



# Purification and characterization of the first bacterial laccase in the rhizospheric bacterium *Azospirillum lipoferum*

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## Abstract

*Azospirillum lipoferum* is a soil bacterium known for its ability to colonize roots and to promote plant growth. Recently, a laccase-like polyphenol oxidase activity has been reported in a strain isolated from the rhizosphere of rice. Purification to apparent homogeneity of *A. lipoferum* laccase was achieved after cell disruption by sonication, protein solubilization by Triton X-100, acetone and protamine precipitation, hydroxyapatite column chromatography in the presence of urea (5 M) and NaCl (2 M), and finally by conventional hydroxyapatite column chromatography. After this final step, laccase was in an inactive form but could be reactivated by adding sodium dodecyl sulfate (SDS). Based on gel electrophoresis results, we suggest that native *A. lipoferum* laccase is composed of a catalytic polypeptide chain with low molecular mass (16.3 kDa) and one or two regulatory/structural chains with high molecular mass (81.5 kDa). The purified enzyme was found to be thermostable up to 70°C for 10 min, had an optimal pH of 6.0, and was inhibited by tropolone, a known inhibitor of metal-containing enzymes. Although the natural substrate of this enzyme is unknown, its biochemical characterization may facilitate further investigations on the ecological role of this laccase in the process of root colonization by *A. lipoferum*. © 2000 Elsevier Science Ltd. All rights reserved.

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## 1. Introduction

Laccases (benzenediol:oxygen oxidoreductases; E.C. 1.10.3.2) are oxidases that catalyse the reduction of oxygen to water, the oxidized substrate being typically a *p*-diphenol. This unique ability can be used to distinguish laccase activity from that of other members of the polyphenol oxidases (PPO) as follows.

1. Catechol oxidases (E.C. 1.10.3.1), which catalyse the hydroxylation of monophenols (monophenolase or cresolase activity) and the oxidation of *o*-diphenols to *o*-quinones (*o*-diphenolase or catecholase activity) in two distinct reactions.

2. Monophenol monooxygenases or tyrosinases (E.C.1.14.18.1), which catalyse only the hydroxylation of monophenols (Nicolas et al., 1994).

The full range of compounds that can be affected directly or indirectly by laccase activity is not known. Polyphenols, methoxy-substituted phenols, diamines and a considerable range of other compounds can be used as substrates, but syringaldazine (4-hydroxy-3,5-dimethoxybenzaldehyde azine) has been considered a laccase-specific substrate (Harkin et al., 1974). Although laccases have been studied for many years, their natural substrate(s) *in vivo* are not known and their function(s) in cell metabolism remain unclear. Interest in laccases has increased recently because of their potential use in the detoxification of pollutants (Bollag et al., 1988).

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Most laccases originate from fungal sources and are located or functionally present extra and/or intracellularly (at least at some stages of their development) in almost every fungus examined, including mycorrhizal species. Generally, fungal laccases are described as copper containing glycoproteins with varying carbohydrate content, and are classified as members of the blue copper proteins or blue copper oxidases, other members of this group being plant ascorbate oxidase, mammalian plasma protein ceruloplasmin and phenoxazinone synthase from *Streptomyces antibioticus* (Freeman et al., 1993; Thurston, 1994). In rare cases, laccases have been found in some higher plants. First discovered in the exudates of the Japanese lacquer tree *Rhus vernicifera*, laccases are also known to be present in Loblolly pine xylem (Bao et al., 1993), and excreted in the growth medium of liquid cultures of *Acer pseudoplatanus* (sycamore) cells (Bligny and Douce, 1983).

Recently, a polyphenol oxidase activity was described in a non-motile strain of *Azospirillum lipoferum* isolated from rice rhizosphere (Bally et al., 1983). Using a combination of substrates and inhibitors, this PPO was identified as a laccase (Givaudan et al., 1993). Comparative studies of *A. lipoferum* and *Pyricularia oryzae* laccases showed that their substrate ranges and sensitivities to several inhibitors were similar (Faure et al., 1995). To date *A. lipoferum* remains one of the only two bacterial species in which laccase activity has been detected (Solano et al., 1997).

In this paper, we describe the isolation of the laccase of *A. lipoferum* strain 4 T. This isolation was mainly based on the property of the laccase to bind tightly to the nucleic acid-protamine complex and on its resistance to high urea and NaCl concentrations. The structure of three laccase active bands revealed by electrophoresis was also investigated.

## 2. Materials and methods

### 2.1. Bacterial strain and growth conditions

*A. lipoferum* non-motile strain 4 T was isolated from rice rhizosphere (Bally et al., 1983). Cells were grown and harvested as described previously (Givaudan et al., 1993).

