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INDUCTION OF LACCASE IN BASIDIOMYCETES:
THE LACCASE-CODING MESSENGER

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Formation of the mRNA specific for the inducible forms of laccase was evidenced in Coriolus versicolor, Pleurotus ostreatus and Pholiota mutabilis.

The half-life time of these mRNAs in the fungi species studied were, respectively, 30, 37 and 24 min.

Molecular weight of the newly synthesized mRNA in Pleurotus ostreatus was about $4.5 \times 10^4$, consistently with the size of the inducible laccase protein.

The polysome obtained from the ferulic acid-treated mycelium, synthesized in vitro a polypeptide with the electrophoretic mobility similar to that of laccase.

It has been previously demonstrated that new forms of laccase (EC 1.14.18.1) are induced by ferulic acid in Coriolus versicolor, Pleurotus ostreatus and Pholiota mutabilis (Leonowicz & Trojanowski, 1975a); the constitutive forms of the enzyme and other cytosolic proteins did not undergo major changes (Leonowicz & Trojanowski, 1975b; Leonowicz, 1975). It has also been found that, in fungi, induction of laccase by phenol and ferulic acid is preceded by increased synthesis of mRNA (Leonowicz et al., 1972; Leonowicz & Trojanowski, 1975b).

The aim of the present work was to characterize the mRNA synthesized de novo during induction of laccase.

MATERIALS AND METHODS

Organisms and culture conditions. The source and storage of the pure cultures of Coriolus versicolor, Pleurotus ostreatus and Pholiota mutabilis were the same as previously described (Leonowicz & Trojanowski, 1975a). The organisms were cultivated in 20-litre bottles in the medium described by Trojanowski & Leonowicz (1969). The experiments with pre-grown mycelial mats and ferulic acid were performed according to Trojanowski & Leonowicz (1969).
Isolation and determination of laccase. The mycelium was washed with cold 0.1 M-phosphate buffer, pH 6.0, and the procedure described by Leonowicz & Trojanowski (1975a) was applied.

Determination of molecular weight of mRNA. Ribonucleic acids containing the [3H]uridine-labelled mRNA were isolated (Leonowicz et al. 1972), and the molecular weight of mRNA was determined by polyacrylamide-gel electrophoresis (Kimmel & Wang, 1972).

Isolation of polysomes. Mycelium (50 g) was washed with a bentonite-saturated buffer containing 100 ml of 0.2 M-Tris/HCl, pH 7.6, 0.4 M-sucrose, 0.01 M-magnesium acetate, 0.06 M-KCl, 0.01 M-dithiothreitol, 0.003 M-glutathione (reduced) and spermine (40 μg/ml), then 100 g of alumina (type 305) was added and the mixture was ground in a mortar at 5°C; during grinding, 50 ml of the same buffer was gradually added. The suspension was centrifuged for 10 min at 4000 g, the sediment was discarded, and to the supernatant Triton X-100 was added to a final concentration of 1%. The mixture was shaken for 1 min and centrifuged for 20 min at 20 000 g. The sediment was discarded, the supernatant was recentrifuged under the same conditions, and then it was digested for 10 min with α-amylase (Peters et al., 1970) to remove polysaccharides. The digest was applied on 4 ml of 2 M-sucrose in the buffer used for washing of mycelium (except that it contained no sucrose, and a tenfold lower, 0.02 M, concentration of Tris/HCl buffer). The preparation was centrifuged for 150 min at 150 000 g in an 8 - 11 aluminium rotor of the VAC 601 ultracentrifuge; the gelatinous sediment obtained was washed with 0.02 M-Tris/borate buffer, pH 7.6, containing 0.01 M-magnesium acetate, 0.06 M-KCl, 0.01 M-dithiothreitol, 0.003 M-glutathione (reduced) and spermine (40 μg/ml). Finally, the sediment was diluted with 0.5 ml of the same buffer, centrifuged for 10 min at 10 000 g to remove impurities, and stored in small portions at a temperature not higher than −20°C.

