STUDIES ON THE FUNGAL PROTEINS IN *PIRICULARIA ORYZAE* CAV.*

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Introduction

One may predict the relative virulence of a given pathogenic organism by the manifestation of its biochemical nature. Otsuka *et al.* (1965) indicated that their works on the strains of *Piricularia oryzae* were rather successful in correlating the relative strength of pathogenicity of the fungus with their utilization of certain carbon and nitrogen compounds as well as requirements of vitamins. Keitt *et al.* (1959) also correlated the pathogenicity of *Venturia inaequalis* with its nutrition. These differences in the nutritional requirements are certainly the indication of metabolic distinctness among the microorganisms in question. Moreover, phylogenetic significance of the fungal metabolites was explored (Udagawa, 1962).

The relationship between races and host genotypes in both *Cladosporium fulvum* and *Phytophthora infestans* suggested that genetic systems, comparable to those controlling the resistance in the hosts, might determine the virulence in these two fungi (Fincham and Day, 1963). Suzuki (1965) believed that variation and formation of new races in *P. oryzae* seemed to originate from heterocaryosis, heterocytosome, and parasexuality. Recently, he has proved the persistent heterocaryosis with living cells under the interference phase contrast microscope whereas this phenomenon was previously observed in the fixed and stained preparations by the same author. The gene-for-gene relationship was suggested to imply the genetic complementarity between each host and pathogen which evolved with disease development, namely the association

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between the two organisms (Fincham and Day, 1963). The gene is basically a functional unit since genes are known to control the primary structure of individual and specific protein molecules. The amino acid replacements within the polypeptide chain determine the biological activity and other properties of the protein molecule (Hartman and Suskind, 1965). It has been known for a long time that enzymes are required to mediate the activity in living cells. Subsequently, the studies on the proteins of *P. oryzae* are plausible in as much as the major components of enzymes, biological unit, are mainly protein.

The present investigation is undertaken to serve as a preliminary experiment on mycelial proteins of the seleted physiologic races of *P. oryzae* which may add to knowledge of the pathogen-suscept relation through biochemical nature of relative pathogenicity.

Materials and Methods

Eleven isolates grouped into six physiologic races of *Piricularia oryzae* Cav. were kindly furnished by Mr. Chin-chung Chien (1965) of the Taiwan Agricultural Research Institute for the present experiment. They are Race #1 (2T–82S), Race #2 (2T–32S), Race #5 (2K–24Sb), Race #12 (2S–74S, 2T–80S, 3C–47S), Race #13 (0'S–45Sa), and Race #17 (2K-82S, 3C–40S, 4K–30S, 4C–31S). The compositions of the Misato's and Tanakas' media, cultural conditions, and procedures for obtaining spore suspensions were described elsewhere (Tseng *et al.*, 1965; Wu *et al.*, 1966) except that spore was germinated and grown in Tanaka's medium for 2 days before further tranfer to 500-ml Erlenmeyer flasks containing 100 ml of Tanaka's medium for another 4 or 5 days incubation to secure good mycelial growth in submerged cultures. Mycelial clumps were harvested by pouring the cultures into a Buchner funnel with a filter paper, washed several times with distilled water, dried, and kept in a freezer at –15°C before use.

The frozen mycelium was placed in a chilled mortar with the same weight of sea sand and then ground to a paste. This paste was frozen overnight at –15°C before extration of soluble protein. The extract was made by adding a volume of 0.1 M phosphate buffer, pH 7.0, three times the weight of the mycelium to this frozen paste, ground, thawed, and allowed to stand at room temperature for 30 minutes. The extract was centrifuged at 1,500×g for 10 minutes. The supernatant was recentrifuged at 10,000×g for 20 minutes, then placed in a cellulose sac of dialyzer tubing (Arthur H. Thomas Co., Philadelphia) and dialysed against glass distilled water for 24 hours. The extract thus obtained was concentrated in cold stream and afterwards centrifuged at 10,000×g for 30 minutes. The clear supernatant was used in the electrophoretic separation

and chromatographic fractionation. In a typical experiment, 5 g of frozen mycelium were ground with 5 g of sand and extracted with 15 ml phosphate buffer. Upon dialysis, concentration, and high speed centrifugation, about one ml of clear supernatant was obtained. This extract contained 10 to 15 mg of soluble protein. Protein was assayed by the Biuret method (Layne, 1957).

