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The influence of copper on the induction of tyrosinase and laccase in *Neurospora crassa*

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The influence of copper on the cycloheximide-induced synthesis of the copper-containing enzymes tyrosinase and laccase in *Neurospora crassa* was studied by enzyme activity measurements and immunological means. The amount of active enzyme molecules is far higher when the culture medium is copper-supplemented before cycloheximide induction. The synthesis of the apoproteins is not dependent on the presence of copper. This suggests the existence of a copper-storage protein for which metallothionein is a likely candidate.

Copper; Tyrosinase; Laccase; Metallothionein

1. INTRODUCTION

The copper-containing enzymes tyrosinase (monophenol-L-dopa:oxygen oxidoreductase, EC 1.14.18.1) and laccase (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) are produced solely during sexual differentiation of the ascomycete *Neurospora crassa* [1]. In this fungus, tyrosinase is responsible for the formation of melanin pigments which give the fruiting bodies their black color [2]. The function of laccase, however, is at the moment less clear. As shown earlier by Horowitz et al. [3], these enzymes can also be induced in vegetatively growing cultures by the addition of various compounds such as cycloheximide and DL-ethionine or merely by starvation in phosphate buffer. As a model for the induction mechanism, they proposed a repressor protein with a rapid turnover rate [3]. Here, we studied the influence of copper on the induction of tyrosinase and laccase. The results demonstrate that the amount of active enzymes is far higher when the culture medium is supplemented with copper before induction.

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2. MATERIALS AND METHODS

2.1. *Organism*

N. crassa wild-type strain FGSC 321 (Fungal Genetic Stock Center, Kansas City, KA) was used in all experiments.

2.2. *Culture conditions*

Cells of *N. crassa* were grown at 25°C on a rotatory shaker in half-strength Vogel N medium [4] containing 1% sucrose before induction. The medium was rendered copper-free by passage through a Chelex 100 column. Copper was added as $CuSO_4$ according to the needs of the experiments (fig.1). For radiolabeling experiments $^{35}SO_4$ (5 μCi/ml) was added 8 h after inoculation to the culture medium.

2.3. *Preparation of samples*

Mycelia were harvested by filtering through cheesecloth, rinsed with cold water and disrupted by grinding with acid-washed quartz sand in

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50 mM sodium phosphate buffer (pH 7.5). After centrifugation, the supernatant was used directly in further experiments. The culture filtrate was concentrated 20 times by flash evaporation.

2.4. *Determination of enzymatic activities*

Tyrosinase and laccase activities were determined as in [5,6]. The activity of L-amino acid oxidase was assayed spectrophotometrically using L-phenylalanine as substrate [7]. Buffers were rendered copper-free by Chelex 100 treatment.

2.5. *Production of antisera*

Tyrosinase and laccase from cycloheximide-induced cultures were purified as described [6,8]. Antisera against SDS-denatured proteins were produced in rabbits according to [9]. The presence of specific antibodies in the antisera was tested by the Ouchterlony double-diffusion test in 2% agar in PBS buffer (137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 2 mM KH_2PO_4).

2.6. *Preparation of fixed* S. aureus *cells*

S. aureus cells for immunoprecipitation were prepared by the procedure of Kessler [10] using 1% glutaraldehyde instead of formalin for fixation. They could be stored as a 10% suspension at 4°C for several months.

2.7. *Immunoprecipitations*

Samples (0.5–1.0 ml) were denatured by dissolving in 0.5% SDS and boiling at 100°C for 5 min. Proteins were immunoprecipitated essentially as in [11]. The final precipitate was dissolved in SDS-polyacrylamide gel electrophoresis buffer [12].

2.8. *Electrophoresis*

Immunoprecipitates were analysed on 10% SDS-polyacrylamide gels using a discontinuous buffer system [12]. Gels were prepared for fluorography by soaking in Enlightning and dried under reduced pressure. For fluorography, Kodak XAR 5 film was exposed for 10–20 days.

3. RESULTS AND DISCUSSION

At present, very little is known about the biosynthesis of copper-containing enzymes. Here, we investigated the biosynthesis of *N. crassa* tyrosinase and laccase in relation to the time of copper addition to the culture medium. To this end, cells were grown in a chemically defined culture medium and supplemented with 25 μM copper as indicated in fig.1. After reaching the stationary growth phase, all cultures were treated with 1 μM cycloheximide, a concentration shown to induce efficiently tyrosinase and laccase without inhibiting protein synthesis excessively [3]. Samples from mycelia and media were removed periodically and assayed for tyrosinase and laccase activity, respectively (fig.2). As a control to follow the extent of induction in the different cultures, the activity of the cycloheximide-inducible flavoenzyme L-amino acid oxidase [13] was also measured.

