LOCALIZATION OF ENZYMES IN THE MYCELIUM AND MICROCONIDIA OF *FUSARIUM OXYSPORUM*¹

YOSHIHARU MARUYAMA² AND MARTIN ALEXANDER

Laboratory of Soil Microbiology, Department of Agronomy, Cornell University, Ithaca, New York

Received for publication February 21, 1962

ABSTRACT

MARUYAMA, YOSHIHARU (Cornell University, Ithaca, N.Y.) AND MARTIN ALEXANDER. Localization of enzymes in the mycelium and microconidia of *Fusarium oxysporum*. J. Bacteriol. **84**:307-312. 1962—Extracts prepared from mycelium and microconidia of *Fusarium oxysporum* f. *cubense* were fractionated into a soluble and four particulate fractions by differential centrifugation, and the distribution of several enzymes in the isolated cell constituents was examined. Succinic dehydrogenase, cytochrome oxidase, and a large amount of the reduced diphosphopyridine nucleotide (DPNH) cytochrome *c* reductase and reduced triphosphopyridine nucleotide cytochrome *c* reductase were associated with one of the particulate fractions prepared from the hyphae; fumarase and DPNH oxidase activities were largely found in the soluble and in a second particulate fraction. The highest recovery and concentration of diphosphopyridine nucleotidase was observed to be bound to a third type of hyphal granule. Aldolase, aconitase, glucose-6-phosphatase, and uricase were recovered entirely with the soluble mycelium constituents. Similar enzyme-distribution patterns were observed in microconidia. Several enzymatic activities of the mycelial extracts were compared with those in the extracts of microconidia.

By comparison with the extensive information available on the metabolic function of subcellular structures of bacteria, little is known concerning the biochemical role of the intracellular structures of either fungus mycelium or spores. Cytological investigations of a number of fungal genera, however, have revealed the presence of nuclei, mitochondria, an endoplasmic reticulum, and other as

yet uncharacterized morphological entities. To ascertain the metabolic role of certain of these structures, Zalokar (1960) examined by cytochemical means the localization of enzymes in *Neurospora*. Biochemically active particles, on the other hand, have been isolated from extracts of the mycelium of *Aspergillus oryzae* (Iwasa, 1960) and *Fusarium oxysporum* f. *lini* (Kikuchi and Barron, 1959), and the distribution of several enzymes has been described.

In a previous report (Maruyama and Alexander, 1962), the isolation and chemical composition of particles from hyphae and microconidia of *F. oxysporum* f. *cubense*, the cause of a wilt of bananas known as Panama disease, were described. The present communication is concerned with the localization of enzymes in hyphae and microconidia of the fungus and a comparison of enzymatic activities of the mycelium and spores. For purposes of relating specific structures with individual metabolic functions, the three requirements of Alexander and Wilson (1955) were considered, namely, that the recovery of enzymatic activities of the isolated fractions should approach 100% of that found in the original extract, that a high percentage of the total activity must reside in the fraction to which the metabolic activity is being attributed, and that the concentration of the enzyme in the isolated fraction must be equal to or greater than that in the initial cell extract.

MATERIALS AND METHODS

Two media were used: a sucrose-nitrate solution and a V-8 juice medium. The former medium contained sucrose, 20 g; KNO₃, 5 g; K₂HPO₄, 2 g; KH₂PO₄, 0.5 g; yeast extract, 2.5 g; MgSO₄·7H₂O, 0.45 g; FeCl₃·6H₂O, 0.05 g; ZnSO₄, 0.02 g; CuSO₄, 1 μg; Na₂MoO₄·2H₂O, 1.5 μg; and distilled water to 1 liter. The V-8 juice medium was prepared by diluting 1.5 liters of V-8 juice (Campbell Soup Co.) to about 3.0 liters with distilled water, adjusting the pH to 7.2 with NaOH, and bringing

¹ Agronomy Paper No. 546.

² Present address: Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo, Tokyo, Japan.

the liquid to a boil. After cooling, the solution was filtered and the clear filtrate made to 10 liters with distilled water.