Paper electrophoresis was carried out with the Spinco Model R eight-strip Durrum-type apparatus with Whatman No. 1 paper (Williams *et al.*, 1958; Block *et al.*, 1958). Electrophoretic separation was achieved by using the Veronal buffer at pH 8.6 with ionic strength of 0.075 (Spinco Buffer B-2). A current of 5 mA/cm and a duration of 6 hours at 2°C were routinely used. The protein on the strips was stained with bromphenol blue (Spinco Dye-4). The stained paper strip was evaluated with Spinco Model RB Analytrol. Six samples, 0.01 ml each, were analyzed at a time to ensure an accurate comparison of different physiologic races or different isolates of the same race.

Both anion exchanger, diethylaminoethylcellulose (DEAE-cellulose) and cation exchanger, carboxymethylcellulose (CM-cellulose), were prepared and packed according to the procedure described by Peterson and Sober (1962). Five-tenth ml of the sample containing 5 to 7.5 mg protein were applied to the DEAE-cellulose column (1.0×20 cm with 1.5 g adsorbent) and equilibrated with 0.001 M phophate buffer, pH 7.0. The protein was eluted successively with sodium chloride at the concentrations of 0, 0.001, 0.005, 0.01 0.05, 0.1, 0.5, and 1.0 M, in 0.001 M phosphate buffer at pH 7.0. Fraction of 5 ml was collected at a flow rate of 2.5 ml per minute. Tne first peak, peak A, obtained from DEAE-cellulose column was applied to a CM-cellulose column (1.5×12 cm with 3.5 g adsorbent), previously equilibrated with 0.001 M phosphate buffer, pH 7.0, and the protein was eluted as described for the elution of DEAE-cellulose column. The flow rate was about 1.0 ml per minute in this case. The reading of each fraction was made at 280 mμ by a spectrophotometer of the Unicam SP. 500. The same butch of cellulose exchangers were used to secure the accurate comparison of the elution patterns.

Results

Electrophoretic separation

It is apparent that mycelial proteins of *P. oryzae* was resolved into apporoximately 2 to 3 components at 5 mA for 5 hours (Fig. 1-B) while at the lower current, 2.5 mA for 5 hours, only one component on the positive site relatively closer to the origin was detected and little significant difference was observed (Fig. 1-A). The electrophoretic pattterns obtained at lower current were very similar to those obtained by Tatsuyama (1961). However, a distinct pattern was revealed by each race when the current of 5 mA was used.

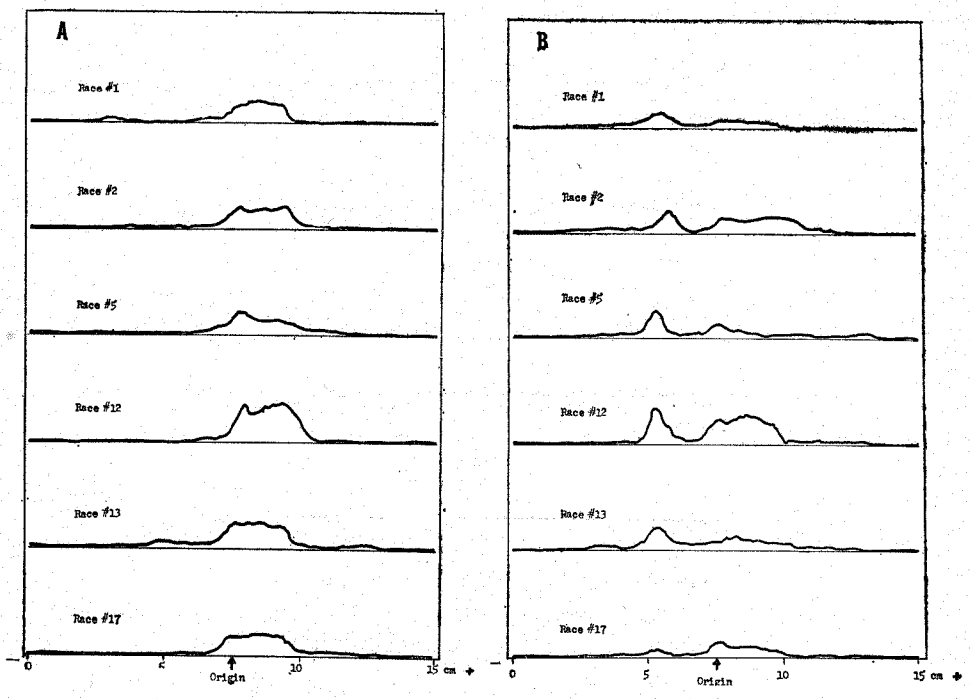

Fig. 1. Comparison of paper electrophoretic patterns of mycelial proteins prepared from six physiologic races of *Piricularia oryzae* at 2.5 mA (A) and 5 mA (B) for 5 hours.