As shown in table 1, there is a clear difference in enzymatic activities between expts A and B on the one hand and C and D on the other, although the induction level is more or less the same as revealed by the measurements of L-amino acid oxidase activity. The highest tyrosinase and laccase activities are found in expts C and D, suggesting that only copper stored during the vegetative growth period is incorporated into apoenzymes (fig.1). Furthermore, expt B indicates that addition of copper after induction to a culture grown under copper-free conditions during vegetative growth leads to markedly decreased synthesis of active enzyme molecules. As expected, virtually no activity was

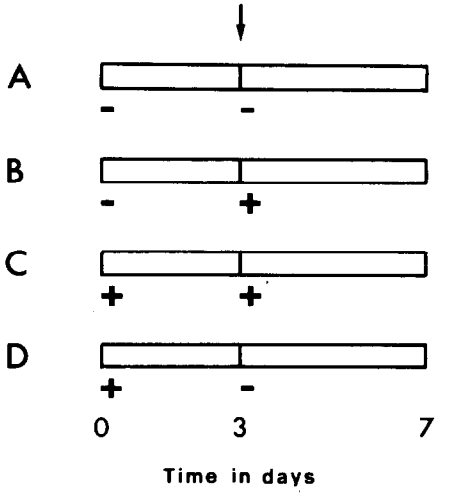

Fig.1. Schematic representation of the four induction experiments A–D. (+) Denotes the addition of copper (25 μM); (−) no copper added. All cultures were induced with cycloheximide (1 μM) after 3 days (arrow).

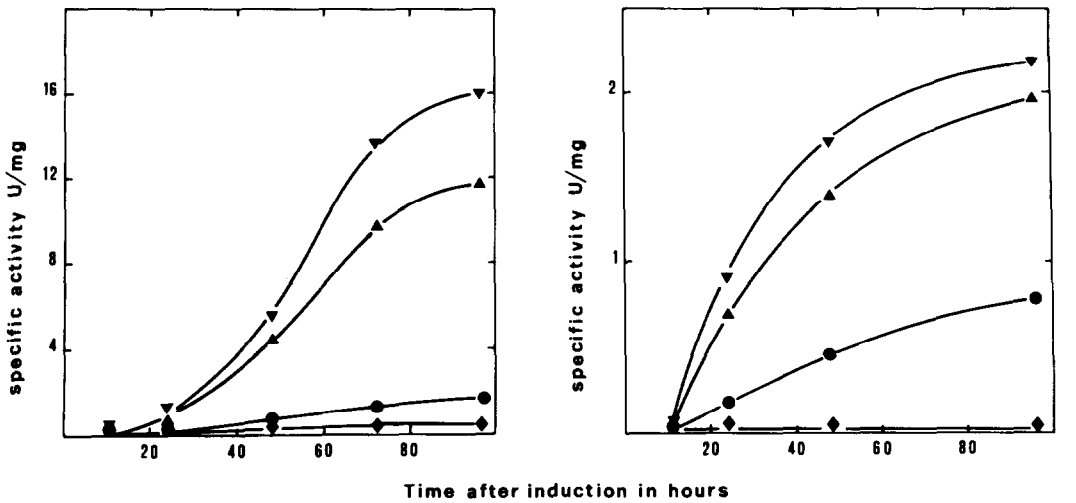

Fig.2. Time course of tyrosinase (left) and laccase (right) activity after cycloheximide induction. Expt A (♦), B (●), C (▲) and D (▼); see fig.1.

detected in a copper-free culture medium (expt A; fig.1, table 1).

Fig.2 shows a kinetic study of the synthesis of tyrosinase and laccase. In all experiments each enzyme follows similar induction kinetics. However, the laccase activity generally increases faster than that of tyrosinase over the first 50 h after induction. The largest differences are observed in expt D.

To obtain further information on the biosynthesis of the two enzymes, induction was studied at the protein level. Polyclonal antisera raised against denatured tyrosinase and laccase were added to SDS-treated samples of mycelia and culture media and the resulting immunocomplexes precipitated by fixed *S. aureus* cells. The immunoprecipitates were analysed on 10% SDS slab gels (fig.3). They clearly indicated that in all experiments (fig.1), the synthesis of apotyrosinase and apolaccase was elicited by cycloheximide to virtually the same extent irrespective of the amount of copper in the culture medium. Furthermore, the presence of copper is not a prerequisite for the secretion of laccase as shown in expt A. Similar findings were reported for the biosynthesis of laccase in sycamore cells [14] and plastocyanin in *Scenedesmus acutus* [15] and *Chlamydomonas reinhardtii* [16].

