solution. The levels of total protein in the precipitate was determined by the method of Lowry (1951). Determination of the uptake rates of radioactive tracers (Amersham, England) into RNA and Protein was held with use of liquid scintillation counter(Aloka 601, Japan). The radioactive C-14 uracil labeling was performed in 5 minutes within shake flask into which the the fungus at a defined developing stage was transfered. Labeling was acompanied by the non-radioactive uracil after 30 seconds from it at ratio of 0.1 ml. of 2.5μ Ci. with 1ml. of 20μg normal uracil. Samples of 0.1ml. involving fungus and culture medium were transfered on to the filter pads, then those were placed gently on gauze which was previously immerersed with ice-cold 5% TCA (20ml.) and 0.01% uracil, then left for overnight at 0°C. The pads were washed by dropping 5% TCA five times, three times with 95% ethyl alcohol and dried with acetone. Pads were put into scintillation vial, then 10ml. scinti-fluid were poured. The scinti-cocktail was prepared with POPOP 50mg. and POP 4g. in

1*l*. of toluene (Burk, D.J. et al. 1972, Michaels, G.A. 1972, Doolittle, W.F. 1972, and Griffin, D.H.and Breuker, C., 1969).

Labeling of the C-14-L-glutamate (2.5μ Ci. in 0.1 ml.) was performed within shake flask in which the fungus at a defined stage of development was transfered.

Pulse labeling was done for 5 minutes then 20g. of mon-radioactive L-glutamate was put into labeling culture 30 seconds later the labeling.

The samples (0.1ml) was transfered on to filter paper pad, then washed with cold 5% TCA involing 0.01% L-glutamic acid. The pad was laid on the gauze which was immeersed of 5% TCA solution, then left for overnight at 0°C, then washed again with 5% TCA solution gently five times, three times with 95% ethyl alcohol then dried up with acetone. The sample was counted for five minutes with liquid scintillation counter, the scinti-cocktail in the vials was the same as stated above(Harvey, R.J. 1970).

Polyacrylamide gel electrophoresis of the extracellular proteins, those have been prepared by precipitation with cold acetone and chilling over-night, was carried out. The precipitation was washed again with cold acetone four times then spun out, and redissolved in phenol: acetic acid: water (2:1: 0.5) solution (Jakusch, B.M. et al. 1970, and Codner, R.C, and Chesson, A. 1971).

The concentration of the gel was 7.5%, and the above acid-phenol extract those were determined by Lowry's methods (1951) was layered on the gel top (250μg. of protein/tube), then it was run in 10% V/V acid running buffer (Codner, R.C. 1971) at 15°C at constant current of 5mA/gel for 5 hours. After electrophoresis the gels were stained with Amido black 10B to check the protein bands in it, then destained (Gordon, A.H.1971, and Davies, B.J. 1964)

Liquid surface culture was performed by inoculating the washed spores into broad mouth jar (9cm diameter×19cm height) containing Czapek-Dox medium. The samples were collected by pipetting the culture medium after gentle mixing on the magnet-stirrer plate.

Results

The levels of extracellular proteins are shown in figure 1. In the D medium the extracellular proteins seemed to be excreted immediately after replacement, however, the levels of the proteins increased along with the proceedings of sporulation.

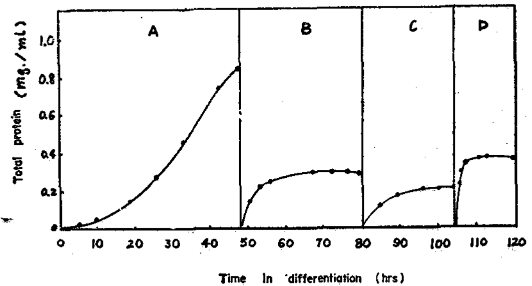


Fig. 1. Changes of the levels of total proteins leaked out of differentiating body of *Aspergillus niger* cultured in Czapek-Dox medium and modified replacement media with techniques of synchronizing and submerged culture in laboratory scale jar fermenter.

A: Medium for germination of spores and vegetative hyphal growth with shaking incubator.

B: Replacement medium for the formation of conidiophores in the jar fermenter.