A duration for electrophoresis may be also influential to the resultant pattern of the electrophoretic separation. Trials on three different duration, e. g. 5 mA for 5, 6, and 8 hours, were attempted. As shown in Fig. 2, the longer the duration for electrophoresis the more the components obtained. Yet, the concentration of each protein component tended to dilute. This resulted in flat peaks which defaced their characteristic patterns. Among the above-mentioned durations for electrophoresis, 6 hours seemed to be suitable for the electrophoretic separation of the mycelial proteins of *P. oryzae* under the experimental condition. Temperature is another effective factor. In comparing electrophoretic patterns presented in Fig. 1-B and Fig. 2-A, slight difference between the two was observed though these electrophoretic separations were carried out under identical condition except that the temperature during electrophoresis were different, namely 26° and 2°C, respectively.

Since each race gave characteristic pattern, electrophoretic patterns of different isolates within a single physiologic race were examined. In the case of Race #12 and Race #17, as shown in Fig. 3, three isolates of each race appeared to be more or less the same in their electrophoretic patterns though dissimilarity within the Race #12 can not be entirely ruled out.

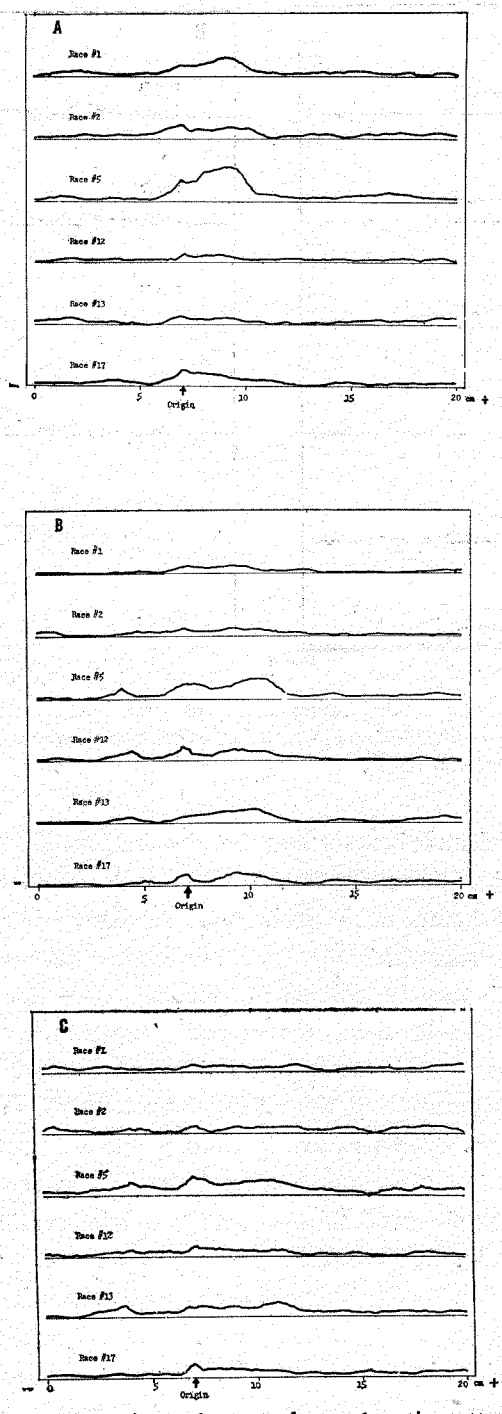

Fig. 2. Comparison of paper electrophoretic patterns of mycelial proteins prepared from six physiologic races of *Piricularia oryzae* at 5 mA for 5 (A), 6 (B), and 8 (C) hours.

