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Iron-regulated proteins in *Phanerochaete chrysosporium* and *Lentinula edodes:* Differential analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis and two-dimensional polyacrylamide gel electrophoresis profiles

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and two-dimensional gel electrophoresis (2-DE) were used to identify iron-responsive proteins in the white-rot species (*Phanerochaete chrysosporium* and *Lentinula edodes*), by comparing the differential patterns of cellular and membrane proteins obtained from iron-sufficient and iron-deficient mycelia. Six cellular proteins induced by iron restriction have been observed in SDS-PAGE for *P. chrysosporium* and twelve for *L. edodes.* In 2-DE, the numbers of iron-restricted induced proteins were 12 and 9, respectively, in a resolution range of 15–60 kDa and p*I* 4.5–8.1. SDS-PAGE for the plasma membrane protein did not show differences, whereas the outer-membrane protein profile showed 6 and 5 proteins induced by iron depletion in *P. chrysosporium* and *L. edodes,* respectively. The results presented here are important data to unravel mechanisms of biosynthesis and/or transport of the iron-complexing agents in ligninolytic fungi and to further correlate them to the ligninolytic processes.

Keywords: Iron-responsive proteins / White-rot fungi / White-rot iron responsive fungal proteins
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1 Introduction

The technological application of wood biodelignification represents a process of potential importance for the economy especially for cellulose pulp industries. Due to the environmental pollution generated by chemical processes, nowadays, researchers have pursued less polluting biological pretreated pulp and biobleaching processes that still produce good-quality cellulose pulp. Within this context, the understanding of the lignin microbiological degradation promoted by fungi (basidiomycetes), which inhabit the wood, plays an essential role. *Phanerochaete chrysosporium* and *Lentinula edodes* are white-rot basidiomycetes bearing different degrees of ligninolytic activity depending on the substrate [1]. Several extracellular oxidative enzymes (lignin-peroxidases (Lips), Mn-peroxidases (MnPs), laccases, and others) produced by different species of white-rot fungi have been characterized and tested for lignin biodegradation activity [2, 3]. However, the lignin biodegradation places a mechanistic problem to the fungal action because in nature this polymer is not susceptible to normal hydrolytic mechanisms [4]. The main question that has been arisen against the direct action of enzymes is related to the small-sized wood pore that would prevent the diffusion of LiPs [5, 6]. Therefore, the process should be a result of combined action between ligninolytic enzymes and low-molecular-mass compounds (LMMCs) [7, 8]. Several papers describing the participation of LMMC iron-chelating and iron-reducing compounds produced by fungi in the wood degradation have been published [9–14]. Among LMMCs, it has been proposed that the iron-chelating compounds (like siderophores) play an important role in lignin biodegradation [15–18].

High affinity iron-chelating compounds, normally called siderophores, are produced by bacteria and fungi under conditions of iron-deficiency [19]. Depending on the strain, the medium and the cultivation conditions, generally, fungi synthesize a variety of hydroxamate siderophores with different structural backbones such as ferrichromes, coprogens, and fusigens (fusarinins) [20]. Unlike most fungi, the wood degrading species, those for which some study has already been accomplished, seem to produce phenolates-catecholates siderophores [12, 14, 21]. Conversely, as well as *Escherichia coli,* which produces enterobactin, a catecholate, and aerobactin, a hydroxamate, there are evidences that ligninolytic fungi produce iron-chelating compounds with different siderophore structures (Rodríguez, J., personal communication). In different bacteria, several operons have been characterized coding proteins involved

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Abbreviation: OMP, outer membrane protein

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in the biosynthesis and transport of siderophores [22]. The characterization of proteins and their respective genes, which participate on the siderophore production and transport in fungi has become more intensive during the last ten years [23–29]. In *Ustilago maydis,* a basidiomycete, the production of ferrichrome depends on the expression of two genes, *sid1* and *sid2,* of which *sid1* represents the ornithine-N^5-oxygenase and *sid2,* possibly has a function in the biosynthesis of the peptide ring [30]. Regulating the whole biosynthesis appears the protein URBS 1 that is a GATA-type transcription factor containing two putative zinc finger motifs [31]. Ascomycetous fungi such as *Aspergillus nidulans, Penicillium chrysogenum* and *Neurospora crassa* produce the SREA, SREP and SRE proteins, respectively, which are iron-regulated GATA-type transcription factors that regulate siderophore biosynthesis [25–27]. As a first approach to the study of siderophore biosynthesis and transport in white-rot fungi, this report describes the iron-regulated protein profiles in SDS-PAGE and 2-DE produced by two species of wood degrading fungi. Cellular as well as membrane proteins were induced as a response to different iron concentrations.