Table 1

Enzymatic activities after induction

Expt	Tyrosinase[a]	Laccase[a]	L-Amino acid oxidase[a]
A	0.6 ± 0.1 (4%)	0 (0%)	0.27 ± 0.09
B	1.6 ± 0.2 (10%)	0.8 ± 0.2 (36%)	0.18 ± 0.05
C	11.8 ± 2.7 (74%)	2.0 ± 0.4 (91%)	0.25 ± 0.05
D	15.9 ± 3.0 (100%)	2.2 ± 0.2 (100%)	0.17 ± 0.05

[a] Mean values and standard deviations (from four independent experiments) of enzymatic activities in U/mg measured 96 h after induction. In parentheses are given the values as a percentage; expt D is taken as 100%

The present data clearly show that the biosynthesis of active tyrosinase and laccase molecules depends strongly on the uptake and storage of copper during vegetative growth. As shown earlier, most of the copper taken up during this growth period is confined intracellularly to the small copper-binding protein metallothionein which is induced exclusively by copper [17]. From these observations it follows that copper ions must be mobilised from copper metallothionein before incorporation into the active sites of the apoenzymes. Such copper transfer has been described recently for copper metallothionein and apotyrosinase in vitro [18]. Furthermore, copper metallothionein is oxidatively degraded with the concomitant release of copper ions after ad-

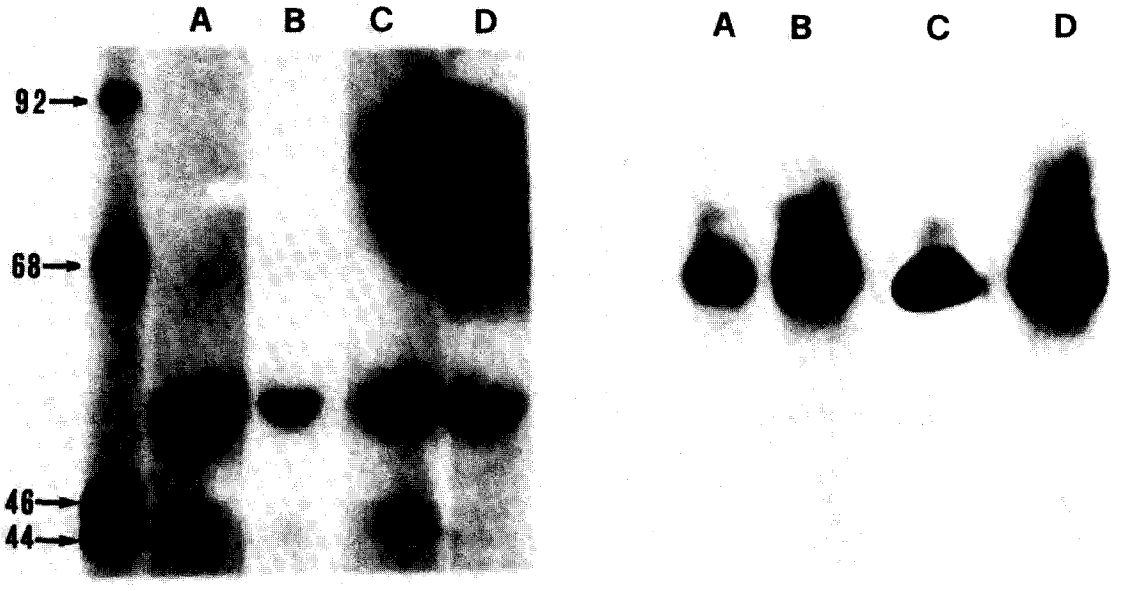

Fig.3. SDS-polyacrylamide gel electrophoresis of ^{35}S-labeled proteins immunologically extracted 96 h after cycloheximide induction with anti-tyrosinase antiserum (left) and anti-laccase antiserum (right). The numbers give the molecular masses of the marker proteins (in kDa).

ministration of cycloheximide [19]. It is therefore suggested that *N. crassa* copper metallothionein serves an important role as a metal-storage protein to provide metal ions for the biosynthesis of tyrosinase and laccase.

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