### 2.2. Protein solubilization and extraction

All steps were carried out at 4°C, except hydroxyapatite chromatography, which was run at room temperature (24 ± 2°C). Exponentially growing cells were harvested by centrifugation (12,000 g, 10 min), resuspended at a concentration of 25 ml g<sup>-1</sup> wet weight in buffer A [10 mM Tris-HCl (pH 7.2)], and cell walls were disrupted by ultrasonic treatment (Vibracell

VC60, Bioblock, France) at 50 W for 8 min (1 s pulses) with cooling in an ice bath. After addition of 50 ml buffer A, total cell extracts were incubated for 1 h at 4°C under gentle agitation in the presence of 1% (v/v, final concentration) Triton X-100. Homogenates from 3 to 4 g bacterial cells treated as above were pooled and used for further purification of the enzyme.

### 2.3. Purification procedure

The homogenates were precipitated twice by adding about six volumes of cold acetone (–20°C), and the precipitated material was collected on the glass container walls. After air drying, the precipitates were suspended in 50 ml buffer B [1 mM Na-phosphate (pH 7.2)] and Salmon protamine sulfate (10 mg g<sup>-1</sup> bacterial cells) was added slowly. After thorough mixing, the solution was centrifuged for 10 min at 12,000 g. The pelleted material was resuspended in 50 ml buffer B and washed four times in the same buffer in order to eliminate the detergent and any non-bound proteins. The final pellet was then solubilised in 50 ml buffer C [1 mM Na-phosphate (pH 7.2), 5 M urea, 2 M NaCl] and centrifuged at 20,000 g for 20 min. The supernatant was applied to a hydroxyapatite (Bio-gel HTP, Bio-rad) column (8 × 2 cm), prepared as described elsewhere (MacGillivray et al., 1972), which had been equilibrated with buffer C. The column was developed with the same buffer at a flow rate of 30 ml h<sup>-1</sup>, and 2.5 ml fractions were collected. Protein and nucleic acids contents of eluates were monitored spectrophotometrically at 280 and 260 nm, respectively. Bound proteins and nucleic acids were further eluted by increasing the concentration of the Na-phosphate buffer to 400 mM. Laccase containing fractions were pooled and dialysed against buffer D [50 mM Na-phosphate buffer (pH 7.2)]. The dialysed laccase active homogenate was clarified by centrifugation at 20,000 g for 20 min and was then applied to a second hydroxyapatite column (2 × 10 cm) which had been previously equilibrated with buffer D. After washing with 50 ml of the same buffer, the column was developed with a Na-phosphate buffer (pH 7.2) step gradient ranging from 50 to 400 mM at a flow rate of 30 ml h<sup>-1</sup>, and 3 ml fractions were collected. Laccase containing fractions were pooled, dialysed extensively against buffer D, and stored at –20°C until further use.

### 2.4. Laccase activity assay and kinetic studies

Laccase activity was measured at 30°C by the end-point method using syringaldazine as substrate in a total volume of reaction medium of 1 ml (Harkin and Obst, 1987). Absorbance at 525 nm was measured

using a Kontron (Uvicon 930) spectrophotometer. Appropriate amounts of laccase preparations were first incubated at 30°C for 10 min in 100 mM MES [2-(*N*-morpholino)-ethanesulfonic acid] buffer (pH 6.0) containing 1.7 mM SDS (see results section for justification). Then the reaction was initiated by addition of 50 µl of 1 mM syringaldazine dissolved in absolute ethanol and was stopped by adding 1 ml absolute ethanol. Rate linearity was checked by carrying out a number of assays at different reaction times (5, 10 or 15 min) with different extract volumes. One unit of activity was defined as the amount of laccase required to produce a  $\Delta\text{OD}_{285}$  increase  $\text{min}^{-1}$  of 1 under standard assay conditions.

The optimum pH for laccase activity was determined as described above in 100 mM buffers between pH 3.5 and 8.2: citrate (pH 3.5–6.0), MES (pH 5.1–7.1) and MOPS (pH 6.2–8.2). Inhibition of activity was studied by incubating enzymatic preparations for 10 min at 30°C in the presence of increasing concentrations of tropolone, prior to substrate addition.