2 Materials and methods

2.1 Fungal species and growth conditions

Wood degradation fungi *P. chrysosporium,* strain BKM-1767, and *L. edodes,* strain UEC-2019, were maintained in cultures on 2% malt-extract-agar (MEA) plates grown at 28°C and stored at 5°C. Cultures to obtain fungal mycelia were made by inoculating several 250 mL Erlenmeyer flasks containing 75 mL of 2% malt-extract liquid broth (ME). To remove iron traces, all glass flasks were dipped in 10% HCl for 48 h, autoclaved at 1 atm for 15 m with 10 mM EDTA, and rinsed with deionized sterile water. Differential mycelial growth conditions were: (i) ME medium (iron-deficient); (ii) ME medium containing 90 μM $FeCl_3 \cdot 6H_2O$ (iron-sufficient). Cultures were incubated at 28°C under agitation at 120 rpm for 11 days. Mycelia were recovered by vacuum filtration, frozen in liquid nitrogen and stored at –80°C until use. The two different culture conditions did not seem to interfere significantly with fungal growth as verified by the mycelium dry weight curves obtained for each species during the 11-day culture period.

2.2 Cellular protein extraction

Cellular proteins were extracted from each growth condition by grounding mycelium to a fine powder in liquid nitrogen with a pestle and a mortar. The protein extraction procedure was according De Mot and Vanderleyden [32]. Originally, the method was described for *Azospirillum* species; recently, it had been used for *Thiobacillus ferrooxidans* [33] and for plant tissues [34]. This method was adapted here for fungal mycelia. The powdered mycelia from each experimental condition were transferred to 2 mL Eppendorf tubes and weighted. To the powdered mycelia it was added a weight-equivalent volume of the extraction buffer containing 0.7 M sucrose, 0.5 M Tris, 30 mM HCl, 50 mM EDTA, 0.1 M KCl, and 40 mM dithiothreitol. The samples were incubated on ice for 15 min. Before eliminating cellular debris by centrifugation at 14 000 rpm for 10 min at room temperature in a microfuge, the suspension was homogenized in a glass tissue grinding tube. After centrifugation, supernatants were transferred to a new 2 mL Eppendorf tube and homogenized with an equal volume of phenol, pH 8.0 (Sigma Chemical Co., St. Louis, MO, USA) which was agitated in a Vortex-Gene 2 for 10 min at room temperature. After centrifugation at 14 000 rpm for 10 min, the extraction procedure was repeated three times by adding the extraction buffer (equal v/v) to the phenolic phase. The precipitation of the cellular proteins had been made by the addition to the phenolic phase of five volumes of 0.1 M ammonium acetate dissolved in methanol, the mixture was then incubated at –20°C for 2 h. After centrifugation at 14 000 rpm for 10 min the pellet was washed twice with 0.1 M ammonium acetate and twice with ice-cold 80% acetone. For SDS-PAGE the pellet was air-dried and dissolved in 30–50 μL lysis buffer (9.8 M urea, 2% v/v Nonidet P–40, 100 mM dithiothreitol). For 2-DE experiments the lysis buffer was 9.8 M urea, 2% v/v Nonidet P-40, 100 mM dithiothreitol and 2% v/v carrier ampholytes (Amersham-Pharmacia Biotech, Uppsala, Sweden), pH 5–7 and pH 3–10 (5:1). In all cases protein concentration was determined by the Bradford method. Samples were stored at –70°C until loaded in a gel.

