Paper Electrophoretic Studies on the Diastatic Enzymes of Molds

Part I. Electrophoretic Technique and the Behaviours of Diastatic Enzymes Observed on Papers.

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Received June 25, 1955

A detailed procedure for the separation of diastatic enzymes of molds was established, which was based on their electrophoretic behaviours on a paper strip. For the detection of enzymes, a bioautographical method was newly devised. Comparing the enzymatic images appeared on the paper strip, the authors classified mold enzyme systems into five types.

In Japan and other Asian countries, various kinds of molds such as *Aspergillus*, *Penicillium*, *Rhizopus*, and *Mucor* are known to be valuable converting agents for the preparation of foods and wines from grains and potatoes. Among numerous reports concerning the mold enzyme system, the following two reports are outstanding owing to their systematic studies. According to Kitahara¹⁾, who investigated the diastatic enzyme systems of molds by using iodine color reaction and saccharifying activity against soluble starch as the assay methods, these molds might be classified into six types in respect to their enzymatic composition, in contrast to Okazaki's view²⁾ who classified the mold enzyme systems into five types by using different strains and another biometric technique.

The experiments which are reported here and the following one were carried out with the same idea as those referred to above, but in a different way to elucidate the actual composition of mold

enzyme systems by making them visible on a filter paper.

Methods

Preparation of Enzyme Solution—The following twelve kinds of mold were studied in this experiment,

Aspergillus oryzae (0-8-1), *Asp. sojae* (0-13-8), *Asp. glaucus* (A-1-8), *Asp. usamii* (1-1 R15-0635), *Asp. niger* (A-9-9), *Asp. awamori* (K-2 0913), *Asp. inui* (6911), *Rhizopus tonkinensis* (R-6-4), *Rh. javanicus*, *Rh. peha* (IR-2-4), *Penicillium chrysogenum* (Q-174), *Mucor spinescens* (Mu-3)

The wheat-bran, inoculated with the mold and incubated at 30° for 4 days, was extracted for 15-17 hours with 3 parts of distilled water in the presence of toluene. The enzyme fraction was precipitated from the filtrate by adding absolute alcohol to the final concentration of 75%. The precipitate thus obtained was washed with absolute alcohol and dried in a vacuum-desiccator.

1, filter paper strip; 2, buffer vessel; 3 carbon electrode vessel; 4, 1 M KCl-3% agar bridge; 5, cock; 6, carbon electrode; 7, glass cover.

Fig. 1. Apparatus employed for Electrophoretic Purpose.

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1) K. Kitahara M. Kurushima, *J. Ferm Tech. Japan* **27** 1, 44, 182, 213, 254 (1949), **28** 106, 388, 422 (1950).

2) H. Okazaki, *J. Agr. Chem. Soc. Japan*, **24**, 88 (1950).

Fig. 2. Bioautographic Technique.

About 0.01ml. aliquot of the enzyme solution was used in the electrophoretic experiment.

Electrophoresis—The apparatus employed in this experiment is shown in Fig. 1. The buffer vessels and the carbon electrode vessels were connected by means of KCl-agar bridges to keep the paper clean from electrode products. By operating the apparatus at the potential gradient of 10 V per cm, satisfactory results were obtained. The strips of paper (Toyo filter paper No. 50), wetted with 0.05 M phosphate buffer and blotted on a dry filter paper to remove any excess moisture, were assembled to the apparatus. When equilibrium reached after thirty minutes, 0.01~0.03 ml. aliquots of the sample were spotted on the starting line of strips, and electromigration was carried out for five hours at room temperature.

Detection of the Enzyme Locus—Two methods were employed for this purpose. The first, rely-

ing on the iodine-starch reaction, served for the detection of dextrinogenic amylase* and the other, relying on bioautographic procedure and being devised by the authors, served for the detection of saccharogenic amylase* and maltase*.

For the detection of saccharogenic amylase, the paper strip was placed on the surface of a thin layer of 2% starch agar plate (medium-D) in which the cells of *Saccharomyces cerevisiae* Rasse II had been previously suspended. After two hours contact, the paper strip was removed. The agar plate thus printed was placed again in an incubator for twenty hours at 30°. (see Fig. 2) During this period, the enzymatic reaction proceeded and the places corresponding to enzyme activities were made visible by the growth of yeast only on saccharified portions of the agar plate. On the whole, it was difficult for us to

* Further definition of these amylases will be given in the following paper.

Table I
Composition of the Media tested for Bioautography.