The fungus was grown with continuous aeration in 6.0 liters of medium at 30 C from a 200-ml inoculum. Cultures were harvested from the synthetic medium at the end of 2 days, and after 3 days from the V-8 juice medium. Large quantities of microconidia were obtained from the V-8 juice culture, and mycelium from the sucrose-nitrate broth. The separation of microconidia and mycelium was achieved by filtering the cultures through 10 sheets of cheesecloth that had been placed on a 250-mesh sieve. Mycelium contaminated with some spores was retained on the cloth, but the adhering conidia were removed from the mycelium by washing. Microconidia were recovered from the original filtrate by centrifugation, and the spores as well as the hyphae were washed with a cold 20% sucrose solution containing 0.01 M $MgCl_2$ and 0.01 M phosphate buffer (pH 7.4). Extracts of the washed cells were prepared by manual grinding, and the extracts were fractionated into four particulate fractions— P_1 (3,000 $\times g$ for 10 min), P_2 (17,000 $\times g$ for 30 min), P_3 (100,000 $\times g$ for 60 min), and P_4 (144,000 $\times g$ for 120 min)—and into a soluble portion (S), by differential centrifugation. Details of the procedures for disruption and fractionation have been described by Maruyama and Alexander (1962).

For each enzyme assay, the extract, supernatant fluid, or particles were diluted in the solution used for cell disruption to a concentration suitable for measurement. In these diluted preparations, the activity was proportional to the quantity of enzyme solution used in the assay. When the endogenous activity for a particular determination was high, the preparations were dialyzed overnight against a 20% sucrose solution at 4 C.

All spectrophotometric measurements were performed in a Beckman model DU spectrophotometer with cuvettes having a light path of 1.0 cm, the unit of activity being taken as equal to a change in optical density of 1.0 per min at room temperature. A unit was considered as equivalent to the μ liters of O_2 consumed per hr at 30 C for ascorbic acid oxidase, μ moles of substrate turnover per 10 min at 30 C for diphosphopyridine nucleotidase, μ moles of phosphate liberated in 10 min at 30 C for aldolase, and

μ moles of phosphate released in 30 min at 37 C for phosphatase. The specific activity was expressed in terms of units per mg of protein in the fraction tested.

Cytochrome oxidase was assayed essentially by the method of Cooperstein and Lazarow (1951), using the units proposed by these authors. Fumarase and aconitase activity were determined by Racker's (1950) procedure, aldolase as described by Taylor (1955), uricase by the method of Schneider and Hogeboom (1952), ascorbic acid oxidase essentially by Mandels' (1953) method, isocitritase by a spectrophotometric assay (Dixon and Kornberg, 1959), and pyridine nucleotidase activities essentially by the method of Kaplan (1955).

Succinic dehydrogenase was determined manometrically and spectrophotometrically, with 2,6-dichlorophenol indophenol, methylene blue, or potassium ferricyanide as electron acceptors, but the spectrophotometric technique using 2,6-dichlorophenol indophenol was the final method adopted. The reaction mixture contained 0.1 ml of 0.2 M sodium succinate, 0.1 ml of 0.1 M KCN, 0.1 ml of a 0.03% solution of the indicator, 1.0 ml of 0.1 M phosphate buffer (pH 7.3), 0.1 to 0.2 ml of the cell fraction, and water to a final volume of 3.0 ml.

Reduced diphosphopyridine nucleotide (DPNH) oxidase was estimated by following the decrease in optical density at 340 $m\mu$ in a reaction mixture that contained 0.1 ml of 0.003 M DPNH (Sigma Chemical Co.), 0.5 ml of phosphate buffer (pH 7.0), 0.1 ml of enzyme solution, and water to 3.0 ml. DPNH cytochrome *c* reductase and reduced triphosphopyridine nucleotide (TPNH) cytochrome *c* reductase were determined by measuring the change in optical density at 550 $m\mu$ in a solution that contained 0.2 ml of 0.006 M DPNH or TPNH (Sigma), 1.0 mg of cytochrome *c*, 0.3 ml of 0.01 M KCN, 0.5 ml of 0.1 M phosphate buffer (pH 7.4), enzyme solution, and water to 3.0 ml. Catecholase content was estimated by observing the increase in optical density at 410 $m\mu$ in a reaction mixture made up of 0.5 ml of 0.5 M catechol, 1.0 ml of 0.1 M phosphate buffer (pH 6.0), enzyme preparation, and water to 3.0 ml.