Preparation of the post-polysomal supernatant. The mycelium was washed and homogenized as described for the isolation of polysomes, except that the buffer contained no bentonite and was supplemented with DNase (3 μg/ml). The homogenate was centrifuged twice (without the addition of Triton X-100) for 20 min at 20 000 g, and once more for 30 min at 30 000 g. The resulting supernatant was applied to a DEAE-SS-cellulose column (diameter 2 cm, adsorbent content 6 g), equilibrated previously with the buffer used for suspending polysomes. Protein was eluted from the column with 0.25 M-NH₄Cl in the equilibration buffer, and precipitated with ammonium sulphate at 1.0 saturation (2 h at 4°C). The precipitate (about 25 mg of protein) was dissolved in 0.3 ml of the same buffer, dialysed three times (45 min each) against 1000 volumes of the same buffer, and purified by centrifugation for 10 min at 1000 g. The preparation was stored in small portions under the same conditions as described for polysomes.

Isolation of tRNA. tRNA was isolated from the post-polysomal supernatant subjected to 30-min incubation with DNase (3 μg/ml, 30°C) according to Clemens & Tata (1972).

Protein synthesis in vitro. The cell-free system synthesizing mycelial protein contained in 1 ml: 60 μM-Tris/HCl, pH 7.6, 30 μM-NH₄Cl, 18 μM-MgCl₂, 2 μM-
-dithiothreitol, 0.1 μm-spermine tetrahydrochloride, 6 μm-ATP, 0.2 μm-GTP, 10 μm-phosphoenolpyruvate, 10 μg of pyruvate kinase, non labelled aminoacids (except aspartic acid) at 0.1 μm concentration, 360 μg of tRNA, the post-polyosomal supernatant (2.5 mg protein), polysomes (3 mg protein), and 0.3 μCi of [14C]aspartic acid or 1 μCi of [3H]aspartic acid, supplemented with non-radioactive aspartic acid at a concentration of 0.1 μm. The incubation was carried out at 35°C for 30 min, then the reaction mixture was subjected to electrophoresis on polyacrylamide gel according to Sugiyama & Nakada (1968). Radioactivity was determined directly in the gel by the method of Muto (1968), as described by Zagórski et al. (1972).

Reagents. The reagents used were from the following sources: ferulic acid, Koch-Light (Colnbrook, England); dithiothreitol, spermine tetrahydrochloride, Alumina type 305 and phosphoenolpyruvate, Sigma Chem. Corp. (Saint Louis, Mo., U.S.A.); Triton X-100, bentonite SE, DEAE-SS-cellulose and cycloheximide (actidione), Serva (Heidelberg, G.F.R.); glutathione, reduced, T. Schuchardt (München, G.F.R.); α-amylose from Aspergillus oryzae, Calbiochem (La Jolla, Calif., U.S.A.); DNase I from beef pancreas, Worthington (Freehold, N.J., U.S.A.); pyruvate kinase, Fluka AG (Bern, Switzerland); actinomycin D, Merck (Darmstadt, G.F.R.); [14C]aspartic acid (120 mCi/mmole) and [U-14C]uridine (400 μCi/mmol), Institute of Radioisotopes (Prague, Czechoslovakia); [G-3H]aspartic acid (178 mCi/mmol), the Radiochemical Centre (Amersham, England).

RESULTS AND DISCUSSION

Induction of laccase. Time-course of the induction of fungal laccase by ferulic acid, is illustrated in Fig. 1. In the three Basidiomycetes species studied, phases I and II of induction lasted about ten times longer than in Ascomycetes fungi (Turner et al., 1970; Cybis & Węgleński, 1972), and 100 times longer than in bacteria (Kepes, 1963; Kepes & Beguin, 1966). The level of laccase in the cultures untreated with ferulic acid remained unchanged under our experimental conditions.