### 2.5. Electrophoretic procedures

Analytical SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was performed with a Mini-Protein II cell (Bio-Rad Laboratories, Inc.) according to the procedure described by Laemmli (1970), using acrylamide concentrations of 7.5 or 12.5% for the separating gels and 3% for the stacking gels. Laccase active samples were concentrated by ultrafiltration (Millipore Ultra-free-CL, cut-off 10,000 NMWL), dissolved in sample buffer containing 6.25 mM Tris–HCl (pH 6.8), 2% SDS, 5%  $\beta$ -mercaptoethanol, 10% glycerol and 0.002% bromophenol blue as tracking dye, and boiled for 2 min just before use. Protein bands were visualized by silver staining using the Pharmacia Biotech silver stain kit according to the manufacturer's instructions. The calibration proteins used for molecular mass determination were myosin from rabbit muscle (200 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase from bovine erythrocytes (29 kDa), trypsinogen (24 kDa), trypsin inhibitor from soybean (20.1 kDa) and  $\alpha$ -lactalbumin (14.2 kDa).

Nondissociating PAGE was performed as described above, except that  $\beta$ -mercaptoethanol was omitted, and the unreduced samples were incubated at 60°C for 10 min prior to electrophoresis. Laccase specific activity staining of the gels was carried out after rinsing the slabs twice with distilled water for 1 min, by incubating the gels in 0.1 M MES buffer (pH 6.0) containing 1.7 mM SDS and 40 µM syringaldazine. Active bands appeared within a few minutes. Because of the instability of the product of the reaction, coloration was stabilized and intensified by further incubating the

gels in the presence of 0.04% (w/v) ABTS [(2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid))] for 1 h, followed by a second 1 h incubation in the presence of 0.02% (w/v) *p*-phenylenediamine. For the purpose of re-electrophoresis, active bands were sliced out from the slabs, and after protein elution into non-reducing sample buffer, they were submitted to a second SDS–PAGE using 7.5% acrylamide resolving gels.

### 2.6. Protein determination

Protein concentration was determined by the dye binding method of Bradford (1976) using bovine serum albumin as standard. The protein content of diluted purified samples was determined using the microassay method of Peterson (1983).

## 3. Results

### 3.1. Purification of *A. lipoferum* laccase

Laccase from *A. lipoferum* 4 T was purified with a 24% yield by a combination of acetone and protamine precipitation, hydroxyapatite chromatography in the presence of 5 M urea and 2 M NaCl, followed by conventional hydroxyapatite chromatography. An outline of the procedure is illustrated in Table 1. The reproducibility was excellent, and similar elution profiles as well as recovery yields were obtained from several independent experiments.

Treatment of cell-free extracts by Triton X-100 for 1 h and subsequent acetone precipitation was found to be necessary, without any negative effect on laccase activity. Curiously, *A. lipoferum* laccase activity was pre-

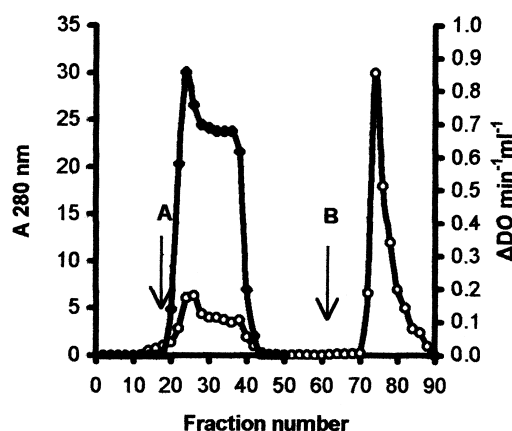


Fig. 1. Hydroxyapatite column chromatography of *A. lipoferum* laccase in the presence of 5 M urea and 2 M NaCl. The column was developed with (A) 1 mM Na-phosphate (pH=7.2), 5 M urea, 2 M NaCl and (B) 400 mM Na-phosphate (pH=7.2), 5 M urea, 2 M NaCl. Fractions of 2.5 ml were assayed for absorbance at 280 nm (○-○) and for activity (●-●).

Table 1

Purification steps of *Azospirillum* laccase. 3 g centrifuged bacterial cells was treated as described in Materials and methods (sections 2.1.–2.3.)

Enzyme fraction	Total activity (U)	Total protein (mg)	Specific activity (U mg <sup>-1</sup> )	Yield (% of extract)	Purification (fold)
Crude extract	184	210	0.9	100	
Acetone precipitation	184	190	1	100	1
Hydroxyapatite chromatography (5 M urea + 2 M NaCl)/dialysis	84	70	1.2	45	1.2
Hydroxyapatite chromatography/dialysis	45	1.9	23.7	24	27

precipitated by protamine sulfate. The enzyme was tightly bound to the precipitated protamine-nucleic acid complex and no activity was present in the supernatant even after gentle centrifugation of the mixture. Activity was still bound to the complex even after three or four washes with low molarity Na-phosphate buffer, resulting in the elimination of almost half the proteins, with a total activity loss of only 20% (result not shown).