Glucose-6-phosphatase activity was assayed according to the method of Swanson (1955). The reaction mixture contained 0.1 ml of 0.1 M glucose-6-phosphate, 0.5 ml of 0.1 M glycylglycine buffer (pH 6.5), dialyzed enzyme solution, and

water to a final volume of 1.1 ml. The mixture was incubated at 37 C for 15 min and, after the reaction was terminated by the addition of 1.2 ml of 20% trichloroacetic acid, the concentration of inorganic phosphate was determined by the method of Fiske and SubbaRow (1929). Phosphatase activity was measured using α - and β -glycerophosphate as substrates. The reaction mixture contained 0.2 ml of 0.2 M substrate, 0.4 ml of 0.2 M tris(hydroxymethyl)aminomethane buffer, 0.1 ml of 0.1 M $MgCl_2$, dialyzed enzyme solution, and water to 2.0 ml. The mixture was incubated at 37 C for 30 min, at which time 2.0 ml of 20% trichloroacetic acid were added. After centrifugation, inorganic phosphate in the mixture was determined by the method of Fiske and SubbaRow (1929).

Samples for protein analysis were extracted with cold 7% trichloroacetic acid and 95% ethanol. The protein was determined as described by Lowry et al. (1951).

RESULTS

The percentage of activity of the mycelial extract recovered after fractionation varied, depending upon the enzyme in question. In certain instances, not all of the initial activity was recovered after centrifugal fractionation; for example, only some 70% of the DPNH cytochrome *c* reductase was found in the various particulate and nonparticulate fractions. By contrast, the succinic dehydrogenase recovery was significantly greater than 100%.

The succinic dehydrogenase and cytochrome oxidase of the mycelium were associated largely with the P_2 granules, and only a small percentage of the recovered activity was observed in the largest particle type (Table 1). Assay of the supernatant fluid failed to show the presence of these two enzymes. Almost three-fourths of the DPNH cytochrome *c* reductase also resided in the P_2 fraction. On the other hand, roughly equal amounts of the TPNH cytochrome *c* reductase activity was found in the P_2 and P_3 fractions.

Among the several hyphal enzymes examined (Table 1), the only one which was recovered in higher yield in the P_3 than in other fractions was diphosphopyridine nucleotidase, but cleavage of the coenzyme occurred with preparations from other cell fractions as well. The optimal pH range for this nucleotidase was in the vicinity of pH 8.0 to 8.5, and the fungal enzyme was not inhibited by 0.05 M nicotinamide; only at 0.33 M nicotinamide was there a significant (70%) inhibition. Extracts of *F. oxysporum* hyphae and conidia also exhibited triphosphopyridine nucleotidase activity, its specific activity being roughly half that of diphosphopyridine nucleotidase.

All of the uricase and the bulk of the aldolase, aconitase, and glucose-6-phosphatase were recovered in the supernatant fraction. Although the P_4 and nonparticulate constituents were not separated prior to assay, in a subsequent examination 115% of the aldolase activity was not sedimented by centrifugation at $144,000 \times g$ for 2 hr. With three of the enzymes, DPNH

TABLE 1. Recovery of enzyme activity in fractions of *Fusarium oxysporum* mycelium

Enzyme	Recovery, % of activity of extract				
	P_1	P_2	P_3	P_4	S
Diphosphopyridine nucleotidase.....	20*		46	9.4	5.8
DPNH oxidase.....	51*		15	0.58	41
DPNH cytochrome <i>c</i> reductase.....	2.3	49	6.0		13†
TPNH cytochrome <i>c</i> reductase.....	—	31	28		17†
Succinic dehydrogenase.....	11	110	18		0†
Cytochrome oxidase.....	2.3	67	23		0†
Fumarase.....	4.7	31	1.2		66†
Aldolase.....	1.7	3.2	0		106†
Aconitase.....	6.8	9.2	0		68†
Uricase.....	0	0	0		84†
β -Glycerophosphatase.....	3.9	28	22		52†
Glucose-6-phosphatase.....	0	5.4	10		72†

* The P_1 and P_2 fractions were collected and analyzed together.