Constituent	Medium				
	A	B	C	D	E
Asparagine			0.25%		
Peptone				0.5%	1.0%
Beef ext.					1.0%
NH ₄ H ₂ PO ₄		0.2%			
K ₂ HPO ₄	0.5%		0.1 %		
KH ₂ PO ₄		0.2%		0.5%	
MgSO ₄ ·7H ₂ O	0.2%	0.2%	0.3 %	0.2%	
NaCl					0.5%

Soluble starch (as in Table II.) and agar (2%) were supplemented to the individual medium named A~E.

Table II
Selection of Suitable Medium for the Detection of Saccharogenic Amylase.

Medium	Time (hours)	Concentration of Soluble Starch			Remark
		none	1%	2%	
A	20	—	±	±	
B	20	—	+	++	
C	20	—	+	++	
D	20	—	+++	++++	suitable
E	20	—	+++	++++	contamination

The growth rate of *Saccharomyces cerevisiae* Rasse II on these agar plate were recorded, using Taka-diastase as a converting agent.

Table III
Selection of Suitable Test-organism for the Detection of Maltase.

Test-organism	Growth on the agar plate	
	20 hrs.	40 hrs.
<i>Zygosacch.</i> sp. No. 1	+	+++
<i>Zygosacch. sake</i> var K	++	+++
<i>Torulopsis</i> sp. No. 28	+	++
<i>Sacch. presisus</i>	+++	++++

Medium D supplemented with maltose (2%) and agar (2%) was used in this experiment.

detect saccharogenic amylases on a paper strip by the iodine-reaction, but this difficulty was easily overcome by the employment of bioautographic technique, the identification of enzymes being extremely simplified. The compositions of starch agar medium and other related media are shown in Tables I and II.

For the detection of dextrinogenic amylase, the paper strip removed from the agar plate was dried at 110° for five minutes, and then sprayed with 0.01 N iodine solution. The position of dextrinogenic amylase was indicated as a well-defined clear spot depending on the reaction product.

For the detection of maltase, another bioautographic technique was employed. In this case, maltose was added to the medium D instead of soluble starch, and *Sacch. presisus* was employed as test-organism in place of *Sacch. cerevisiae* Rasse II. As shown in Table III, *Sacch. presisus* proved to be the most suitable among these five strains tested, because it showed good growth on the medium only after the added maltose was converted to glucose by maltase.

Results

Electrophoretic Behaviors of Dextrinogenic Amylases—Electromigration was carried out at room temperature for five hours in 0.05 M phosphate buffer (pH 7.6) at the potential of 300 D.C. volts and at the current of 0.3 m. amp/cm. Fig. 3 shows the colored zones of dextrinogenic amylases which appeared on paper strips treated with iodine solution. In contrast to the fact that the spots corresponding to the dextrinogenic amylases of *Asp. oryzae* (+41 mm), *Asp. sojae* (+41 mm),

Asp. glaucus (+40 mm), *Asp. usamii* (+39 mm), *Asp. awamori* (+41 mm), *Asp. inui* (+40 mm), *Rh. pèka* (+41 mm), *M. spinescens* (+39 mm), and *Pen. chrysogenum* (+41 mm) were detected on the anode side with or without tailing effect, those of *Rh. tonkinensis* (−18 mm) and *Rh. javanicus* (−18 mm) were found on the cathode side.

Electrophoretic Behaviors of Saccharogenic Amylases—Electromigration was carried out in the same condition as mentioned above, and the bioautographic technique using *Sacch. cerevisiae* Resse II as the test-organism was employed in this experiment. As shown in Fig. 4, saccharogenic amylases of *Asp. oryzae* and *Asp. sojae* moved towards the anode side (+12 mm and 12 mm) although with lower mobilities than the dextrinogenic amylases of those molds. So far as *Asp. usamii* is concerned, three spots were detected on the anode side (+60 mm, +37 mm, +24 mm), among which two (+60 mm and 24 mm) were saccharogenic amylases without dextrinogenic activity and one (+37 mm) was the mixture of the two enzymes. One component each of the saccharogenic amylases of *Asp. awamori* and *Asp. inui* moved independently from their dextrinogenic amylases towards the anode (+22 mm). But the spots corresponding to saccharogenic amylases of *Rh. tonkinensis* (−40 mm), *Rh. javanicus* (−40 mm), *Rh. pèka* (−12 mm), *M. spinescens* (−14 mm),

Fig. 3. Electrophoretic Behaviours of Dextrinogenic Amylases (detected by iodine-starch reaction).

Fig. 4. Electrophoretic Behaviours of Saccharogenic Amylases (detected by bioautography).