Solubilization of laccase activity from the protamine-nucleic acid complex by use of increasing concentrations of sodium chloride and/or phosphate was not satisfactory. The most efficient treatment for enzyme solubilization was achieved with Na-phosphate containing 5 M urea and 2 M NaCl, with a recovery yield of about 100%.

Laccase active preparations were then submitted to hydroxyapatite chromatography under high ionic strength conditions in the presence of a chaotropic agent. Activity as well as other nucleic acid-free proteins, was not retained by the column (Fig. 1). Nucleic acids and bound proteins (63% of the bulk proteins) were further eluted by increasing the concentration of the phosphate buffer to 400 mM.

The final step of *A. lipoferum* laccase purification

was conventional hydroxyapatite chromatography (Fig. 2). The enzyme was eluted as a symmetric peak with 0.2 M phosphate, and the apparent specific activity of pooled fractions was increased to 24 U mg<sup>-1</sup> protein. The purification factor was about 27-fold and the recovery around 24%, and laccase purity was checked by SDS-PAGE (Fig. 5).

Similar chromatography elution patterns were obtained with extracts that were not treated with protamine. In this case, subsequent purification of the enzyme to homogeneity by conventional hydroxyapatite chromatography was not satisfactory because numerous contaminant proteins were still present in the laccase active fractions as judged by SDS-PAGE in denaturing conditions (results not shown). Therefore, despite the low specific activity after protamine precipitation and hydroxyapatite chromatography in the presence of urea and NaCl, these steps were found to be essential for the purification of *A. lipoferum* laccase.

### 3.2. Properties of *A. lipoferum* laccase

After the final hydroxyapatite chromatography step, the enzyme was in an inactive form. Laccase was reactivated by adding SDS to the reaction mixture (Fig. 3). A sharp increase in activity was obtained up to 0.4 mM SDS and apparent maximal rates occurred in the range from 0.7 to 1.7 mM SDS. At higher concen-

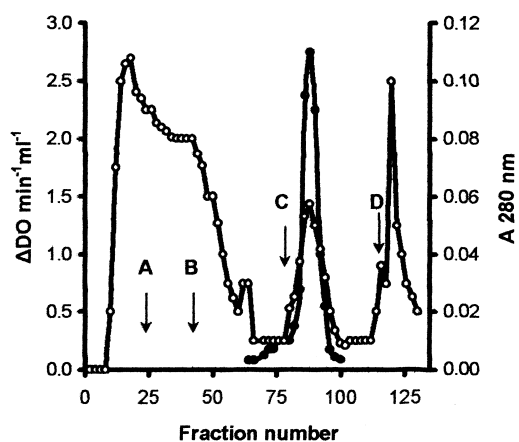


Fig. 2. Conventional hydroxyapatite column chromatography of *A. lipoferum* laccase. The elutants were: A = 50 mM Na-phosphate buffer pH = 7.2; B = 150 mM Na-phosphate buffer pH = 7.2; C = 200 mM Na-phosphate buffer pH = 7.2; and D = 400 mM Na-phosphate buffer pH = 7.2. Fractions of 3 ml were assayed for absorbance at 280 nm (○—○) and for activity (●—●).

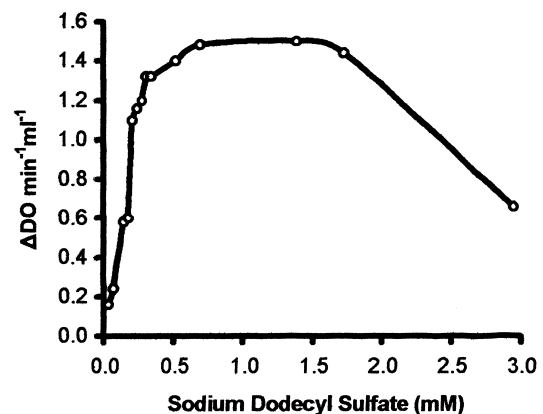


Fig. 3. Activation of purified laccase by sodium dodecyl sulfate (SDS).

trations of SDS, the reaction rate declined, although the enzyme retained 50% of its maximal activity at 3 mM SDS.

SDS-activated *Azospirillum* laccase showed a remarkable thermal stability (Fig. 4). Activity was not affected and remained stable after preincubation for 10 min at temperatures ranging from 25 to 70°C. At 80°C laccase had 40% residual activity but was completely inactivated after 2 or 3 min of preincubation at higher temperatures. The half-life of the enzyme at 70°C was estimated to be 43 min.