† The P_4 and supernatant fractions were not separated.

TABLE 2. *Enzyme concentration in fractions of Fusarium oxysporum mycelium*

Enzyme	Specific activity in cell extract	Enzyme concn*				
		P ₁	P ₂	P ₃	P ₄	S
Diphosphopyridine nucleotidase...	1.3	1.3†		3.2	0.69	0.15
DPNH oxidase.....	0.060	3.9†		1.2	0.040	0.38
DPNH cytochrome <i>c</i> reductase....	0.43	1.1	3.6	0.30		0.19‡
TPNH cytochrome <i>c</i> reductase....	0.46		2.5	1.5		0.24‡
Succinic dehydrogenase.....	0.012	2.1	6.4	1.5		0.0‡
Cytochrome oxidase.....	0.013	1.1	5.2	1.1		0.0‡
Fumarase.....	1.21	1.1	4.0	0.90		1.0‡
Aldolase.....	1.2	0.52	0.35	0.0		1.6‡
Aconitase.....	0.040	1.22	1.25	0.0		0.95‡
Uricase.....	0.012	0.0	0.0	0.0		1.5‡
β -Glycerophosphatase.....	0.40	2.5	2.5	1.8		0.68‡
Glucose-6-phosphatase.....	0.26	0.0	0.90	1.6		0.96‡

* The enzyme concentration is defined as the ratio of specific activity of the enzyme in the fraction to that in the cell extract.

† The P₁ and P₂ fractions were collected and analyzed together.

‡ The P₄ and supernatant fractions were not separated.

TABLE 3. *Localization of enzymes in fungal microconidia*

Enzyme	Criterion	Fraction			
		P ₁ -P ₂	P ₃	P ₄	S
Diphosphopyridine nucleotidase	Recovery, %	24	40	11	16
	Concn	4.0	5.5	1.2	0.71
DPNH oxidase	Recovery, %	48	26	11	25
	Concn	3.4	1.5	0.64	0.47
DPNH cytochrome <i>c</i> reductase	Recovery, %	64	54	5.0	2.1
	Concn	4.7	3.2	0.52	0.050
Succinic dehydrogenase	Recovery, %	60	32		0*
	Concn	3.1	2.3		0
Cytochrome oxidase	Recovery, %	62	43		11*
	Concn	4.1	2.9		0.17
Catecholase	Recovery, %	19	19		48*
	Concn	1.2	1.2		0.70
Aldolase	Recovery, %				131
	Concn				1.8

* The P₄ and supernatant fractions were not separated.

oxidase, fumarase, and glycerophosphatase, significant proportions of the activity were found among both the sedimented and nonsedimented hyphal constituents.

The values for relative enzyme concentration, i.e., the ratio of specific activity in an isolated fraction to that in the original cell extract, point more specifically to an association between a biochemical capacity and an individual cell structure. Thus, the highest concentrations of succinic dehydrogenase, cytochrome oxidase, DPNH cytochrome *c* reductase, TPNH cytochrome *c*

reductase, and fumarase were noted in the particles comprising the P₂ fraction (Table 2). It is possible that the comparatively high concentrations of these enzymes among the P₁ and, to a lesser extent, among the P₃ constituents result from incomplete separation. Also found in the large particles is the DPNH oxidase, although the percentage recovery indicates the enzyme was distributed with the nonsedimented portions of the hyphae as well. Diphosphopyridine nucleotidase was once again noted to be an attribute of the P₃ particles.