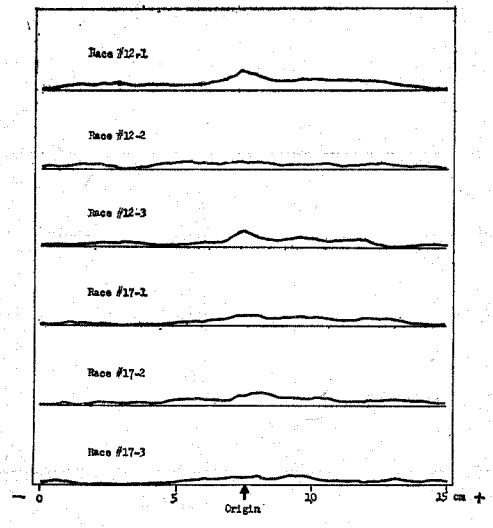

Fig. 3. Comparison of paper electrophoretic patterns of mycelial proteins prepared from different isolates of Race #12 and of Race #17 of *Piricularia oryzae* at 5 mA for 6 hours.

Rather low resolution of paper electrophoresis in separation of the mycelial proteins may be due to low concentration of the proteins applied. The extraction of fungal protein encountered the difficulties that fungi are resistant to disruption which is a unique problem due to the nature of its cell wall (Stine *et al.*, 1964). In short, we might be able to understand the relationship between physiologic races and their virulence by means of paper electrophoresis if we could prepare the extract of fungal protein in higher concentration.

Chromatographic fractionation

Fractionation of the fungal proteins of *P. oryzae* was accomplished by the stepwise elution on column chromatography of cellulose exchangers since stepwise elution was suggested to be an advantageous method for separation of components in low concentration with great sensitivity and rapidity (Fasold *et al.*, 1963).

Elution patterns of the mycelial protein shown in Fig. 4 indicates that all six races are more or less the same in certain respect, but none of them are entirely the same. They can be divided into two groups: one included Races #1, #2, #13, and #17; and the other group, Race #5 and #12. Peak A fraction of each race gave similar pattern when it was chromatographed on CM-cellulose column (Fig. 5).

Since elution patterns of the six physiologic races can be divided into two groups, different isolates of two representative races, Race #12 and Race #17, were selected for the chromatography on DEAE-cellulose column. The resultant