The  $K_m$  of the SDS-activated enzyme was 34.65  $\mu\text{M}$  as determined by using syringaldazine as substrate in the range from 3 to 100  $\mu\text{M}$  (result not shown). Optimal pH was 6.0 as determined in either citrate or MES or MOPS buffers (result not shown).

Bacterial SDS-activated laccase was inhibited by tropolone, a specific inhibitor of metal containing enzymes. The  $\text{IC}_{50}$  value was 80  $\mu\text{M}$ .

### 3.3. Electrophoresis and multiple forms of bacterial laccase

SDS-PAGE under unreduced conditions revealed three active bands, designed as  $\alpha$ ,  $\beta$  and  $\gamma$ , with an apparent molecular mass of 179.3, 97.8 and 48.9 kDa, respectively. During the coloration procedure, band  $\beta$  appeared first and band  $\gamma$  last. Silver staining of the same gels revealed three bands with  $R_f$  values similar to those of the active bands (Fig. 5, lane I.A and I.B). Activity zymograms were always the same regardless of the purification step and treatment of the acetone crude extract with protamine or not. When extracts were heated at 60°C for 10 min before electrophoresis, a new protein band was revealed with an apparent molecular mass of 81.5 kDa while the activity of the 48.9 kDa band was enhanced. Under totally reducing conditions, two protein bands were obtained, with

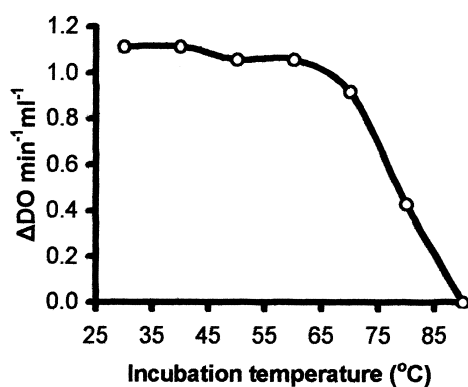


Fig. 4. Thermostability of SDS-activated *A. lipoferum* laccase upon preincubation at different temperatures for 10 min. Activity was measured at 30°C as described in Materials and methods (section 2.4).

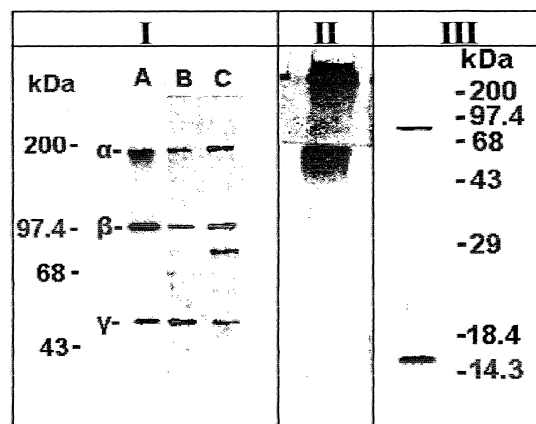


Fig. 5. Polyacrylamide gel electrophoresis of *A. lipoferum* laccase. I. SDS-PAGE under non-reducing semi denaturing conditions at 7.5% polyacrylamide resolving gel. II. Native PAGE at 7.5% polyacrylamide resolving gel. III. SDS-PAGE under fully reducing conditions at 12.5% polyacrylamide resolving gel. IA: gel stained for laccase activity with syringaldazine, ABTS and *p*-phenylenediamine, activity bands designed as  $\alpha$ ,  $\beta$  and  $\gamma$ . IB: gel stained for proteins. IC: sample was preincubated at 60°C for 10 min prior to electrophoresis, gel stained for proteins. II: native PAGE of laccase preparation, gel stained as lane IA in the presence of 1.7 mM SDS (in the photo stacking gel is also viewing). III: SDS-PAGE under fully denaturing conditions (presence of  $\beta$ -mercaptoethanol in the sample buffer), gel stained for proteins. Left and right of gels image: molecular masses in kDa of markers.

apparent molecular masses of 81.5 kDa (which corresponds to the new band appearing following preincubation at 60°C for 10 min) and 16.3 kDa, respectively (Fig. 5, III). When the active bands were extracted from the gels and submitted to a second electrophoresis, bands  $\alpha$  and  $\beta$  were each resolved into two bands (band  $\alpha$  to  $\alpha$  and  $\beta$ , and band  $\beta$  to  $\alpha$  and  $\beta$ ), and the