An intracellular localization of enzymes somewhat similar to that of the mycelium was found in the microconidia (Table 3). Essentially all of the succinic dehydrogenase, cytochrome oxidase, and DPNH cytochrome *c* reductase was particulate. However, although the combined P_1 - P_2 fraction contained somewhat larger percentages and higher relative concentrations of these enzymes than the succeeding particles, no distinct biochemical differentiation was possible. Aldolase was clearly soluble, whereas diphosphopyridine nucleotidase, DPNH oxidase, and catecholase were observed in all fractions. On the other hand, as with the hyphal extracts, the subcellular fraction containing the largest percentage and the highest concentration of diphosphopyridine nucleotidase was the P_3 particle type.

The results of a comparative study of several enzymes in mycelial and conidial extracts are summarized in Table 4. Because the metabolic activity of the fungus was affected by growth conditions, two media were used and the results of three different experiments described. Neither filament nor spore was richer in DPNH oxidase and aldolase, but the mycelium exhibited higher specific activities than the conidium with diphosphopyridine nucleotidase, cytochrome oxidase,

and ascorbic acid oxidase. In particular, the diphosphopyridine nucleotidase content of mycelial extracts exceeded that of conidial extracts; however, the pH optima of the catalysts from the two sources were identical, namely, pH 8.0 to 8.5. Further, the data indicate a greater content of succinic dehydrogenase in the spores.

The pH optima for the aldolase of hyphae and conidia were at pH 8.0. The DPNH oxidase, which functioned optimally in the vicinity of pH 7.0, was inhibited more markedly by KCN when in a particulate than in a soluble form. For example, 0.001 M KCN totally abolished DPNH oxidase activity of all particle types, but the same concentration inhibited the soluble enzyme only by 20%. The pH optimum for phosphatase was found at 8.5 when the activity was measured using β -glycerophosphate as substrate; however, when phosphatase was assayed with α -glycerophosphate as substrate, the pH optimum was at 5.0 to 7.0.

DISCUSSION

Despite the fact that the isolated particles were not purified to minimize possible enzyme inactivation, distinct associations between certain of the catalysts and fungal cell structures were observed. Succinic dehydrogenase and cytochrome oxidase, typical mitochondrial enzymes in liver cells, were characteristic of the P_2 components of *F. oxysporum*. Such observations may indicate that the particles of this fraction are the mitochondrial equivalents of this fungus, at least from the metabolic viewpoint. Moreover, though much of the DPNH cytochrome *c* reductase and TPNH cytochrome *c* reductase was found in the P_2 fraction, activity was also observed in the P_3 and soluble fractions; similar results have been reported by Kikuchi and Barron (1959).

Particularly noteworthy is the fact that only one of the enzymes investigated, diphosphopyridine nucleotidase, was concentrated in the P_3 fraction. The association of the succinic dehydrogenase with one fraction and diphosphopyridine nucleotidase with another suggests the existence of two separate functional entities in the microorganism, although artifacts arising from particle rupture or protein absorption may conceivably account for the differences in the distribution patterns. The distribution of deoxyribonucleic acid and the isolation of a P_4 granule rich in pro-

TABLE 4. Comparison of enzymatic activities in extracts of mycelium and microconidia

Enzyme	Expt	Growth medium*	Specific activity† of enzyme	
			Mycelium	Microconidia
Diphosphopyridine nucleotidase	1	V-8	1.9	0.076
	2	SM	1.3	0.076
	3	SM	0.93	0.073
DPNH oxidase		V-8	0.042	0.040
Succinic dehydrogenase	1	V-8	0.015	0.050
	2	V-8	0.012	0.021
	3	SM	0.023	0.028
Cytochrome oxidase	1	V-8	0.024	0.0026
	2	SM	0.021	0.0016
Aldolase	1	V-8	0.41	0.42
	2	SM	0.91	0.25
Ascorbic acid oxidase	1	V-8	43	8.5
	2	V-8	65	32
	3	SM	17	8.0
Isocitratase		SM	0.0045	0.0060

* SM represents the synthetic medium; V-8, the V-8 juice medium.

† Units per mg of protein (see Materials and Methods).

tein and ribonucleic acid (Maruyama and Alexander, 1962) but poor in the enzymes investigated to date suggest the presence of additional structural units within the fungus.