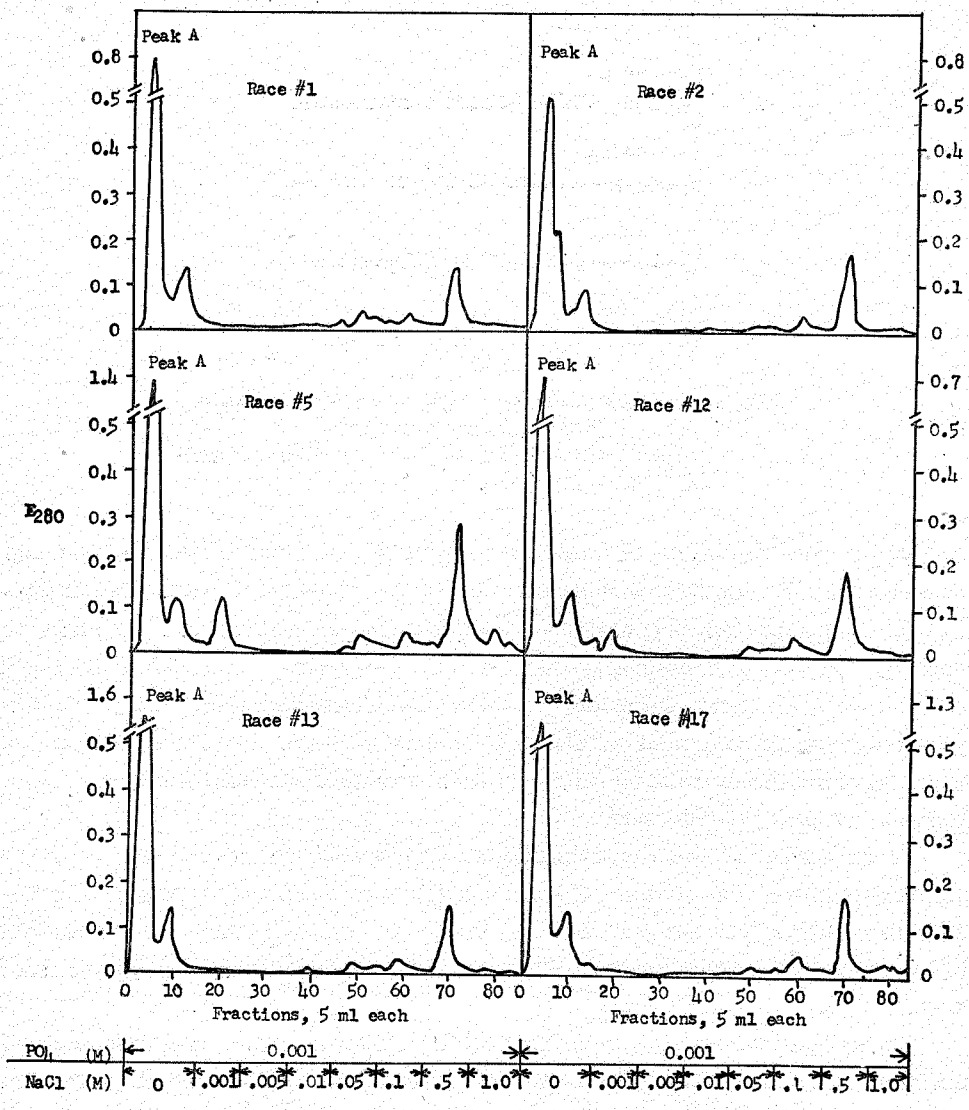

Fig. 4. Elution patterns of the mycelial proteins prepared from six physiologic races of *Piricularia oryzae*. Column, DEAE-cellulose, 1.0 × 20 cm.