![Fig. 1. Effect of ferulic acid on the activity of intracellular laccase in the fungi: A, Corticis versicolor; B, Pleurotus ostreatus; C, Pholiota mutabilis. Laccase activity: O, control (without ferulic acid); •, in the presence of 0.2 mm-ferulic acid. I, II, III, successive phases of induction. Laccase activity is expressed in arbitrary units.](image-url)
Synthesis of specific mRNA. Synthesis of mRNA on induction of laccase was followed by two methods: indirect, by measuring the enzymatic activity in the presence of "cumulative messenger" (Kepes, 1967; Cybis & Węgleński, 1972), and direct, by labelling of mRNA in vivo with radioactive precursors.

Fig. 2. The response of laccase activity to the accumulated messenger. The activity was measured in the mycelium preincubated (▼) or non-preincubated (●) with ferulic acid. For conditions of synthesis of the cumulative messenger, see text. A, Coriolus versicolor; B, Pleurotus ostreatus; C, Pholiota mutabilis.

In the first case, the mycelia of Coriolus versicolor, Pleurotus ostreatus and Pholiota mutabilis were incubated with ferulic acid for 60, 75, and 75 min, respectively (phase I of induction, corresponding to mRNA synthesis), then were transferred to the medium free of the inducing agent, and incubated in this medium until laccase activity attained a complete plateau. When the mycelium was transferred again to

Fig. 3. ¹⁴C-Labelling of RNA, and laccase activity on induction of laccase with ferulic acid. [¹⁴C]RNA (■) and laccase activity (○) in the cultures grown in the presence of ferulic acid. A, Coriolus versicolor; B, Pleurotus ostreatus; C, Pholiota mutabilis.

the ferulic-acid-containing medium, the synthesis of laccase protein started immediately due to the presence of the existing specific accumulated messenger (Fig. 2).

For direct measurement of specific mRNA, the mycelium was incubated for the time indicated with ferulic acid and [³H]uridine, then the [³H]uridine-containing
RNA was isolated. Radioactivity of RNA and laccase activity were measured parallelly in the mycelium (Fig. 3). As it can be seen from Figs. 1, 2 and 3, mRNA synthesis demonstrated by both methods preceded laccase synthesis by the time corresponding to the induction phases I plus II.

Effect of antibiotics. Mycelium was incubated with ferulic acid for time corresponding to the induction phase I, then it was transferred parallelly to the two media free of ferulic acid, one of which contained actidione (5 μg/ml). Incubation was continued as previously, except that the incubation medium contained no ferulic acid (Fig. 4). In the medium devoid of the antibiotic, induction of laccase took place for some time linearly due to the presence of specific mRNA, then gradually passed to a plateau (Fig. 4). The effect of induction was abruptly abolished by actidione, an antibiotic which blocks translational ribosomes and thus stops biosynthesis of laccase protein at the elongation step.

Fig. 4. Effect of actidione on induction of laccase in: A, Coriolus versicolor; B, Pleurotus ostreatus; and C, Pholiota mutabilis. Laccase activity was measured in the mycelium preincubated with ferulic acid (●) and transferred to the medium free of ferulic acid, containing (△) or not containing (○) actidione.

Fig. 5. Effect of actinomycin D on mRNA and the activity of intracellular laccase in: A, Coriolus versicolor; B, Pleurotus ostreatus; and C, Pholiota mutabilis. The mycelium was cultivated in the presence of 0.2 mM-ferulic acid, and actinomycin D (5 μg/ml) was added at the time indicated by arrow. [14C]RNA, in the absence (●) and presence (△) of actinomycin D. Laccase activity, in the absence (●) and presence (△) of actinomycin D.
Actinomycin D, an inhibitor of transcription, when added to the cell-free system simultaneously with ferulic acid, completely abolished the induction of both mRNA and laccase (Fig. 5); the addition of actinomycin D to the system incubated with ferulic acid for 1 h caused a partial inhibition, and after 2 h had practically no effect. The experiment with actinomycin D indicates that inhibition of laccase induction was due to blocking of the specific mRNA synthesis, and the induction did not result from activation of the pre-existing form.

