Differentiation of *Aspergillus sojae* from *Aspergillus oryzae* by Polyacrylamide Gel Disc Electrophoresis

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SUMMARY

Alkaline proteinases from strains of *Aspergillus oryzae* and *A. sojae* migrated with species-specific mobility in electrophoresis on polyacrylamide gels; the protein patterns were not affected by age or cultural conditions. The results support the establishment of *A. sojae* as a species distinct from *A. oryzae*.

INTRODUCTION

Biochemical characters have been used by many investigators to supplement other kinds of taxonomic data. For example, three *Penicillium* species were distinguished by their respective protein patterns on acrylamide gels (Bent, 1967) and zymograms provided a means of identifying 15 species of the genus *Aspergillus* (Nealson & Garber, 1967).

Sakaguchi & Yamada (1944) separated *Aspergillus sojae* from *A. oryzae* on differences in conidial surface; *A. sojae* having echinulate conidia and *A. oryzae* smooth to roughened conidia. In the present investigation polyacrylamide gel disc electrophoresis was used to characterize alkaline proteinases from these same species.

METHODS

Organisms. Tables 1 and 2 show the organisms used. Most of them were identified and kindly supplied by Dr H. Murakami, Research Institute of Brewing, Tokyo (RIB). Some were obtained from the Culture Collection of the Institute of Applied Microbiology, University of Tokyo (IAM).

Culture methods. The fungus was usually grown at 30 °C for 72 h in stationary flasks on a solid medium or in shaken flasks on a liquid medium. The solid medium consisted of 5 g of wheat bran moistened with 4 ml of tap water in 150 ml conical flasks. The liquid undefined medium consisted of defatted soybean powder, 0.5 g; wheat bran, 2 g; and tap water, 100 ml. The liquid semidefined medium contained glucose, 1 g; peptone, 0.4 g; KH_2PO_4 , 3 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 10 mg; ZnCl_2 , 1 mg; and distilled water, 100 ml, adjusted to pH 6.5 with NaOH, in 500 ml shaking flasks.

Browning of rice koji, production of kojic acid and pink coloration of conidia on medium containing anisic acid were examined by the methods described by Murakami *et al.* (1965).

Preparation of samples for electrophoresis. The enzymes were precipitated from cooled distilled water culture extracts or culture filtrates by addition of two volumes of ethanol at -20°, dissolved in a small volume of distilled water and used for gel electrophoresis after removal of insoluble substances by centrifugation at 4000 g for 5 min.

Electrophoresis. In general, the procedure described by Davis (1964) was followed for disc electrophoresis on polyacrylamide gels. However, samples were applied in 20% (w/v)

Table 1. *Source and some physiological properties of Aspergillus oryzae strains*

The strains designated by their accession numbers in the culture collections of the Research Institute of Brewing, Tokyo (RIB) and the Institute of Applied Microbiology, University of Tokyo (IAM). Browning of rice koji (a) was scored after 40 h incubation at 30 °C. Production of kojic acid (b) was examined by addition of 1% FeCl₃ solution to 15-day culture on the rice koji extract medium. Pink coloration of conidia (c) was observed after culture for 30 days on slants of the modified Czapek's agar containing anisic acid.

Strain	Recovered from or same as	Rice koji browning (a)	Kojic acid production (b)	Colour shift of conidia (c)
RIB 67	'Sake' koji	—	+	+
RIB 81	'Sake' koji	+	+	+
RIB 83	'Sake' koji	—	+	+
RIB 127	'Sake' koji	—	—	+
RIB 216	'Sake' koji	+	—	—
RIB 333	'Miso' koji	+	—	—
RIB 430	Fukuda's strain	+	—	—
RIB 609	'Sake' and 'Miso' koji	+	—	+
RIB 1031	<i>A. oryzae</i> WB447 (Thom no. 113)	+	+	—
RIB 1032	<i>A. flavus</i> WB482 (Thom no. 108)*	—	+	+
IAM 2600	.	—	+	+
IAM 2625	.	+	—	+
IAM 2630	.	—	—	—
IAM 2633	.	—	—	—
IAM 2639	.	+	—	—
IAM 2645	.	+	+	—
IAM 2651	.	+	+	+
IAM 2681	.	—	+	—
IAM 2683	.	—	—	—
IAM 2684	.	—	—	+
IAM 2691	.	+	—	+
IAM 2706	.	+	+	—
IAM 2722	.	+	+	+

* Identified as *A. oryzae* by Dr H. Murakami.

sucrose solution and spacer gels were omitted. After a run at 4 mA per gel column at 4 °C for an appropriate period, frozen gels were sliced laterally into 2 mm strips, extracted for 4 h to overnight at 4 °C with 2 ml of 0.05 M-phosphate buffer at pH 7.3 and assayed for alkaline proteinase. Comparisons were limited to tubes run simultaneously.