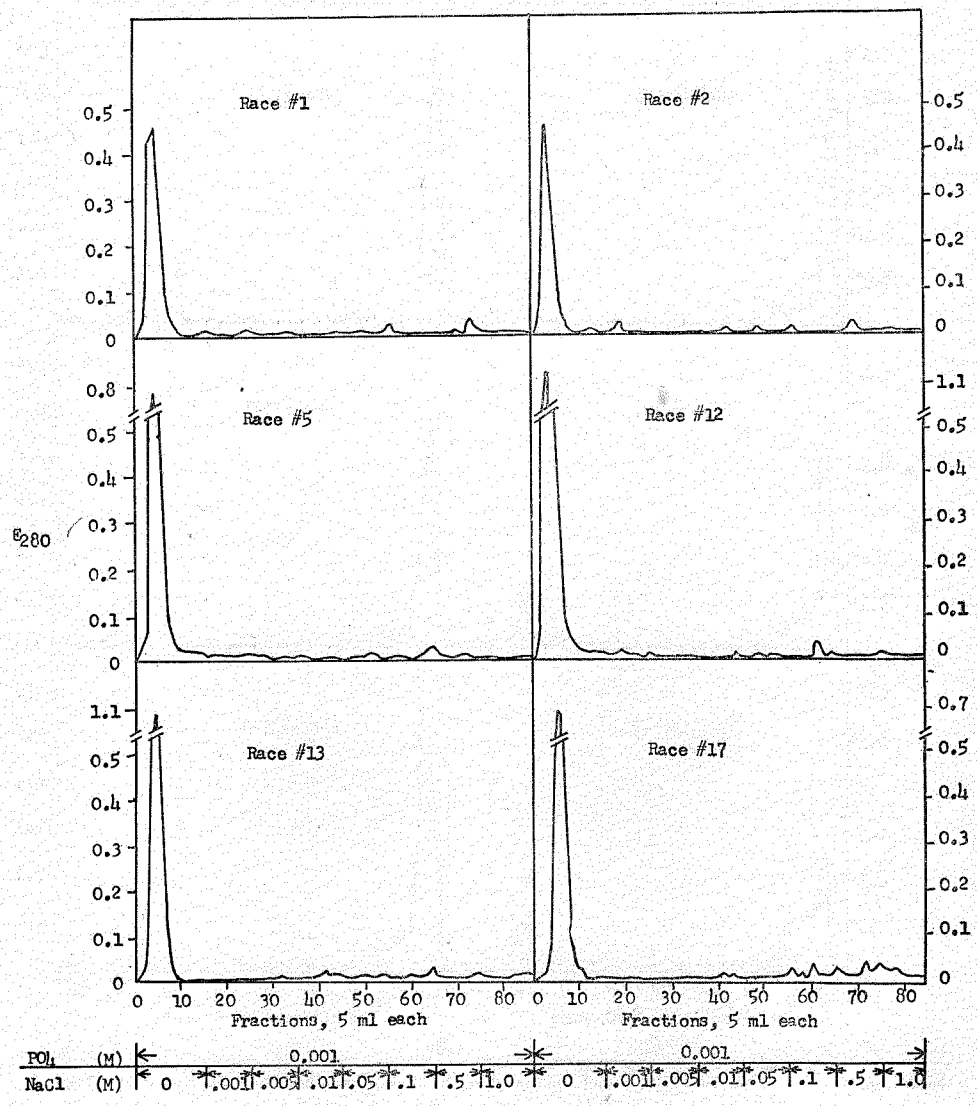

Fig. 5. Chromatography of peak A in Fig. 4 on CM-cellulose column, 1.5×12 cm.

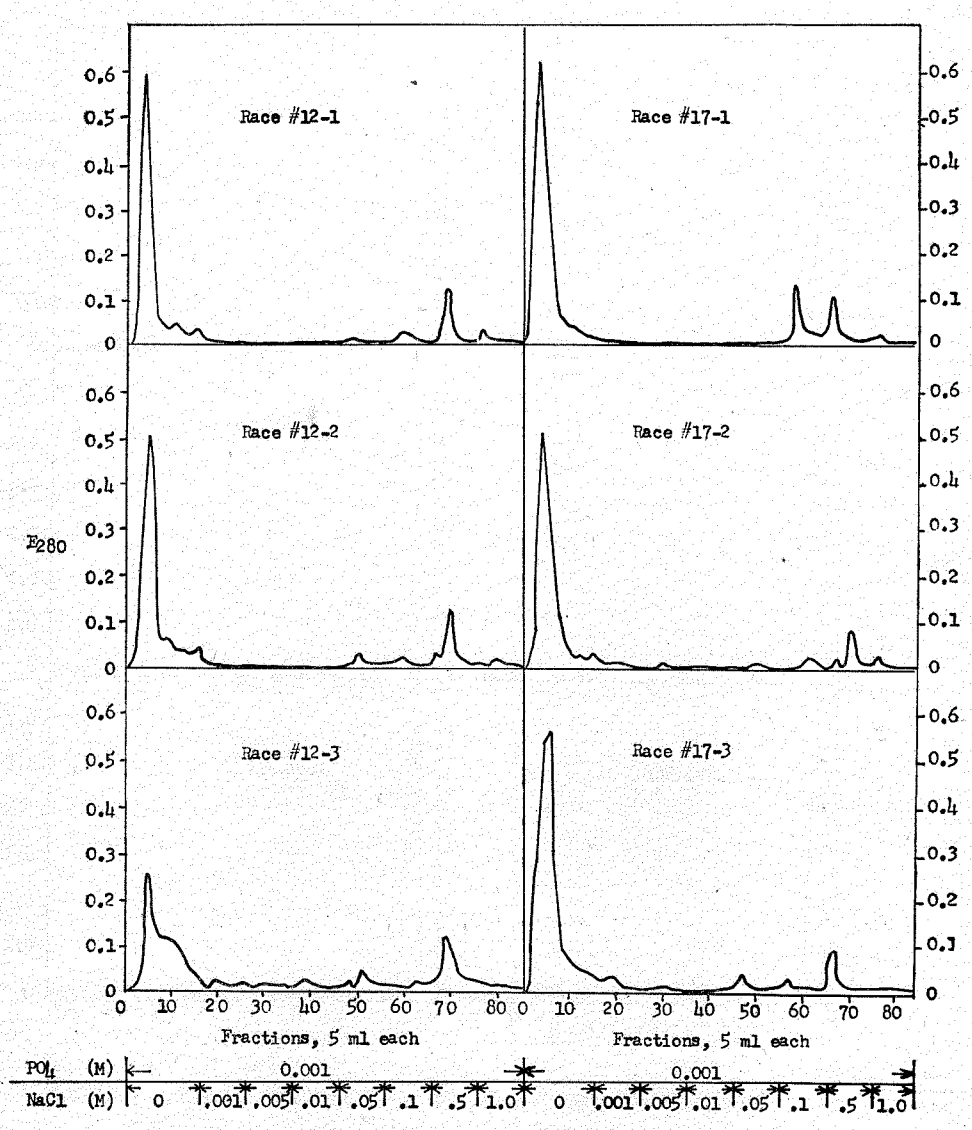

Fig. 6. Elution patterns of the mycelial proteins prepared from different isolates of Race #12 and of Race #17 of *Piricularia oryzae*. Column, DEAE-cellulose, 1.0 × 20 cm.

