EFFECT OF NYSTATIN ON THE FORMATION
OF
EXTRACELLULAR PROTEASE BY *ASPERGILLUS NIGER*

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(Received February 29, 1968)

The effect of nystatin on the acid protease formation by *Aspergillus niger* as induced by sulfur deficiency was studied.

Nystatin at the concentration of 10 μ g/ml was found to inhibit the growth and the induced protease formation. The leakage of free amino acids from mycelia was also noted at this concentration. When nystatin was added to S-deficient cultures at different intervals in the induction period, a complete inhibition of protease formation was observed when added within the first 2.5 hr. Later additions caused less effective inhibition.

After the protease induction in sulfur-deficient media, mycelia were either treated or not treated with nystatin and they were further cultured for protease formation on S-deficient media containing ¹⁴C-amino acid mixture. It was found that the nystatin-treated mycelium produced protease of higher specific radioactivity than the non-treated mycelium. This suggests that a fungal mycelium which has already established the protease-forming system can produce protease at the expense of exogenous nutrients, irrespective of the loss of the pool amino acid from the mycelium.

Our previous finding (1) that the formation of extracellular acid protease by *Aspergillus niger* is induced by sulfur deficiency has led us to investigate the mechanism of this phenomenon. TOMONAGA (2) observed that the formation of protease under such conditions requires the presence of nitrogen source and that exogenous amino acids are incorporated into protease protein *via* pool amino acid. This suggests that the pool amino acid under sulfur deficiency plays an important role in the formation of protease.

Nystatin, a polyene antibiotic active against fungi, has been known to attack the cell membrane and cause the leakage from fungal cells of low molecular weight cellular materials including free amino acids (3). The present investigation was carried out with special attention on the role of pool amino

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acid in the protease formation by *A. niger* under S-deficiency, using nystatin as an inhibitor.

MATERIALS AND METHODS

Organism and cultivation. *Aspergillus niger* strain 1617 was used throughout the experiments. Culture medium used had the following composition: Monosodium L-glutamate, 0.3 g; DL-alanine, 0.3 g; L-aspartic acid, 0.3 g; NaNO₃, 2.0 g; KH₂PO₄, 1.0 g; KCl, 0.5 g; MgCl₂·6H₂O, 0.25 g; Na₂SO₄, 0.25 g; FeCl₃·6H₂O, 0.01 g; glucose, 20 g; water to 1 liter (pH 6.0). This medium will be referred to as the normal medium. S-deficient medium excluded Na₂SO₄ from normal medium.

In the primary culture, the dry conidia of the fungus formed on the filter paper culture (4) were inoculated as uniformly as possible on the surface of the normal medium which was incubated at 30° (1). Sampling of mycelial mat with a sterilized cork borer was performed as described previously (2). Circular pieces (1.4 cm in diameter) of the mat thus collected were transferred gently onto sulfur-deficient medium contained in test tubes (1.5 cm in diameter) to induce the protease formation.

Chemicals. Nystatin was obtained by courtesy of Mr. Y. Yagisawa of Japan Antibiotic Research Association.

Estimation of cellular materials. Fungal mycelia were fractionated into the pool amino acid and soluble protein fractions. The pool size was estimated by the method of YEMM and COCKING (5) and expressed by leucine equivalents. The amount of the soluble protein fraction was determined by the method of LOWRY *et al.* (6) and expressed by serum albumin equivalents. The dry weight of mycelia was determined after drying at 110° for 1 hr.

Enzyme assay. The protease activity in media was assayed by the method of HAGIWARA (7) employing casein as substrate as described previously, pH of the assay system being kept at pH 3.0 with 0.1 M lactate buffer.

Chromatography of protease. In the chromatographic separation of protease, culture filtrate was treated with ammonium sulfate of 80% saturation and the precipitate was subjected to chromatography with Sephadex G-25 column (1.5×30 cm) to remove salt. Fractions containing protease were combined and buffered with 0.01 M lactate buffer (final concentration) with the addition of 0.05 M NaCl. This enzyme solution was subjected to SE-cellulose column chromatography (1×15 cm) using an eluting solvent of 0.1 M lactate buffer containing a linear gradient concentration (0.05–0.3 M) of NaCl. Amount of each fraction was 6 ml.

Labeling of mycelial mats. Pieces of mycelial samples were labeled with ¹⁴C-amino acid mixture (¹⁴C-*Chlorella* hydrolysate obtained from Prof. B. Maruo of this Institute) as described previously (2). Radioactivity of cellular fractions was expressed by cpm per unit area (cm²) of mycelial mat.