Assay of enzyme activity. Alkaline proteinase activity was determined by the modified Anson's method (Anson, 1938). The reaction mixture contained 2 ml of gel extract and 1 ml of 1.5% milk casein (Hammersten grade, E. Merck Ag, Darmstadt, Germany) in 0.05 M-phosphate buffer at pH 7.3. The reaction mixture was incubated at 30 °C for a suitable period depending on enzyme activity of the sample applied to electrophoresis. The reaction was stopped by addition of 3 ml of 7.5% trichloroacetic acid (TCA) solution. The amount of TCA-soluble non-proteinous substances showing positive reaction to the Folin's phenol reagent (Folin & Ciocalteu, 1927) was determined at 660 nm. One unit of proteinase activity is the amount of enzyme which will liberate an amount of the above non-proteinous substances equivalent to 1 µg of tyrosine/min.

Electron microscopy. To obtain mature conidia for electron microscopy the fungi were grown on Czapek's slopes at 30 °C for 21 days. Electron micrographs of dry conidia on collodion-coated grids were taken with a Hitachi HS-6 type electron microscope.

Table 2. Source and some physiological properties of *Aspergillus sojae* strains*

Strain	Recovered from or same as	Rice koji browning (a)	Kojic acid production (b)	Colour shift of conidia (c)
RIB 328	'Shoyu' koji	—	+	+
RIB 401	'Shoyu' koji	—	+	+
RIB 405	'Shoyu' koji	—	+	+
RIB 410	'Shoyu' koji	—	+	+
RIB 1011	<i>A. oryzae</i> IAM 2654†	+	—	—
RIB 1023	<i>A. sojae</i> IAM 2666	—	+	+
RIB 1024	<i>A. sojae</i> IAM 2678	—	+	+
RIB 1040	<i>A. parasiticus</i> IAM 2150†	+	+	—
RIB 1041	<i>A. sojae</i> IAM 2631	+	+	—
RIB 1042	<i>A. sojae</i> IAM 2664	—	+	—
RIB 1044	<i>A. sojae</i> IAM 2668	—	—	—
RIB 1045	<i>A. sojae</i> IAM 2669	+	+	—
RIB 1046	<i>A. sojae</i> IAM 2674	—	+	+
RIB 1047	<i>A. sojae</i> IAM 2677	—	+	—
RIB 1048	<i>A. sojae</i> IAM 2687	+	—	—
RIB 1049	<i>A. sojae</i> IAM 2703	+	+	—
RIB 1050	<i>A. sojae</i> IAM 2718	—	+	+
RIB 1051	<i>A. sojae</i> IAM 2723	—	—	—
RIB 1052	<i>A. parasiticus</i> HUT 2076†	+	+	—
RIB 1054	<i>A. parasiticus</i> HUT 2148†	—	+	+
RIB 1059	<i>A. parasiticus</i> IFO 4204†	+	+	+

HUT = Faculty of Engineering, Hiroshima University.

IFO = Institute for Fermentation, Osaka.

* See the sub-legend of Table 1 for notes.

† Identified as *A. sojae* by Dr H. Murakami. Fig. 1. Electrophoretic mobility of alkaline proteinases from *Aspergillus oryzae* (O) and *A. sojae* (●) at different periods of electrophoresis. (a) 30 min, (b) 60 min, (c) 120 min.

RESULTS AND DISCUSSION

Aspergillus oryzae had conidia with smooth surfaces whilst *A. sojae* had conidia with echinulate surfaces. Physiological properties could not be used to distinguish these two species (Tables 1, 2).

Fig. 1 shows the time course of electrophoresis of alkaline proteinases from *Aspergillus oryzae* and *A. sojae*. The difference in electrophoretic mobility of the enzymes increased with time. This difference was not due to experimental variation.

The electrophoretic mobility of alkaline proteinase was found to be species-specific, since the mobility was identical for all strains of the same species. The only exception was the enzyme from *Aspergillus sojae* RIB 1048, which showed the mobility of the *A. oryzae* enzyme. Since this strain was very similar to *A. oryzae*, except that it formed echinulate conidia, it may be a form intermediate between the two species (Neurath, Walsh & Winter, 1967; Smith, 1970).

It is well known that the quantitative production of alkaline proteinases by these species is affected by cultural conditions. However, neither age nor cultural conditions altered the electrophoretic properties of the enzyme of either species. Such constancy of the electrophoretic mobility was also observed for samples of several strains of both the species prepared from culture filtrates of the liquid undefined medium and liquid semidefined medium.

The alkaline proteinases from *Aspergillus oryzae* and *A. sojae* have been purified by Subramanian & Kalnitsky (1964), Nordwig & Jahn (1968) and Hayashi, Fukushima & Mogi (1967). Although the enzymes had many properties in common they differed in amino acid composition and molecular weight. These results are consistent with the observed differences in electrophoretograms of the enzymes since electrophoretic mobility is considered to reflect the net charge, shape and dimension of an enzyme protein molecule.

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