2.3 SDS-PAGE and 2-DE

Approximately 40 μg of cellular proteins were loaded in 15% SDS-PAGE according to Laemmli [35]. The 10 kDa Protein Ladder (Gibco-BRL, Bethesda, USA) was used as molecular weight standard to estimate differentially expressed mycelial protein molecular weights. The protein profiles were visualized by silver staining as described by Blum *et al.* [36]. The 2-DE experiments were conducted according to De Mot and Vanderleyden [32]. Approximately 360 μg of cellular proteins were loaded in the isoelectric focusing gel. The upper reservoir was loaded with 20 mM NaOH and the lower reservoir with 10 mM H_3PO_4. Prerunning conditions were 15 min at 200 V, 30 min at 300 V and 60 min at 400 V. The first-dimensional gels were run at 400 V for 18 h. Second-dimensional analyses

were performed according to Laemmli [35] on 12% polyacrylamide gels. The 10 kDa Protein Ladder was loaded together with the 1-D separated protein strips in the second-dimensional gels to estimate the relative molecular weights of differentially expressed mycelial proteins. After electrophoresis, the second-dimensional gels were silver-stained [36]. The p*I* of the protein spots were estimated based on the pH gradient formed in one unloaded first-dimensional gel, which ran simultaneously, and calculated by measuring the pH of every 1 cm sliced gel strips. Induced proteins in 2-DE gels were comparatively quantified by digitalizing the gel photographs using an image scanner (HP ScanJet 3200C) and differential spots were quantified by densitometry using the ImageMaster 2D Elite 3.0 software (Pharmacia). To eliminate differences generated by both the amount of protein loaded in each gel and the silver staining method, the spots whose intensity was increased in iron-deficient or iron-sufficient mycelia were identified by comparing four different protein extractions and four different gel runs.

2.4 Plasma membrane protein extraction

The protocol described by Bowman *et al.* [37] for extraction of *Neurospora crassa* plasma membrane proteins was used to isolate the mycelial membrane proteins of the two white-rot species. After grounded to a fine powder in liquid nitrogen, mycelia were suspended in solution A (0.59 M sucrose, 5 mM EDTA, 50 mM NaH_2PO_4, 70 μL β-mercaptoethanol, 133 mg/mL *Helix pomatia* β-glucoronidase type H-1 enzyme (Sigma Chemical) and incubated for 1 h at 30°C. The suspension was homogenized in a glass tissue grind tube before centrifugation at 4000 × *g* for 10 min at 4°C. The pellet was washed with 0.68 M sucrose and recentrifuged. The pellet was suspended in solution B (0.33 M sucrose, 1 mM EGTA, 0.3% bovine serum albumin, pH adjusted to 7.1 with KOH), homogenized as described above, and centrifuged at 1000 × *g* for 10 min. The resulting pellet was suspended in solution B and submitted to a third round of homogenization and centrifugation. The supernatant was centrifuged in a Beckman JA 20 rotor for 30 min at 11 700 × *g* and the supernatant was recentrifuged for 40 min at 39 400 × *g*. Finally, the pellet was and suspended in 6 mM Tris-HCl, pH 6.8, 2% SDS, and 5% β-mercaptoethanol. The membrane protein profiles were observed in silver-stained 10% SDS-PAGE as mentioned above [36].

2.5 Outer membrane protein extraction

The procedure described by Mezence and Borion [38] was used for outer membrane protein (OMP) extraction with some modifications. Mycelia obtained from iron-sufficient and iron-deficient cultures were grounded in liquid nitrogen, suspended in PBS containing 1 mM PMSF and centrifuged at 9000 × *g* for 10 min at 4°C; this procedure was repeated twice. The pellet was resuspended in PBS containing 1 mM PMSF and homogenized in a tissue glass grinder for 10 min at 4°C. Undisrupted cells and large fragments were removed by centrifugation at 9000 × *g* for 10 min at 4°C. The centrifugation procedure was repeated five times, finally the supernatant was centrifuged at 53 000 × *g* for 60 min at 4°C in a Beckman L8 80M ultracentrifuge using the SW50 rotor. The pellet (OMPs) was suspended in Laemmli solubilization buffer [35] and heated at 100°C for 5 min. Prior to load the SDS-PAGE, the samples were centrifuged at 10 000 × *g* for 5 min. The proteins were visualized by silver staining [36].