RESULTS

Effect of nystatin concentration on protease formation

The fungus was grown on normal medium for 50 hr and pieces of the mycelial mats were cut out from the mycelium, then transferred onto sulfur-deficient media containing various concentrations of nystatin. After 10 and 20 hr of cultivation, amounts of amino acid pool and soluble protein, together with dry weight of each sample, were measured and protease activity in each medium was also determined.

The results summarized in Table 1 clearly show that the growth of mycelia as judged from increases in dry weight and soluble protein was inhibited by more than 10 $\mu\text{g/ml}$ of nystatin. Protease formation was also inhibited almost completely at these concentrations. It is worth mentioning that the indication of the leakage of pool amino acid was also observed at these concentrations. In the following experiments, 10 $\mu\text{g/ml}$ of nystatin was used throughout.

Table 1. Effect of various concentrations of nystatin on the growth and protease formation of *Aspergillus niger* mycelia cultured on sulfur-deficient media.

Mycelia grown on normal media for 50 hr was transferred onto sulfur-deficient media containing various concentrations of nystatin, and various parameters were estimated after 10 and 20 hr of cultivation.

Nystatin conc ($\mu\text{g/ml}$)	Culture time (hr)	Dry wt. (mg/cm^2)	Sol. protein (μg albumin eq./ cm^2)	Pool amino acid (μg leucine eq./ cm^2)	Protease in medium (unit/ml)
0	0	4.15	696	480	0
	10	7.05	1082	685	0.4
	20	8.75	1542	902	5.9
1	10	5.00	1080	632	0.4
	20	6.45	1464	859	2.8
10	10	3.60	960	532	0.1
	20	4.25	1110	316	0.1
30	10	3.05	930	210	0.1
	20	3.60	1086	158	0.1

Effect of nystatin added at different intervals in sulfur-deficient media

The effect of delayed addition of nystatin on mycelial growth and protease formation in the course of protease induction was examined. Experimental procedures are schematically presented in Fig. 1. From each one of 5 sheets of mycelial mat grown on the normal media for 40 hr, 6 circular pieces were cut out. Five groups of mycelial mats, each consisting of 6 pieces, were

cultured on sulfur-deficient media for 0, 2.5, 5, 7.5, and 10 hr (primary cultures). After the primary cultures were over, two pieces of mycelia in each group were sampled and subjected to measurements of dry weight, amino acid pool size, soluble protein content of the mycelia, and protease activity in the medium. The other 4 pieces were divided into two subgroups and cultured for 20 hr on sulfur-deficient media in the presence and absence of 10 $\mu\text{g/ml}$ of nystatin (secondary cultures). At the end of the secondary culture, the above-mentioned four parameters were estimated on each subgroup.

Fig. 1. Schematic representation of experimental procedures.

This figure shows procedures carried out on one group of mycelial mat. Slanting arrows ($\nearrow$) indicate the sampling procedure of mycelial pieces. Numbers indicated on these arrows are the following parameters which were estimated after samplings: 1, Dry weight of mycelium; 2, size of amino acid pool; 3, amount of soluble protein; 4, protease unit in culture medium. Duration of the primary culture for each group was 0, 2.5, 5, 7.5, or 10 hr. For further explanations see text.

In the primary culture, dry weight, soluble protein content, and amino acid pool size increased gradually, indicating the occurrence of mycelial growth in sulfur-deficient media, if not active, as already shown by YANAGITA and NOMACHI (8). Protease was also produced after about 5 hr of lag period as described previously (1).

In the secondary cultures, starting from the end of varied induction periods in the primary cultures, increments in these parameters during 20 hr of cultivation in the presence and absence of nystatin changed characteristically (Fig. 2). Effect of nystatin on the increment in dry weight became somewhat

Fig. 2. Changes in amounts of cellular materials and in extracellular protease activity in the primary and secondary cultures under sulfur-deficiency as influenced by the presence and absence of nystatin.

For experimental procedures see Fig. 1 and text. Changes in the amounts of parameters in the primary cultures represent those in mycelia grown in sulfur-deficient media in the absence of nystatin. Changes in amounts of parameters in the secondary cultures represent changes in increments or decrement during 20 hr cultivation in sulfur-deficient media in the absence (—○—) and presence (---×---) of 10 μ g/ml of nystatin following the primary cultures of different durations.

less marked as the primary induction period became longer. The increment in soluble protein content in the absence of nystatin decreased markedly as the induction period became longer, while that in the presence was kept at an almost constant low level. The increment and decrement in amino acid pool size in the absence and presence of nystatin, respectively, changed almost in parallel as the primary induction period became longer.