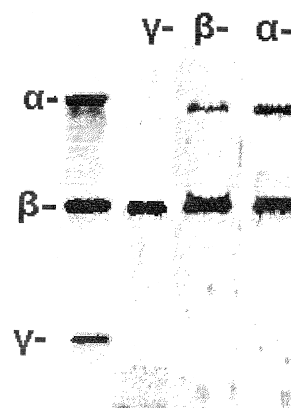


Fig. 6. SDS-re-electrophoresis of three electrophoretic active forms of *A. lipoferum* laccase referred as  $\alpha$ ,  $\beta$  and  $\gamma$  (left side lane). Each active band was extracted from the gel and re-subjected to electrophoresis under non reducing conditions. Lane  $\gamma$ : re-electrophoresis of  $\gamma$  form, lane  $\beta$ : re-electrophoresis of  $\beta$  form, lane  $\alpha$ : re-electrophoresis of  $\alpha$  form.

faster one (band  $\gamma$ ) to one active band with the same mobility as band  $\beta$  (Fig. 6).

#### 4. Discussion

The aim of the present study was to characterize the enzyme responsible for the laccase activity in a variant strain of the rhizospheric bacterium *A. lipoferum*. To our knowledge, this is the first report describing the purification of a bacterial laccase. Protamine precipitation and hydroxyapatite chromatography in the presence of urea and NaCl were the key steps in the purification of *A. lipoferum* laccase to apparent homogeneity. Contrary to other reports, the zymograms remained the same at all steps of purification, and gave three active bands referred as  $\alpha$ ,  $\beta$  and  $\gamma$  with constant  $R_f$  values. Therefore, the three active bands revealed by electrophoresis should be considered as potent native forms of *Azospirillum* laccase. Many attempts with other techniques of protein purification gave unsatisfactory results in respect to enzyme yield, homogeneity and “stability” of activity zymograms.

Surprisingly, laccase was tightly bound to the protamine-nucleic acid complex. Protamine sulfate from Salmon is currently used as a cleaning step for extracts containing large amounts of nucleic acids. The resultant precipitate usually contains mainly nucleoproteins and small quantities of soluble proteins. The binding of enzymes to such complexes has been reported only in rare cases. For example, it has been observed that  $\alpha$ -ketoglutarate dehydrogenase from *E. coli* and yeast phosphofructokinase were totally adsorbed on the protamine-nucleic acids complex and this phenomenon has been used as an important means of purification of these two enzymes (Reed and Mukherjee, 1969; Welch and Scopes, 1981). This peculiar property was also found to be essential for *A. lipoferum* laccase purification.

The recovery of laccase from the protamine-nucleic acid complex was achieved using 5 M urea under high ionic strength conditions (2 M NaCl). Various other attempts to solubilise laccase were not satisfactory. It was surprising that laccase conserved its catalytic conformation in such high salt and urea concentrations, which are often used to denature proteins. The same surprising phenomenon has also been observed for other enzymes of the PPO group, e.g. *o*-diphenol oxidase from potatoes, which was fully active in 4 M urea (Balasingam and Ferdinand, 1970) and tyrosinase from *Vicia faba* (L) for which full activity was also achieved in 4 M urea (Swain et al., 1966). In the latter case, it was concluded that the activity of this enzyme is promoted by mild denaturing conditions, which normally cause a reduction in the activity of many enzymes. The *o*-diphenol oxidase from potatoes has

been characterized as a ribonucleoenzyme since the fully active enzymatic preparation consisted of a mixture of RNA and proteins in equal amounts. Contrary to our bacterial laccase, the enzyme could not be precipitated by protamine and the activity remained in the supernatant (Balasingam and Ferdinand, 1970).

*A. lipoferum* laccase activity was not retained by the hydroxyapatite and was eluted immediately after the void volume in the first fractions of column wash with the equilibration buffer (Fig. 1). Other proteins without any laccase activity, as well as RNA and DNA were eluted by 0.4 M phosphate buffer. Under the special conditions used (presence of 2 M NaCl and 5 M urea), hydroxyapatite acts as an ion-exchanger for macromolecules and can be used for the separation of basic and acidic proteins. This procedure has been used for the fractionation of the non-histone proteins of chromatin from calf thymus and various other tissues (MacGillivray et al., 1972). According to the authors, the proteins that were not retained by the urea–NaCl–hydroxyapatite column in 1 mM phosphate buffer were identified as histones. Thus, based on the results of MacGillivray et al. (1972), microbial laccase elution profile from the urea–NaCl–hydroxyapatite column provides indirect evidence of the basic nature of this enzyme. Further, the use of salt and urea as solubilization agents of laccase from the protamine-nucleic acids complex may also be an indirect proof that the microbial laccase is a basic protein and is bound in vivo to microbial DNA. Similar solubilization conditions (e.g. 2–3 M NaCl and 5–7 M urea) have been used to dissociate and solubilize DNA-binding proteins (Bekhor et al., 1969; Gilmur and Paul, 1969).