C: Replacement medium in which phialides were formed and vesicles were matured (presporulation stage) in the jar fermenter.

D: The last replacement medium for the asexual sporulation in the jar fermenter.

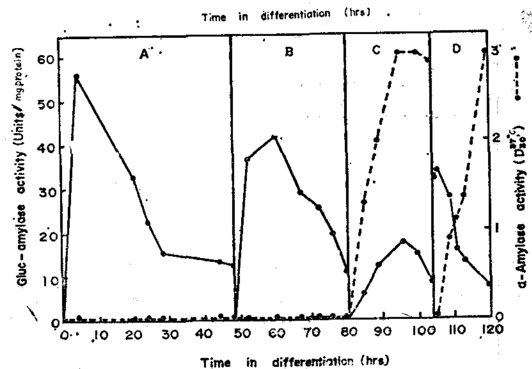


Fig. 2. Changes in the activities of alpha and gluc-amylases during the differentiation of *Aspergillus niger* in submerged culture with replacement media in jar fermenter.

Gluc-amylase activity increased only in the medium C in which the phialide mature. However, the highest peak of alpha-amylase actity is shown in the medium C and D in which presporulation and sporulation occur. (Fig. 2 and Fig. 3)

Activities of the proteinases increased in B and C medium but not increased in D medium. (Fig. 4)

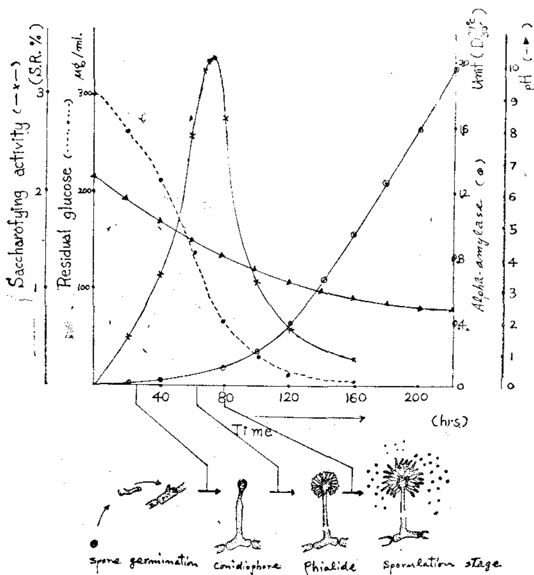


Fig. 3. Changes in activites of alpha and gluc-amylases, in the leveles of residual glucose, and pH levels during the differentiation of *Aspergillus niger* grown on the surface of Czapek-Dox liquid medium. Diluted starch solution (0. 1%) and diluted (0. 01 N.) KI-iodine solution was used for the determination of alpha-amylase activity. Also 0. 1% starch solution was used for gluc-amylase assay.

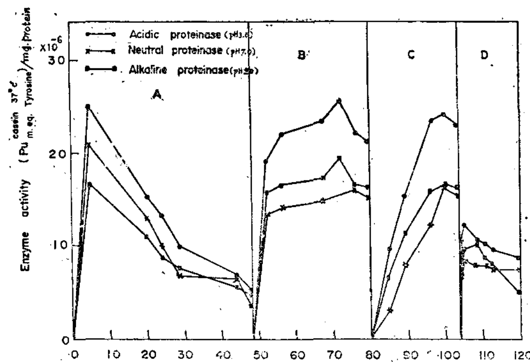


Fig. 4. Changes of activities of the proteinases those are acidophilic, alkalinephilic and neutralphilic during the differentiation of *Aspergillus niger*

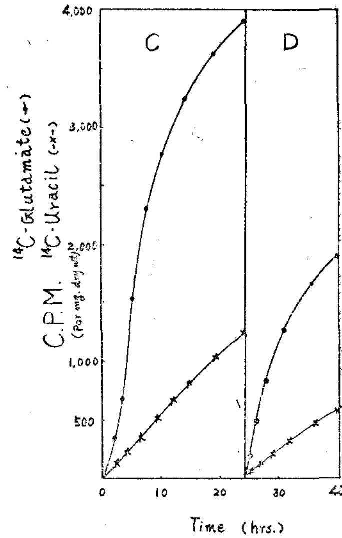


Fig. 5. The uptake rate of C-14 uracil into RNA and C-14 L-glutamic acid into proteins by pulse-chased labeling into *Aspergillus niger* which was in the stages of presporulation and sporulation. Liquid scintillation counter was used for the counting of radioactivities.