3 Results and discussion

3.1 SDS-PAGE cellular protein differential profiles

A large number of proteins with apparent molecular masses (M_r) ranging from 10–100 kDa can be observed in all SDS-PAGE of *P. chrysosporium* and *L. edodes* cellular protein extracts. When grown under conditions of iron limitation, both species synthesized several cellular proteins, which are repressed in mycelia grown in iron-sufficient culture medium. Six differential proteins were distinguished for *P chrysosporium* and eleven for *L. edodes* in preparations from iron-deficient mycelia (Fig. 1). On repeated protein preparations and different SDS-PAGE runs, the apparent M_r for the proteins that had an increased expression in *P. chrysosporium* iron-deficient mycelia corresponded to: M_r = 32, 37, 56, 60, 68, 90 (Fig. 1A). For *L. edodes* iron-deficient mycelia, the apparent M_r for the proteins that have an increased expression were: M_r = 10, 17, 21, 23, 29, 32, 57, 63, 68, 90, 100 (Fig. 1B). Conversely, two proteins with increased expression can be observed in iron-sufficient profiles for *P. chrysosporiun* (M_r = 30 and 49) and three for *L. edodes* (M_r = 8, 24, 70). Those polypeptides are probably produced in the presence of iron due to a mechanism of iron-positive regulation. The FUR protein which is a global regulator that responds to iron concentrations was characterized initially as transcriptional repressor of the siderophore biosynthesis. Proteins homologous to FUR have been found in several fungi as well. The M_r of those proteins deduced from the putative amino acid sequence vary from 55 to 104 kDa [30, 25, 27]. Therefore, we could suppose that among proteins with increased expression in the presence of iron it can be included a FUR-like protein.

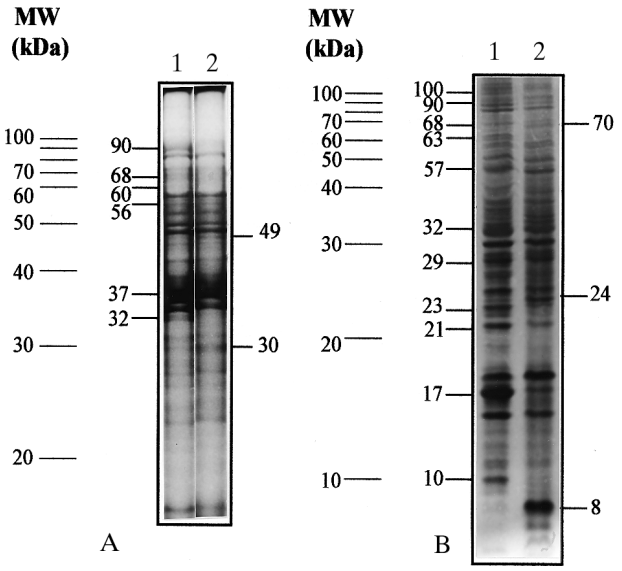

Figure 1. SDS-PAGE profiles of iron-responsive cellular proteins in the white-rot wood degrading fungi *P. chrysosporium* and *L. edodes.* (A) Proteins for *P. chrysosporium* and (B) *L. edodes.* Lane 1, iron-deficient mycelium extracts; 2, iron-sufficient mycelium extracts. Induced proteins in each differential growth condition are denoted with a trace and a number corresponding to the apparent molecular mass in kDa. Molecular weight standard (10 kDa Ladder; Gibco-BRL) used to estimate the apparent M_r of induced proteins is shown for each gel. Differential proteins denoted have been observed by comparing four different protein profiles.

3.2 2-DE cellular protein differential profiles

A careful analysis of the proteins separated in 2-DE led to the identification of several spots whose silver-stained intensity varied when iron-deficient and iron-sufficient mycelium extract were compared. To avoid misinterpretations on differences in protein synthesis due to variations in the mycelia growth rate or in the protein recovery, four different protein extractions and four replicate 2-DE gels were visually compared. For *P. chrysosporium,* in the M_r range of 15–60 kDa and p*I* 4.2–6.5, twelve spots could be identified only in extracts from iron-deficient mycelia (Table 1; Fig. 2A). Among those spots, one denoted as protein 19 (Table 1; Fig. 2A) seems to be an aggregate of four 36 kDa proteins whose p*I* varied from 5.35–5.47. At least four polypeptides were present in extracts from both culture conditions but presented more intensely stained spots in iron-deficient mycelia (Table 1; Fig. 2A). The spot 1 of 16 kDa (p*I* 5.65) has a markedly enhanced intensity clearly indicating a derepressed expression under iron deficiency. The spot named 17 showed also high intensity verified only in iron-deficient conditions (Fig. 2A). On the other hand, eleven polypeptides have also been observed only in extracts from iron-sufficient mycelia indicating that some proteins are iron inducible, and four, although visualized in both culture conditions, presented higher intensity in mycelia grown in the presence of Fe^{3+} (Fig. 2B; Table 1). In both gel conditions some other spots that show differences in intensity can