It has been reported previously that phenoloxidases retain enzymatic activity after separation on SDS–PAGE (Angleton and Flurkey, 1984). *A. lipoferum* extracts analyzed by SDS–PAGE, regardless of the purification step, always gave three active bands (with apparent molecular masses of 179.3, 97.8 and 48.9 kDa) towards syringaldazine, ABTS and *p*-phenylenediamine (band  $\alpha$ ,  $\beta$  and  $\gamma$ , respectively, Fig. 5, lane I.A). The homogeneity of *A. lipoferum* laccase preparation at the end of the purification procedure was confirmed because the three bands of protein on the gel coincided with active bands (compare lane I.A and I.B of Fig. 5). Analysis of the same laccase preparation by SDS–PAGE under totally reducing conditions revealed two polypeptides with molecular masses of 81.5 (H, heavy chain) and 16.3 kDa (L, light chain) respectively (Fig. 6, III). These results taken together suggest that the light chain (16.3 kDa) was responsible for the catalytic activity of the aggregates, since band  $\gamma$  could have consisted of three light chains ( $16.3 \times 3 = 48.9$  kDa), band  $\beta$  could have consisted of six light chains ( $16.3 \times 6 = 97.8$  kDa) and band  $\alpha$  could have

consisted of 11 light chains ( $16.3 \times 11 = 179.3$  kDa). The results of re-electrophoresis presented in Fig. 6 support these statements because the bands  $\alpha$  and  $\beta$  were reversibly interconvertible and that band  $\beta$  could be also a product of the band  $\gamma$ . Thus the three active bands could be the product of association-dissociation phenomena of one molecular species. Similar observations have been made with other enzymes of the PPO group, like mushroom tyrosinase (Jolley et al., 1969; Strothkamp et al., 1976), particulate tyrosinase of human malignant melanoma (Nishioka, 1978) and polyphenol oxidase from spinach thylakoids (Golbeck and Cammarata, 1981). In all cases, authors based on electrophoretic or chromatographic findings noted that the results tend to support the dynamic conversion of one form of the enzyme to another rather than the existence of separate molecular species.

However, the active bands  $\alpha$  and  $\beta$  of *A. lipoferum* appear to consist of two different molecular species: band  $\alpha$  could be a trimeric aggregate of two heavy and one light chain ( $2 \times 81.5 + 16.3 = 179.3$  kDa) and band  $\beta$  could be a dimeric aggregate of one heavy and one light chain ( $81.5 + 16.3 = 97.8$  kDa). Heavy chains seem to be loosely bound in these aggregates and were separated upon heating ( $70^\circ\text{C}$  for 10 min) and shown to be a distinct band of protein on the gel (Fig. 5, lane I.C). It is therefore likely that the heavy chain is a natural constituent of the native laccase and more studies are necessary to elucidate its role. Studies of the quaternary structure of a mushroom tyrosinase revealed that the enzyme consisted of two polypeptide chains, referred as heavy with  $M_r 43,000 \pm 1,000$  and light with  $M_r 13,400 \pm 600$  (Strothkamp et al., 1976). It is of note that the  $M_r$  of the light chain of *A. lipoferum* laccase matches with this latter value. Therefore, we suggest that native *A. lipoferum* is a multimeric enzyme consisting of one catalytic chain (16.3 kDa) and one or two regular/structural heavy chains (81.5 kDa). This suggestion is also supported by the observation that band  $\beta$  was the first to appear on the gel, band  $\alpha$  the second, while in heated preparations band  $\gamma$  appeared first.

*A. lipoferum* laccase eluted from the last hydroxyapatite column in an inactive form. This ability of the *A. lipoferum* laccase to exist in an inactive or latent form seems to be a common feature of enzymes of the PPO group (Moore and Flurkey, 1990; Solano et al., 1997). A variety of treatments or agents have been used for the activation of the latent forms of PPOs, including urea (Swain et al., 1966), SDS (Moore and Flurkey, 1990; Wittenberg and Triplett, 1985), protease (Aso et al., 1985), fatty acids (Golbeck and Cammarata, 1981) and fixation on nitrocellulose membrane (Bray et al., 1991).