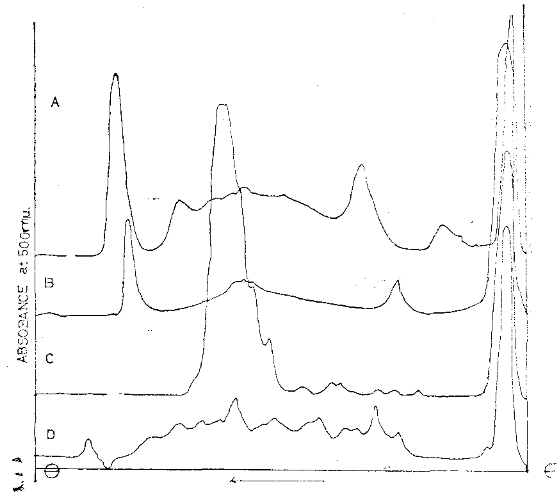
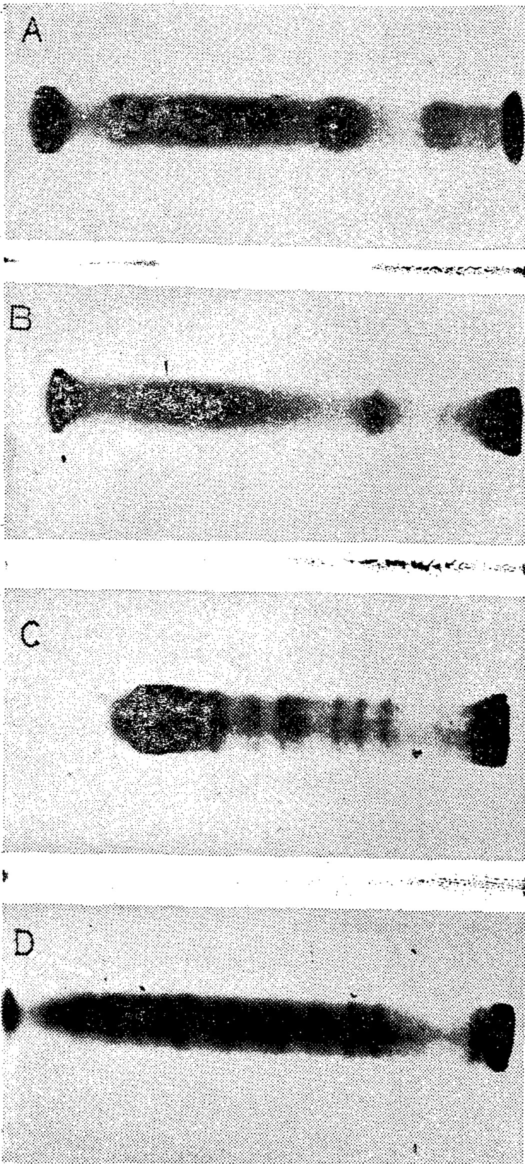


Fig. 6. Comparison of disc gel electrophoretic profiles of exogenus proteins from each differentiated stage of *Aspergillus niger* grown in raplacement media. Polyacrylamide gel electrophoresis was done in the conditions of 5 mA per tube for 4 hours, 7. 5% gels and acid buffer solution. The proteins were prepared by addition of 2 volumes of cold acetone into culture filtrates to precipitate, then this was extracted with phenol-acid solution. The proteins in the gels were stained with Amido black 10B, then destained. The photographic prints with extrordinary thin paper were put into the recording densitometer in order to compare qualitative analysis of protein bands in mumber.



Explanation of the plates

Fig. A. The profiles of proteins by the running of polyacrylamide gel electrophoresis from mycelial body of *Aspergillios niger* in shake culture(Grown in medium A).

Fig. B. The profiles of proteins from *Aspergillus niger* which is in the stage of conidiophore formation(Grown in medium B).