The effect of nystatin on the productivity of protease in the secondary culture is worth noting. As seen in Fig. 2 (bottom right), complete inhibition was observed when nystatin was added in the early phase of the induction period. When nystatin was added after the induction period of about 5 hr, its inhibitory effect was reduced conspicuously. This suggests that, when the protease-forming system had been once established in the primary culture, protease was produced at the expense of exogenous nitrogen and carbon sources, even though the mycelial cells had been deprived of pool amino acid. This consideration was supported by the next experiments.

Chromatographic analysis of protease formed in the presence of nystatin

Two sheets of mycelial mat grown for 30 hr on normal media were transferred onto sulfur-deficient media and incubated for 10 hr for the protease production. These were then cultured for 10 hr on sulfur-deficient media in the presence and absence of nystatin. In the presence of nystatin, the cell should have been deprived of pool amino acid. These mats were further cultured for 20 hr on nystatin-free sulfur-deficient media containing ^{14}C -amino acid mixture and protease formed in the media was isolated and purified by chromatography. The results are shown in Fig. 3.

It is thus clear that the mycelium previously treated with nystatin after the protease induction can form protease as actively as the control mycelium. Specific radioactivities of protease samples produced with and without nystatin pretreatment were 176 and 85 cpm/protease unit, respectively. This suggests that the nystatin-treated mycelium, which has been deprived of a certain amount of pool amino acid, produced protease rather directly from exogenous nitrogen, whereas the non-treated one produced it *via* pool amino acid, resulting in the formation of protease of low specific radioactivity.

DISCUSSION

HORVÁTH (9) reported that the formation of amylase by *Penicillium chrysogenum* as induced by soluble starch was effectively inhibited by 1 $\mu\text{g}/\text{ml}$ of nystatin when added within the first 2 hr and that the later addition of the antibiotic caused less effective inhibition. The present experiments also demonstrated that the formation of protease by *A. niger* as induced by sulfur-deficiency was inhibited 100% when 10 $\mu\text{g}/\text{ml}$ of nystatin was added in the early phase (within the first 2.5 hr) of induction period.

In the interpretation of such phenomena in fungal cultures, it should be

Fig. 3. Elution patterns of SE-cellulose column chromatography of protease produced by *Aspergillus niger*.

Experiments were designed by the following scheme (for details see text). Circles represent mycelial mats transferred from one medium to another and slanting arrows show sampling of media followed by chromatography and radioactivity measurements.

taken into account that fungal hyphae grow only at the apices and that they are more susceptible to various antifungal substances generally in the apical region than in other regions far from the apex. The fact that the absolute amount (the sum of increment in control and decrement in test cultures) of pool amino acid leaking out from the cells was almost constant independent of the duration of the primary culture (see Fig. 2) indeed suggests that the length of hypha attacked by nystatin is almost constant being limited probably to the apical region.

Kinetic analysis by YANAGITA and NOMACHI (8) of protease induction by sulfur-deficiency in *A. niger* demonstrated that the growing tip of the fungal hypha does not produce protease in response to sulfur-deficiency but the neighboring region does, and the region next to the responding region actually produces protease. According to these workers, following the elongation of *ca.* 30 μ at the hyphal tip in sulfur-deficient medium during 5 hr, the neighboring region responds to form protease. The fact that the complete inhibition of protease formation was observed when nystatin was added within 5 hr of induction period strongly suggests that some reaction associated with the tip growth under sulfur-deficiency is responsible for the protease induction and the reaction may not be able to proceed further, when low molecular weight substances including pool amino acid are lost by the action of nystatin. In other words, the pool substances including free amino acids may be required for the construction of a protease-forming system. Once it is established, the pool fraction may not necessarily be indispensable; even the nystatin-treated mycelium can produce protease at the expense of exogenous nutrients, irrespective of the leakage, if any, of pool amino acid. The present finding that a mycelium pretreated with nystatin after the protease induction can produce protease of higher specific radioactivity in radioactive sulfur-deficient medium than that without nystatin treatment supports the above consideration.

The author's thanks are due to Prof. T. Yanagita for his valuable advice and encouragement throughout the course of this study.

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