According to Froehner & Eriksson (1974), actidione and actinomycin D at low concentrations not only do not block laccase synthesis in some fungi (Neurospora crassa) but even have an inducing effect. The authors explain this intriguing effect, in agreement with Horowitz et al. (1970), by partial inhibition of the general synthesis of repressor protein. Consequently, lowering of the repressor concentration causes derepression of the laccase gene. In our experiments, actidione and actinomycin D at low concentrations also caused some induction of laccase in all three fungi studied, it was necessary, therefore, to apply the antibiotics at concentrations as high as 5 μg/ml.

* Determination of half-life time of the mRNA coding for laccase.* In the preliminary experiments it was found that 8-h incubation in the absence of the inducing agent did not cause appreciable losses of the activity of the inducible form of laccase. It was also found that the uptake of ferulic acid by mycelium during phase I of induction has a linear character, which indicates the undisturbed transport of the inducing agent into the cells (in preparation).

The half-life time of mRNA was determined by the logarithmic method of Kepes (1963), further developed by Cybis & Węgleński (1972). The mycelium was incubated with ferulic acid for the time corresponding to phase I of induction, then transferred to the medium free of ferulic acid, and cultivated until the laccase activity attained a plateau (Fig. 6, curve 1). Intercepts (5) designate the half-life

![Fig. 6. Messenger RNA activity during the first phase of laccase induction in the fungi: A, Coriolus versicolor; B, Pleurotus ostreatus; and C, Pholiota mutabilis. 1, Laccase activity; 2, plateau of the laccase activity; 3, mRNA activity; 4, logarithm of mRNA activity; 5, biological half-life time of mRNA. For details, see Cybis & Węgleński (1972).]
time of mRNA. The respective values for mRNA of Coriolus versicolor, Pleurotus ostreatus and Pholiota mutabilis were 30, 37 and 24 min. The half-life time of the mRNA specific for β-galactosidase, determined in the same way for Escherichia coli (Kepes, 1963) is 1 min, and for arginase in Aspergillus nidulans, 2.7 min (Cybis & Węgielński, 1972). Thus, the values obtained for Basidiomycetes are considerably higher. However, even longer half-life time of specific mRNAs (2 h) have been reported for peroxidase of sugar-cane stem (Gayler & Glasziou, 1968).

**Molecular weight of mRNA.** The results of electrophoresis of the Pleurotus ostreatus RNA are presented in Fig. 7. As it can be seen, the molecular weight of the rapidly labelled RNA is 4.5×10⁶. This value is compatible with coding by this RNA species of the inducible form of laccase in this organism (the molecular weight of this enzyme is 4.7×10⁴; Leonowicz & Trojanowski, 1975a).

![Graphs showing electrophoresis results](image)

Fig. 7. Polyacrylamide-gel electrophoresis (A) and molecular weight determination (B) of ribonucleic acids of the fungus Pleurotus ostreatus.

A: ---, absorbance; •, radioactivity.

B: 1, E. coli 23S rRNA; 2, yeast 18S rRNA; 3, E. coli 16S rRNA; 4, [14C]RNA from Pleurotus ostreatus; 5, yeast 4S rRNA. The ribonucleic acids used as standards were from Calbiochem (La Jolla, Calif., U.S.A).