The intracellular localization of many of the fungal enzymes was generally similar to that reported for liver cells. Exceptions are the uricase and glucose-6-phosphatase, the latter being particulate in animal cells. On the other hand, Mitchell (1959) reported that glucose-6-phosphatase in *Staphylococcus aureus* was associated largely with nonparticulate cell constituents, data similar to those reported herein for fungal hyphae. In addition to this enzyme, the distribution patterns of succinic dehydrogenase, cytochrome oxidase, and fumarase in the fusarium resemble those found in bacteria.

F. oxysporum possesses three different spore types, microconidia, macroconidia, and chlamydospores. Only one of the three types has been examined, and the localization patterns were found to be similar to those of the mycelium. The data reveal, however, no distinct differentiation of conidium particle types on the basis of enzyme localizations. This may be a consequence of an inadequate technique of separation by differential centrifugation, but such a procedure of separation should not be difficult to devise. Moreover, investigations of the macroconidia and chlamydospores should provide important additions to knowledge of the biochemistry of fungi.

ACKNOWLEDGMENT

The investigation was supported in part by a grant from the United Fruit Co.

LITERATURE CITED

- ALEXANDER, M., AND P. W. WILSON. 1955. Enzyme localization in *Azotobacter vinelandii*. Proc. Natl. Acad. Sci. U.S. **41**:843-848.
- COOPERSTEIN, S. J., AND A. LAZAROW. 1951. A microspectrophotometric method for the determination of cytochrome oxidase. J. Biol. Chem. **189**:665-670.
- DIXON, G. H., AND H. L. KORNBERG. 1959. Assay methods for key enzymes of the glyoxylate cycle. Biochem. J. **72**:3P.
- FISKE, C. H., AND Y. SUBBAROW. 1929. Phosphocreatine. J. Biol. Chem. **81**:629-679.
- IWASA, K. 1960. Phosphorus metabolism of fungal cells. I. Oxidative phosphorylation by mitochondria from *Aspergillus oryzae*. J. Biochem. (Tokyo) **47**:445-453.
- KAPLAN, N. O. 1955. Animal tissue DPNase (pyridine transglycosidase), p. 660-663. In S. Colowick and N. O. Kaplan [ed.], Methods in enzymology, vol. 2. Academic Press, Inc., New York.
- KIKUCHI, G., AND E. S. G. BARRON. 1959. Electron transport system in *Fusarium lini*. Arch. Biochem. Biophys. **84**:96-105.
- LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
- MANDELS, G. R. 1953. The properties and surface location of an enzyme oxidizing ascorbic acid in fungus spores. Arch. Biochem. Biophys. **42**:164-173.
- MARUYAMA, Y., AND M. ALEXANDER. 1962. Distribution of protein and nucleic acids in hyphae and microconidia of fusarium. Arch. Mikrobiol. **41**:401-407.
- MITCHELL, P. 1959. Structure and function in micro-organisms, p. 73-93. In E. W. Crook [ed.], The structure and function of subcellular components. Cambridge University Press, Cambridge.
- RACKER, E. 1950. Spectrophotometric measurements of the enzymatic formation of fumaric and cis-aconitic acids. Biochim. et Biophys. Acta **4**:211-214.
- SCHNEIDER, W. C., AND G. H. HOGEBOM. 1952. Intracellular distribution of enzymes. IX. Certain purine-metabolizing enzymes. J. Biol. Chem. **195**:161-166.
- SWANSON, M. A. 1955. Glucose-6-phosphatase from liver, p. 541-546. In S. Colowick and N. O. Kaplan [ed.], Methods in enzymology, vol. 2. Academic Press, Inc., New York.
- TAYLOR, J. F. 1955. Aldolase from muscle, p. 310-315. In S. Colowick and N. O. Kaplan [ed.], Methods in enzymology, vol. 1. Academic Press, Inc., New York.
- ZALOKAR, M. 1960. Cytochemistry of centrifuged hyphae of *Neurospora*. Exptl. Cell Research **19**:114-132.