elution patterns show that they are more or less the same within a race but one isolate of Race #12 slightly differs from the other two isolates tested (Fig. 6). Elution condition and pH value of the buffer used may be important since some proteins such as hemoglobins were found to be very strict in this sense (Fasold *et al.*, 1963). Again the protein concentration of the extract may be influential.

Discussion

Following the observation on specific activity of lactic dehydrogenase in five races of *P. oryzae*, it seemed to correlate with relative pathogenicity of the fungus to rice plants (Wu *et al.*, 1966). The soluble proteins of fungal mycelium were studied by means of paper electrophoresis and column chromatography on ion exchange cellulose column, since electrophoresis and chromatography were currently explored for their possible utilization in taxonomy (Alston *et al.*, 1963).

From their studies on the soluble proteins of Neurospora, Chang *et al.* (1962) concluded that electrophoresis was an adequate technique for separating and detecting the various compounds in Neurospora protein. They emphasized that some particular proteins could be recognized in this way and that the studies of their synthesis and metabolic significance would become possible. Distinct patterns were observed with 6 different *Pythium* spp. by starch-gel electrophoresis of their proteins (Clare, 1963) and the same technique was also applied successfully to charactrize different taxa in the *formae speciales* of *Fusarium oxysporum* with respect to their patterns of esterase and phosphatase (Meyer *et al.*, 1964). On the other hand, Suskind and Bonner (1960), by electrophoresis, attributed the alterations in ribonucleic acid, protein, and ribonuclease formation of *Neurospora crassa* to something other than gene mutation. Qualitative differences between the paper electrophoretic patterns of two different races of each fungus, i. e. *P. oryzae*, *Cochliobolus miyabeanus*, and *Gibberella fujikuroi* were not found by Tatsuyama (1961). This might be attributed to low current, since this was also found to be the case in the present experiment with six physiologic races *P. oryzae* (Fig. 1-A). However, increase of current from 2.5 mA to 5 mA raised the resolution.

The data from the current investigation are not complete for the accurate evaluation of the biochemical nature of virulence of *P. oryzae*. Nevertheless, the result showed that electrophoretic pattern and elution pattern of mycelial proteins seemed to correlate to the relative pathogenicity of the physiologic races tested. From the inoculation studies of rice plant, Chien and his associates (1965) indicated that the Japanese, but not U. S., differential varieties of rice could be utilized to obtain satisfactory differentiation of the Taiwan races of

P. oryzae. By U. S. differentials, Races #5 and #12 showed a general similarity in host reaction. In this connection, similarity of elution patterns of mycelial proteins prepared from Races #5 and #12 on DEAE-cellulose in the present experiment might be merely a coincidence. On the other hand, Races #12 and #13 were placed under the name "Group P" by Chien with their similar host reaction on 16 differential varieties used in Taiwan.

These facts suggest that using of different set of differential varieties will cause further complication by the heterogeneity of the physiologic races of *P. orzae* or probably by heterocaryosis. Thus, the determination of the relative virulence of a given pathogenic organism by the manifestation of its biochemical nature is thought to be meaningful.

Summary

Electrophoretic separations of mycelial proteins of six physiologic races and three representative isolates of two races of *Piricularia oryzae* showed their characteristic patterns. This was also demonstrated by stepwise elution of the same materials from ion exchange cellulose columns, particularly the anion exchanger, DEAE-cellulose. The possibility on the utilization of biochemical nature of pathogenic organisms in interpretation of their relative virulence was discussed.

稻熱病菌蛋白質之研究

吳龍溪 施正齡

本試驗以電氣泳動法及色層分析法區別六種稻熱病菌生理小種菌絲所含有蛋白質之組成。生理小種內各菌株之蛋白質組成頗相似，表示各生理小種之蛋白質組成具有特殊性。由此可推想分析稻熱病菌蛋白質可能測定其菌株對寄主植物之致病性，以配合寄主植物接種法。

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