Fig. 5. Electrophoretic Behaviours of Maltases (detected by bioautography).

and *Pen. chrysogenum* (-11mm) were found on the cathode side. (see Fig. 4)

Electrophoretic Behaviors of Maltases—The spots corresponding to maltase of *Asp. oryzae*, *Asp. sojae*, and *Asp. glaucus* were found on the anode side considerably apart from the starting line. On the contrary, the maltase spots of the other strains were discovered on the cathode side (-12~-18 mm). (see Fig. 5)

Effects of Heat-treatment on Enzyme Action—Following the description given by Okazaki, the enzyme solutions were treated at pH 2.5 and at 40° for thirty minutes. The results are summarized in Table IV.

Discussion

The data presented in this paper may be in favor of the following view, in which the mold enzyme systems were classified into five types from enzymologic

point of view. (see Table IV)

Asp. oryzae type—All of the detected amylases, e.g. dextrinogenic amylase (non-resistant against the heat-treatment), saccharogenic amylase (resistant), and maltase (resistant) showed the tendencies to move towards the anode when they were analysed electrophoretically. The enzyme systems of *Asp. sojae* and *Asp. glaucus* may be classified in this type.

Asp. usamii type—Three spots of amylases were found on the anode side, among which two of them (+60 mm and +24mm) may be resistant saccharogenic amylases, and the other one (+37 mm) a less resistant dextrinogenic amylases. The maltase of this type of mold was definitely characterized by its migration towards the cathode.

Asp. awamori type—The dextrinogenic amylase (resistant) and the saccharogenic amylase (resistant) migrated towards the

Table IV
Classification of Molds by their Enzymatic Constitution.

Composition of Enzyme System				Mobility (pH 7.6)	Homologous Strains
Typical Strain					
<i>Asp. oryzae</i>	(1)	(+) type, non-res.,	D. A.	+2.9	<i>Asp. oryzae</i>
	(2)	(+) type, res.,	S. A.	+0.8	<i>Asp. sojae</i>
	(3)	(+) type, res.,	M.	+0.6	<i>Asp. glaucus</i>
<i>Asp. usamii</i>	(1)	(+) type, res.,	S. A.	+4.2	<i>Asp. usamii</i>
	(2)	(+) type, poorly res.,	D. A.	+2.9	
	(3)	(+) type, res.,	S. A.	+1.7	
	(4)	(-) type, res.,	M.	-0.8	
<i>Asp. awamori</i>	(1)	(+) type, poorly res.,	D. A.	+2.9	<i>Asp. awamori</i> <i>Asp. inui</i>
	(2)	(+) type, res.,	S. A.	+1.6	
	(3)	(-) type, res.,	M.	-1.4	
<i>Rh. tonkinensis</i>	(1)	(-) type, non-res.,	D. A.	-1.2	<i>Rh. tonkinensis</i> <i>Rh. javanicus</i>
	(2)	(-) type, res.,	M.	-1.3	
	(3)	(-) type, res.,	S. A.	-2.8	
<i>M. spinescens</i>	(1)	(+) type, non-res.,	D. A.	+2.8	<i>M. spinescens</i>
	(2)	(-) type, non-res.,	S. A.	-0.8	<i>Rh. péka</i>
	(3)	(-) type, non-res.,	M.	-1.3	<i>Pen. chrysogenum</i>
(+) type				migrated towards anode side	
(-) type				migrated towards cathode side	
non-res.				non-resistant against the treatment	
res.				resistant against the treatment	
D. A.				dextrinogenic amylase	
S. A.				saccharogenic amylase	
M.				maltase	
Mobility				cm/V. Sec.	

anode side, in contrast to maltase (resistant) which migrated towards the cathode side. The enzyme system of *Asp. inui* may belong to this type.

Rh. tonkinensis—All of the three spots migrated towards the cathode side. The dextrinogenic amylase was non-resistant, but saccharogenic amylase and maltase were resistant against the treatment. *Rh. javanicus* may be one member of this type.

M. spinescens type—The dextrinogenic amylase migrated towards the anode side. The saccharogenic amylase and maltase of this type appeared on the cathode side, not so far from the starting line. All of them were non-resistant. *Rh. péka* and *Pen. chrysogenum* may be of this type.

It is very interesting that the classifica-

tion presented here shows very good agreement with those of Kitahara and Okazaki in spite of the fact that these three classifications were based on different methods. And the authors believe this work should be evaluated from the view point that the various kinds of diastatic enzyme of molds were successfully separated and became visible on a filter paper.

Acknowledgment

We wish to thank Prof. K. Sakaguchi and Dr. S. Iida for their valuable advice. The test-organisms used in this investigation were kindly supplied from the Institute of Applied Microbiology, University of Tokyo.