The use of SDS as an activating agent is particularly interesting because this anionic detergent is better

known for its deleterious effects on the activity of most enzymes, and only very few cases of enzyme activation have been reported. The activation of *Xenopus* tyrosinase (Wittenberg and Triplett, 1985) and the broad beam polyphenoloxidase (Moore and Flurkey, 1990) by high SDS concentrations, has been reported. Recently it was found that SDS concentrations below 0.05% (w/v) stimulated the L-DOPA oxidase activity, but tended to inhibit the 2,6-dimethoxyphenol and syringaldazine oxidase activity of extracts of the melanogenic marine bacterium strain MMB-1 (Solano et al., 1997). In our case, the *A. lipoferum* laccase was active in the crude extracts as well as after solubilization by Triton X-100, acetone precipitation, in solutions of high ionic strength and urea concentrations, and after protamine precipitation. It is only after the final conventional hydroxyapatite chromatography that the enzyme was transformed into a latent, SDS-activable form. This result suggests that the native enzyme may be associated with a factor (or factors) essential for laccase activity, which was lost during chromatography. According to our results, SDS could at least partially substitute for this factor. Thus, it is possible that incomplete activation by SDS of the latent form of laccase during the last steps of purification may account for the low specific activity together with the small purification factor (Table 1).

Attempts to reconstitute the active form of the enzyme by incubating the latent form with hydroxyapatite elution fractions were unsuccessful. Native PAGE of SDS-activated laccase gave a broad unresolved band in the upper part of the gel after staining with substrate in the presence of SDS (Fig. 5, II). This inability of the enzyme to enter the gel under completely native conditions — together with the fact that enzyme resolution was achieved only in extracts treated with SDS prior to electrophoresis — supports the idea that SDS mainly altered the physical conformation of the enzyme and consequently its catalytic properties. According to Moore and Flurkey (1990) the binding of small amounts (generally below the critical micelle concentration, i.e. 3.5 mM) of SDS on enzymes alters both their enzymatic and physical characteristics, suggesting therefore that limited conformational changes may induce or initiate the activation of latent enzymatic forms. As suggested by Solano et al. (1997), we postulate that similar conformational modifications of latent forms may exist in vivo, which could be responsible for laccase activation in *A. lipoferum*.

Generally, enzymes of the PPO group are relatively thermostable (Coll et al., 1993; Fukushima and Kirk, 1995; Munoz et al., 1997). *A. lipoferum* laccase was also found to be thermostable and its half-life value (43 min) was in the range reported for fungal laccases. Furthermore, we observed that in some cases, the ac-

tivity of SDS-activated laccase preparations could be stimulated by heat, the maximum activation being achieved after a 10 min preincubation period at 70°C (result not shown). This observation reinforces the hypothesis that conformational changes may be responsible for *A. lipoferum* laccase activation.

*Azospirillum* spp. are motile nitrogen-fixing bacteria with the potential to increase the yield of economically important cereals and grasses (Jacoud et al., 1998). Motility and chemotaxis are thought to be important factors for efficient plant colonization (Mandimba et al., 1986). *Azospirillum* move using a mixed flagellation: a single polar flagellum when grown in liquid medium and additional lateral flagella when grown on solid media. Swarming has been suggested to play an important role in tissue colonization. Swimming motility in *Azospirillum* has been suggested to play a role in movement of bacteria toward the plant roots. Swarming across the surface of roots may be important for the long-term colonization.

A motile strain and a non-motile strain of *A. lipoferum* have been isolated at the same frequency in the rhizosphere of rice (Bally et al., 1983). Both strains were able to colonize efficiently rice roots ( $10^8$  cfu g<sup>-1</sup> fresh roots) (Alexandre et al., 1996). Unlike the motile strain of *A. lipoferum*, the non-motile strain exhibits an intracellular laccase activity (Givaudan et al., 1993). The exact role of this intracellular laccase in *A. lipoferum* is unknown. But the ability to oxidize naturally occurring phenolic compounds resulting from lignin metabolism (Faure et al., 1996) could be related to the competitiveness of *Azospirillum* in the rhizosphere. Otherwise, *A. lipoferum* motile strain is able to generate non-motile forms in vitro showing the same phenotypic characteristics as the previously non-motile *A. lipoferum* strain directly isolated from the rice rhizosphere. Inoculation of rice roots with an *A. lipoferum* motile strain showed that rice rhizosphere enhanced the frequency of appearance of stable non-motile forms (Alexandre et al., 1996). Emergence of a non-swimming but swarming strain may be important for colonization of the plant by the bacteria, especially when the oxygen concentration changes and the laccase activity could be important to the survival and activity of the bacterium on the plant roots. The role of the laccase activity in the plant–bacteria interaction is currently under investigation.

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