Table 1. Estimated molecular masses, p*I* and densitometric determination of differential spots in 2-DE gels for *P. chrysosporium*

Protein ID	M_r (kDa)	p*I*	IDef[a)]	ISuf[b)]	Protein ID	M_r (kDa)	p*I*	IDef[a)]	ISuf[b)]
2	17	5.41	7.484	0	5	18	5.65	0	3.150
3	17	5.60	6.162	0	7	20	5.81	0	7.484
6	19	5.89	5.692	0	10	25	5.91	0	3.396
11	26	5.85	2.817	0	13	33	5.70	0	32.500
17	35	6.00	28.384	0	14	33	5.80	0	7.621
19[c)]	36	5.35–5.47	38.812	0	15	35	5.63	0	3.020
18	36	5.88	1.541	0	16	38	6.14	0	9.680
20	37	5.78	3.483	0	22	42	5.45	0	16.025
21	38	5.84	3.667	0	25	44	5.49	0	23.898
24	40	6.00	8.805	0	23	44	5.42	0	8.940
26	43	5.44	10.705	0	27	46	5.58	0	19.584
30	48	5.44	6.578	0	4	18	5.31	5.483	12.553
1	16	5.65	116.725	15.325	28	46	6.15	2.041	24.015
9	21	6.00	10.275	4.223	29	50	5.81	2.570	9.248
8	22	5.87	9.273	2.683	31	58	6.00	17.772	53.328
12	28	5.88	8.400	3.036					

a) IDef, densitometric values in spot volume in iron-deficiency
b) ISuf, densitometric values in spot volume in iron-sufficiency
c) protein aggregate