Fig. C. The profiles of proteins from *Aspergillus niger* which is in the stage of phialides formation and vesicle maturation(Grown in medium C).

Fig. D: The profiles of proteins from *Aspergillus niger* which is in the sporulation stage grown in medium D.

Uptake rate of C-14 uracil into RNA increased up to 15 times much during maturation stage of vesicles and phialide, but only 16% was increased during sporulation. Uptake-rate of C-14 L-glutamate into proteins increased up to 65 times much during vesicles and phialide maturation but only 30% increased during the sporulation(Fig. 5).

Polyacrylamide gel electrophoresis of the extracellular proteins carried out, and their profiles are shown in the plate. The number of protein bands along with the differentiation increased gradually, they showed more and clearer profiles more sharper separation as the development proceeded (Fig. 6).

Discussion

The replacement media in submerged culture are well explained by Smith, J.E.(1973 and 1974). Various intra-cellular enzymes and their activities during the differentiation of this fungus have been investigated with these media and synchronizing techniques those developed by Anderson, Galbraith and Smith(1969 and 1971).

In these experiments with the replacement culture release of the proteins during pre-sporulation and sporulation was found, and the release have continued for more than five hours during sporulation. Injured or moribund cells release substances which act on surviving cells and divert them into a new developmental path (Cochrane, V.W. 1958). The multiplicity of the biologically active substances excreted by spore-formers into their culture medium is well known, but remained unexplained(Schaeffer, 1969) The most interesting phenomenon is that alpha-amylase suddenly appears in the stage of phialide development and its synthesis continned through the stage of sporulation. Synthesis of alpha-amylase by *Aspergillus oryzae* transfered from complete medium containing starch to starchless medium are occured at different stage of biomass formation and post-growth stage. Alpha-amylase forming m-RNA was claimed to be long-lived (Feniksova, R.V. et al, 1969).

Myrothecinm verrucaria can produce cellulase when grown on media containing glucose or glycerol as sole carbohydrate source. The enzyme complex can depolymerize cellulosic substrate. Since cellulase can be produced by reducing the organism's constituent sugars, therefore this enzyme is synthesized in the absence of cellulose or any of nstitutive and that production may be partly controlled by catabolic repression. Spores appeared at coabout the same time that cellulase began to accumulate(Hulme, M.A. and Stranks, D.W. 1971).

As shown in figure 3 the production of alpha-amylase occured at the time of glucose exaustion in the medium. Exhaustion of the carbon supply in the medium enhances the activity of R. glucanase in *Schizophyllum commune* dikaryons. This enzyme is responsible for R. glucan degradation, which appears to be requisite for pileus expansion(Niderpruem, D.J. and Wessels, J.G.H. 1969).

Appearance of gluc-amylase was in the stage of pre-sporulation(Fig. 3) and the curve is quite sharp, therefore it can be suggested that differential appearance of amylolytic enzymes occurs sequentially in different stage of the development. Differentiation is the result of differential gene action(Gross, P.R. 1968).

However, whether the gluc-amylase is induced or repressed (by mechanisms) is still unknown. The activities of proteinase (acidic, neutral and alkaline) incrcased in A,B,and C medium except in D medium. Protease A and B showed highest activities specifically during the sporulation of yeast (Klar and Halvorson, 1975, Warren, S.C. 1968). More than half of the proteins which were produced during the vegetative growth in yeast have been decomposed and subjected to the turnover of the proteins (Tsuboi, 1977). Culture filtrates of sporeforming bacteria, usually studied after the end of growth, have strong proteolytic activity(Davies, R. 1963, Pollock, M.R. 1962).

Bacillus licheniformis showed this activity to increase rapidly at the end of growth as a result of *de novo* synthesis rather than as a result of delayed excretion or activation of performed enzyme (Levison, S. and Aronson, A.I. 1967)

Vigorous uptake of the radioactive uracil and L-glutamate into RNA and protein in pre-sporulation stage (Fig. 5) does mean that the rapid turnover of the protein and RNA synthesis are being held prior to the sporulation. This fact occurs slightly earlier than those in *Bacillus subtilis* reported by Spudich, A and Kornberg, A.(1968).