**Synthesis of laccase protein in vitro.** The results of electrophoresis of the radioactive product synthesized in vitro in the systems from Coriolus versicolor are illustrated in Figs. 8 and 9. In the first system, the supernatant was derived from the mycelium grown in the absence of ferulic acid, and polysomes from the mycelium incubated with ferulic acid, whereas in the other two systems both the supernatant and polysomes were derived from the same mycelium. In both cases when polysomes were derived from the mycelium treated with the inducing agent, electrophoretic mobility of the radioactive polypeptide was closely similar to that of the inducible form of the Coriolus laccase (indicated in the gel by arrows). Thus it appears that polysomes from the mycelium grown in the presence of the inducing agent contain the biologically active mRNA specific for the inducible form of laccase.
The presence of mRNAs specific for inducible enzyme has been repeatedly reported (Kepes & Beguin, 1966; Cybis & Węgleński, 1972). Among others, it is known that synthesis de novo of α-amylase is induced by gibberelin (GA₃) in the

Fig. 8. Polycrylamide-gel electrophoresis of the polypeptides synthesized in vitro in homologous systems from Coriolus versicolor. A, Polysomes and supernatant from control mycelium; B, polysomes and supernatant from the mycelium cultivated for 2 h with ferulic acid; C, difference between B and A. [¹⁴C]Aspartic acid was used as a marker. The arrow indicates position on the gel of the inducible form of laccase.

Fig. 9. Polycrylamide-gel electrophoresis of the polypeptides synthesized in vitro in mixed systems from Coriolus versicolor. A, Polysomes from control mycelium, supernatant from mycelium cultivated in the presence of ferulic acid; B, polysomes from mycelium cultivated in the presence of ferulic acid, supernatant from control mycelium; C, difference between B and A. [¹⁴C]Aspartic acid was used as a marker. The arrow indicates position on the gel of the inducible form of laccase.

aleurone layer of barley and wheat seed (Filner & Varner, 1967; McMaster, 1976). The increased synthesis of α-amylase occurs after a lag phase (Goodwin & Carr, 1972) as in the case of laccase. The inducible forms of α-amylase are polymorphic (Tanaka & Akazawa, 1970), and their biosynthesis de novo was demonstrated with the use of specific inhibitors (Chrispeels & Varner, 1967) and by measuring incorporation of labelled precursors to the RNA containing a poly(A) chain (Jacobsen & Zwar, 1974a, b; Ho & Varner, 1974).

The molecular weight and immunological properties of the polypeptide synthesized in vitro in the system containing the RNA isolated from the wheat seed aleurone cells treated with GA₃, were similar to those of wheat α-amylase (Higgins et al.,
1976). According to those authors, the specific, active mRNA was synthesized de novo.

There are several experiments, with the use of active polysomes, evidencing biosynthesis in vitro of specific proteins, e.g. liver albumin (Hill et al., 1972), hair keratin (Steinert & Rogers, 1971), lactalbumin and lactoglobulin of mammary glands (Gaye et al., 1972). In the examples cited, the specific mRNA was bound with polysomes which were isolated from the tissues specialized in preferential synthesis of particular proteins. It seems possible that in the higher fungi studied, mycelium, similarly as the afore-mentioned specialized tissues, responds to the presence of ferulic acid by preferential synthesis of laccase, an enzyme indispensable for detoxication of the phenol-polluted environment (Grabbe et al., 1968).

REFERENCES

INDUKCJA LAKAZY U BASIDOMYCETES:
mRNA SWOISTY DLA LAKAZY

Streszczenie

Stwierdzono syntezę mRNA specyficznego dla indukujących się form lakazy u Coriolus versicolor, Pleurotus ostreatus i Pholiota mutabilis.

Czas biologicznego półtrwania tych mRNA wynosił odpowiednio 30, 37 i 24 min.

Masa cząsteczkowa nowo-synthetyzowanego mRNA u Pleurotus ostreatus jest ok. $4,5 \times 10^4$, co jest zgodne z wielkością cząsteczki białka indukującej się formy lakazy.

Polisomy uzyskane z grzybni traktowanej kwasem ferulowym syntetyzowały in vitro polipeptyd o ruchliwości elektroforetycznej podobnej do ruchliwości białka lakazy.

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