Electrophoretic Studies of Alkaline Proteinases from Strains of *Aspergillus flavus* Group

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A large number of papers have been published on the taxonomy of *Aspergilli* during the last few decades. As one of the results of such efforts, new publication of "The Genus *Aspergillus*" by Raper and Fennell¹⁾ was emerged from "A Manual of the *Aspergilli*" by Thom and Raper.²⁾ In addition to this, Murakami³⁾ recently completed the classification of Japanese industrial strains of yellow-green *Aspergilli* and related fungi with the aid of multi-component analysis after laborious and extensive studies for more than ten years. However, as far as the classification of strains of *Aspergillus flavus* group (based on the description by Raper and Fennell in this report) is concerned, there are still some questions on the definition of fungal taxa. Since no true sexual life cycle has been found in any strains of this group, species concept is ambiguous and creation of the species is rather arbitrary.

In a previous paper⁴⁾ the author found that alkaline proteinases from most of the *Aspergillus* species showed species-specific mobility in electrophoresis on acrylamide gel columns. Therefore, application of this technique to the species of a certain group was expected to give some clues to support the established classification of imperfect fungi and to provide evidence at molecular level on establishment of some species in that group. For example, although *A. sojae* was distinguished from *A. oryzae* by Sakaguchi and Yamada⁵⁾ in 1944 on the basis of the nature of conidia walls, this separation was recently confirmed to be reasonable by the electrophoretic studies of al-

kaline proteinases produced by these species.⁴⁾ The aim of this note is to report the results of the similar experiments relevant to the taxonomy and phylogeny of the strains in *A. flavus* group.

Fungal strains were kindly supplied by Dr. H. Murakami, Research Institute of Brewing, Tokyo (RIB) and Culture Collection of the Institute of Applied Microbiology, University of Tokyo (IAM). In this paper *A. sojae* and *A. toxicarius* were respectively separated from *A. oryzae* and *A. flavus* according to the classification by Sakaguchi and Yamada⁵⁾ and Murakami.³⁾

Samples for gel electrophoresis were prepared by the method in the previous paper.⁴⁾ Alkaline proteinases, much or less, were produced on wheat bran solid culture for 72 to 120 hr by all the strains examined but *A. zonatus* RIB 1033 which did not form the proteinase under various cultural conditions. Polyacrylamide gel electrophoresis was accomplished by the method of Davis⁶⁾ for pH 9.4 gels and of Reisfeld *et al.*⁷⁾ for pH 4.0 gels. After a run at 4 mA per gel column at 4°C for 90 min (pH 9.4 gels) or 180 min (pH 4.0 gels), gels were fractionated in 2 mm thick with a gel slicer and extracted overnight with 2 ml of 0.05 M phosphate buffer (pH 7.3) at 4°C. The enzyme activities of the fractionated gels were assayed by the method previously reported,⁸⁾ but expressed as arbitrary units.

Electron micrographs of dry conidia on Formvar-coated grids were taken with a JEM-7A type electron microscope. Mature conidia

FIG. 1. Electron Micrographs of Smooth or Roughened Conidia of Some Strains in *Aspergillus flavus* Group.

(a) *A. oryzae* RIB 81, (b) *A. zonatus* RIB 1033, (c) *A. clavato-flavus* RIB 1034, (d) *A. subolivaceus* RIB 1035, (e) *A. avenaceus* RIB 1036, (f) *A. flavus* RIB 1406. ($\times 3,000$).

for this purpose were obtained by incubation of fungi on Czapek's solution agar slants at 30°C for 3 weeks.

Figures 1 and 2 were the electron micrographs of conidia of the strains used. *A. oryzae*, *A. zonatus*, *A. clavato-flavus*, *A. suboli-*

FIG. 2. Electron Micrographs of Echinulate or Tuberculate Conidia of Some Strains in *Aspergillus flavus* Group.

(a) *A. sojae* RIB 401, (b) *A. sojae* RIB 405, (c) *A. tamarii* IAM 2499, (d) *A. parasiticus* RIB 4032, (e) *A. toxicarius* RIB 4001, (f) *A. flavo-furcatis* RIB 3006. ($\times 3,000$).

vaceus, *A. avenaceus*, and *A. flavus* showed the smooth or somewhat roughened conidia, but *A. sojae*, *A. parasiticus*, *A. toxicarius*, *A. tamarii*,

and *A. flavo-furcatis* exhibited echinulate or tuberculate conidia. As described later, the nature of conidia walls highly correlated with

FIG. 3. Electrophoregrams of Alkaline Proteinases from Strains of *Aspergillus flavus* Group on Polyacrylamide Gel Columns at pH 9.4 for 90 min.

Strains: fur=*A. flavo-furcatis*, ave=*A. avenaceus*, cla=*A. clavato-flavus*, par=*A. parasiticus*, fla=*A. flavus*, soj=*A. sojae*, ory=*A. oryzae*, tam=*A. tamarii*, sub=*A. subolivaceus*, tox=*A. toxicarius*.

FIG. 4. Polyacrylamide Gel Electrophoresis Patterns of Alkaline Proteinases from Strains of *Aspergillus flavus* Group at pH 4.0 for 180 min.