During the differentiation of sporangia in the water mold Achlya, inhibition of protein synthesis during the process completely inhibited further differentiation(Timberlake, et al, 1973). Turnover of RNA and its synthesis were also vigorous during the sporulation(Tsuboi, M. 1977). Incorporation of radioactive precursers into RNA continued at a high rate throughout the period of differentiation in Achlya(Griffin and Breuker, 1969). However, RNA synthesis ceases during differentiation of the sporangia in *Blastocladiella emersonii*.(Murphy and Lovett. 1966).

Much clearer separation and diverse bands (in the separation of proteins) throughout polyacrylamide gel electrophoresis means that more completed form and diverse variety of proteins were synthesized in *de novo* during the sporulation of this fungus. At least alpha-amylase, gluc-amylase and proteinases are seemed to be involved in *de novo* synthesis during the presporulation and sporulation. Although, the unknown bands in the gel are to be investigated further to determine the nature and to identify.

There was slight shift towards synthesis of longer polypeptide chains in the radioactivity patterns of the soluble proteins in sporulation cultures(Jackush, B.M. and Rush, H.P. et al, 1970).

Molecular hybridization experiments with pulse-labeled total ribonucleic acid or isolated non-ribosomal rapidly labled RNA species and DNA from growth periods of 8, 16, and 24 hour of *Neurospora crassa* showed differential transcription. Hybridization competition experiments between RNA species isolated from 8, 16, and 24 hour of growth showed qualitative differences in the type of RNA synthesized during these periods(Bhagwat, A.S. and Mahadevan, P.R. 1973).

It seems that quite number of proteins(including enzymes) are being synthesized during the sporulation and pre-sporulation, then these are excreted out of the cell into the medium. Some of these

proteins may contribute to the differentiation by reorganizing the reserve carbohydrate and proteins.

This research was supported partly by a grant from the Ministry of Education, Republic of Korea. The author would like to thank the Ministry and Authority of Ewha Womans University for assistance.

요 약

검정곰팡이(*Aspergillus niger*)의 액침배양과 액체 표면배양을 통한 동조적 형태분화에 있어서 체외효소인 단백질분해효소, 알파 및 글루크아밀라제의 신생적 생합성 상황을 연구하였다. 글루크아미라제는 경자(phialide)가 성숙하는 단계 즉 포자 형성의 전단계에서만 그 활성이 왕성하였다. 단백질분해효소(산성, 중성 및 알칼리성)들은 분생자병의 성장단계에서 활성이 약간 증가하였으나, 경자의 성숙단계에서는 활성이 극히 활발하였다.

알파아밀라제는 경자의 성숙시기와 포자형성기에서 활성이 활발하였으며 그 활성은 장기간 지속 되었다. 알파아밀라제의 활성은 포자형성 기간중 계속 증가하였으므로 신생적으로 생합성된다고 할 수 있으며, 포도당배지에서 많은 량이 생합성되었고, 또 포도당량의 고갈에 조음하여 그 생합성이 개시되었으므로 이 효소는 구성적 효소이며 이화 대사물의 역제작용(catabolite repression)을 받는 효소라고 할 수 있다.

포리아크릴아미드 젤(polyacrylamide gel)을 이용한 전기영동으로서 포자형성기와 그 전단계의 균체로부터 다양하고 선명한 세포외성 단백질을 분리할 수 있었다. 균사형성기나 포자발아기의 균체로부터는 극소수의 선명치 못한 분리상을 얻었다.

C-14 우라실이 균체의 RNA핵산으로 섭취되어 들어가는 비율과 C-14 글루탐산이 균체 단백질으로 섭취되어 들어가는 비율은 포자형성전기에서 왕성하였으며 포자형성 기간중에는 극히 저조하였다.

알파아밀라제의 신생적 생합성과 포자형성이 일치하는 현상은 유전인자의 표현과정이 내포되는 분화(포자형성)라는 점에서 볼 때 의의와 인과관계가 있을 것으로 사료된다.

이 연구의 경비 일부는 1978년도 문교부 학술연구조성비의 지원을 받아서 충당하였으므로 문교부당국과 여러가지 편의를 베풀어 주신 이화여자대학교의 관계제위께 심심한 감사를 드린다.

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