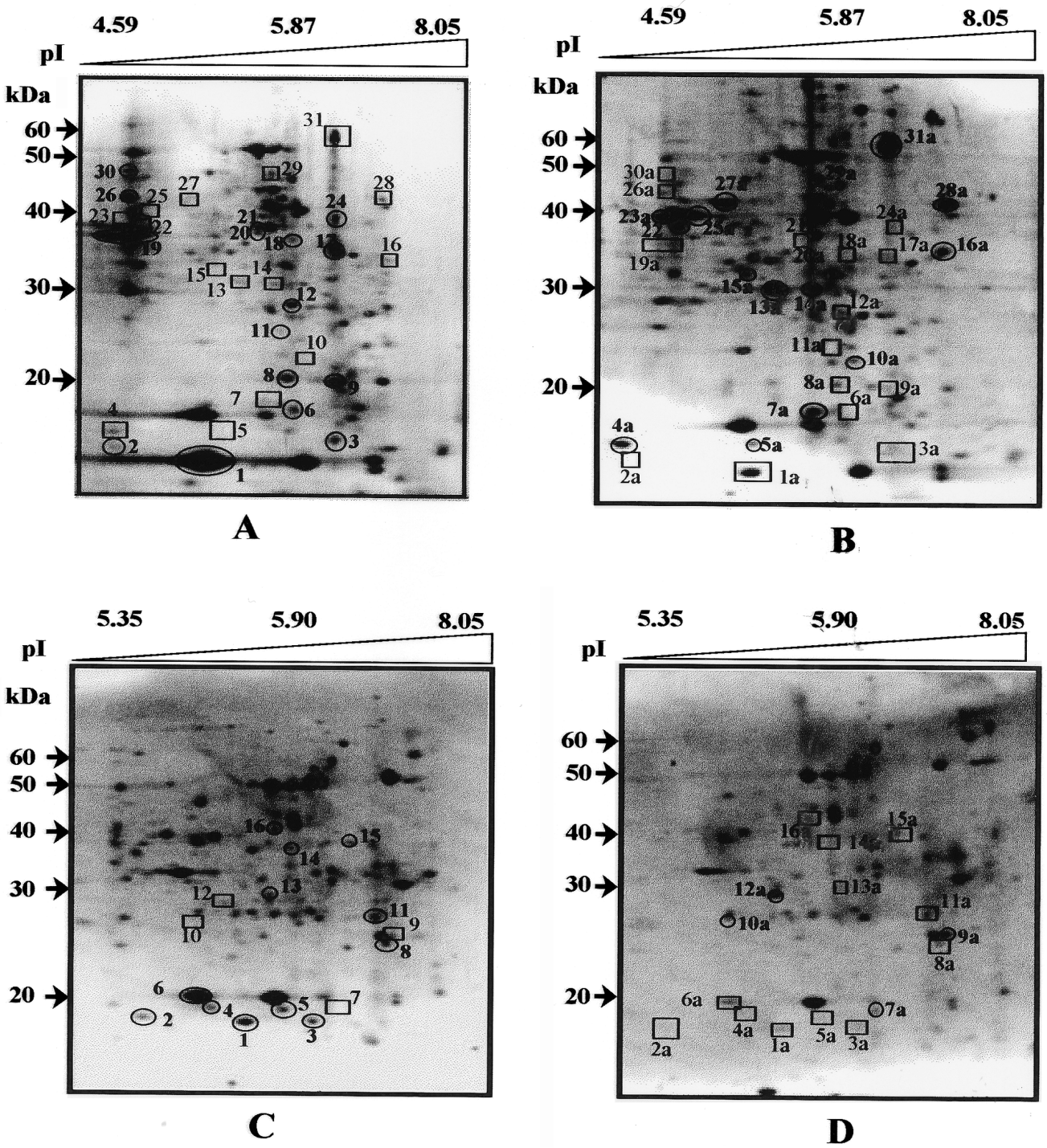

Figure 2. 2-DE analysis of iron-responsive cellular proteins in the white-rot wood degrading fungi *P. chrysosporium* and *L. edodes.* Iron-deficient mycelium extracts for (A) *P. chrysosporium* and (C) *L. edodes;* iron-sufficient mycelium extracts for (B) *P. chrysosporium* and (D) *L. edodes.* In *P. chrysosporium* gels, all proteins that had their synthesis affected by differential growth conditions were numbered from 1 to 31 in the iron-deficient gel and from 1a to 31a in the iron-sufficient gel. In *L. edodes* gels, all proteins that had their synthesis affected by differential growth conditions were numbered from 1 to 16 in the iron-deficient gel and from 1a to 16a in the iron-sufficient gel. Spots denoted by a circle and numbers in bold indicate proteins that were induced or had increased expression. Squares represent either the absence or the diminished expression. The pH of the isoelectric focusing gel ranging from 4.6 to 8.0 is shown on the top of each gel. Molecular weight standard (10 kDa Ladder; Gibco-BRL) used to estimate the M_r of induced proteins is shown on the left of the gels. Differences have been denoted by comparing four different gels.

Table 2. Estimated molecular masses, p*I* and densitometric determination of differential spots in 2-DE gels for *L. edodes*

Protein ID	M_r (kDa)	p*I*	IDef[a)]	ISuf[b]
2	18	5.30	3.270	0
3	18	6.47	4.923	0
4	19	5.80	3.096	0
5	19	6.27	5.727	0
8	25	6.96	2.797	0
13	31	6.18	2.287	0
14	38	6.31	1.995	0
15	39	6.72	2.700	0
16	42	6.21	6.425	0
1	18	6.03	11.526	0.540
6	20	5.70	41.226	7.580
11	28	6.88	6.828	4.693
7	19	6.58	0	3.578
10	27	5.70	0	2.506
9	26	7.00	1.434	7.795
12	30	5.88	1.938	12.218

a) IDef, densitometric values in spot volume in iron-deficiency
b) ISuf, densitometric values in spot volume in iron-sufficiency

be seen (Fig. 2A, B and C, D), but they have not been marked as differentially expressed proteins because after careful inspection over quadruplicated gels they did not show significant differences. Table 2 describes the M_r and the estimated p*I* of differential polypeptides for *L. edodes.* Nine spots are only observed on the iron-deficient mycelia extract gel (Fig. 2C; Table 2) whereas three had clearly an increased expression (Fig. 2C; Table 2). Additionally, two polypeptide spots appear only on the iron-sufficient mycelia extract gel (Fig. 2D; Table 2) and other two had an increased expression (Fig. 2D; Table 2).