Strains: ave=*A. avenaceus*, fur=*A. flavo-furcatis*, cla=*A. clavato-flavus*, par=*A. parasiticus*, soj=*A. sojae*, fla=*A. flavus*, tam=*A. tamarii*, ory=*A. oryzae*, tox=*A. toxicarius*, sub=*A. subolivaceus*.

the electrophoretic mobilities of alkaline proteinases produced by these species.

Figures 3 and 4 illustrate the electrophoretic patterns of alkaline proteinases formed by the representative strains of *A. flavus* group in polyacrylamide gels at pH 9.4 and pH 4.0, respectively. In either case alkaline proteinases from *A. flavo-furcatis*, *A. tamarii*, *A. parasiticus*, and *A. toxicarius* showed the same electrophoretic mobilities as that from *A. sojae*. It is noteworthy that all these strains produce echinulate or tuberculate conidia (Fig. 2). To the contrary, *A. flavus* and *A. subolivaceus* producing smooth conidia (Fig. 1) secreted alkaline proteinases which have the same

mobilities as the proteinase of *A. oryzae* in polyacrylamide gel electrophoresis under the acid or alkaline condition.

Since the electrophoretic mobility is considered to reflect the net charge, shape, and dimension of the enzyme protein and physico-chemical properties of alkaline proteinase from *A. oryzae* are almost the same to those from *A. flavus*,⁹⁻¹³⁾ the enzyme from *A. subolivaceus* seems to be identical or very much homologous to that from *A. oryzae*, although any informations are not yet available about analytical data such as amino acid composition, amino acids at amino- and carboxyl-terminals, and molecular weight of *A. suboli-*

vaceus enzyme. Similarly, the enzymes from *A. tamarii*, *A. flavo-furcatis*, *A. parasiticus*, and *A. toxicarius* may be identical or homologous to that from *A. sojae*.

The electrophoretic mobility of alkaline proteinase from *A. clavato-flavus* was between those from *A. oryzae* and *A. sojae* in pH 9.4 gels and was the smallest of all enzymes examined in pH 4.0 gels. On the other hand, the proteinase from *A. avenaceus* moved fastest at pH 9.4 gels and migrated in the midst of those from *A. clavato-flavus* and *A. oryzae* at pH 4.0 gels. Changes in the order of mobilities of the enzymes from these species in acid and alkaline gels are not strange, since the fact that two proteins possessing identical isoelectric points will not necessarily have identical mobilities throughout the whole pH range is already known by the mobility-pH curves of purified human serum proteins and of bovine serum albumin.¹⁴⁾ Therefore, alkaline proteinases of *A. flavus* group can be electrophoretically classed into 4 types re-

presented by the enzymes from *A. oryzae*, *A. sojae*, *A. clavato-flavus*, and *A. avenaceus* (Table I).

The results of multi-component analyses of the morphological and physiological characters of a number of strains in *A. flavus* group by Murakami¹⁵⁾ indicate that *A. clavato-flavus* is very close to *A. oryzae* and that *A. zonatus*, *A. subolivaceus*, and *A. avenaceus* are close to *A. flavus*. However, the electrophoretic studies on alkaline proteinases produced by these strains apparently differentiated *A. clavato-flavus* and *A. avenaceus* from other species. The electrophoretic classification again supported the rationality of the separation of *A. toxicarius* from *A. flavus* based on the properties of conidial surfaces by Murakami.³⁾

Moreover, the above results suggest some phylogenetic relationship among the species in *A. flavus* group. The species producing the proteinases with the same electrophoretic mobilities may be phylogenetically closer each other than other species producing the proteinases with different mobilities in this group, although more analytical data are necessary to establish this speculation.

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TABLE I. THE NATURES OF CONIDIA WALLS AND ELECTROPHORETIC TYPES OF ALKALINE PROTEINASES PRODUCED BY SPECIES OF *Aspergillus flavus* GROUP

Species classed by Raper & Fennell	Species assigned by Murakami ^{a)}	Conidia	Alkaline proteinase ^{b)}
<i>A. flavus</i>	<i>A. flavus</i>	smooth	ory
	<i>A. toxicarius</i>	echinulate	soj
<i>A. parasiticus</i>	<i>A. parasiticus</i>	echinulate	soj
<i>A. oryzae</i>	<i>A. oryzae</i>	smooth	ory
	<i>A. sojae</i>	echinulate	soj
<i>A. zonatus</i>	(<i>A. flavus</i>)	smooth	—
<i>A. clavato-flavus</i>	(<i>A. oryzae</i>)	smooth	cla
<i>A. tamarii</i>	<i>A. tamarii</i>	tuberculate	soj
<i>A. flavo-furcatis</i>	(<i>A. tamarii</i>)	tuberculate	soj
<i>A. subolivaceus</i>	(<i>A. flavus</i>)	smooth	ory
<i>A. avenaceus</i>	(<i>A. flavus</i>)	smooth	ave

^{a)} The species described by Raper & Fennell are very close to the species in parentheses according to the classification by Murakami.

^{b)} ory=*A. oryzae* type, soj=*A. sojae* type, cla=*A. clavato-flavus* type, ave=*A. avenaceus* type.

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