3.3 Plasma and OMP profiles

Plasma membrane proteins of *P. chrysosporium* and *L. edodes* mycelia that had been grown in iron-sufficient or iron-deficient media were compared. There were no significant differences in SDS-PAGE profile (data not shown). A similar result was reported before for *Neurospora crassa* by Van der Helm and Winkelmann [39]. Conversely, the *P. chrysosporium* and *L. edodes* outer membrane protein (OMP) profiles for mycelia grown in iron-deficient medium presented significant differences when compared to iron-sufficient OMP mycelium extract (Fig. 3). Six proteins (M_r = 22, 30, 38, 55, 58, 65) showed higher silver staining intensity in the iron-deficient *P. chrysosporium* profile, whereas one polypeptide (M_r 50) was expressed only in iron-sufficient mycelia. For *L. edodes,* the numbers of iron-limited and iron-sufficient proteins were 5 (M_r = 35, 38, 42, 48, 55) and 2 (M_r = 34, 39), respectively. Those proteins induced under iron-deficient conditions and repressed under iron-sufficient condition may represent surface receptors, which participate in iron-siderophore complexes translocation through the outer membrane.

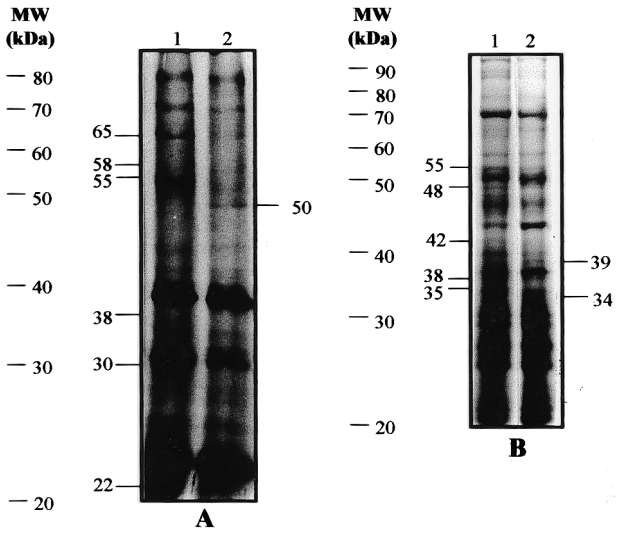

Figure 3. The effect of iron-deficient (lane 1) and iron-sufficient (lane 2) growth conditions on OMP synthesis. (A) *P. chrysosporium,* (B) *L. edodes.* Traces indicate the induced proteins and the numbers correspond to the estimated M_r. Molecular weight standard (10 kDa Ladder; Gibco-BRL) is shown on the left of each gel.

4 Concluding remarks

To our knowledge there is no published information about iron uptake (free or siderophore-chelated) mechanisms in wood degrading fungi. In general, many fungi secrete hydroxamate-like siderophores under iron-deficiency. Normally, fungal siderophore biosynthesis involves an ornithine N^5-hydroxilation and acylation followed by the action of a nonribosomal peptide synthetase [30]. Most detailed studies related to iron transport are on *Saccharomyces cerevisiae* which does not synthesize siderophores, but exhibits two mechanisms of iron capture that respond to the iron concentration [20, 40]. A mechanism of low affinity is activated when a high concentration of the metal exists. This mechanism involves the action of an iron-reductase that reduces Fe^{3+} to Fe^{2+} in the external membrane and, the reduced iron is internalized, in this case a low-affinity transporter (FET4, $\sim$ 60 kDa) is induced. The mechanism of high affinity expresses when the concentration of iron is low, and it is mediated by two

iron reductases (FREI and 2, with molecular masses varying from a 71 to 80 kDa). The Fe^{2+} are oxidized by a multicooper oxidase (FET3, with a molecular mass of approximately 70 kDa), whose expression is regulated by AFTI (75.8 kDa), a transcription factor regulated by iron. The transport is effectuated by a permease FTRI (44 kDa). However, the yeast is capable to assimilate iron-siderophore complexes, which are produced by other microorganisms. It means that iron-siderophore complex receptors exist in different organisms and can be induced by iron-chelating compounds produced by other species. Recently, as a result of the *S. cerevisiae* genome sequencing several genes were identified coding for proteins belonging to the main superfamily of membrane facilitators that are capable to transport iron-siderophore complexes of either hydroxamate or catecholate structures. The estimated sizes of these facilitator proteins vary between 60 and 70 kDa [28, 29, 41, 42]. The iron-deficient or iron-sufficient regulated proteins identified in SDS-PAGE and 2-DE profiles of *P. chrysosporium* and *L. edodes* are important results to further understanding the complex mechanisms of iron uptake in wood degrading species.

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