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Tuber borchii fruit body: 2-dimensional profile and protein identification

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Abstract

The formation of the fruit body represents the final phase of the ectomycorrhizal fungus *T. borchii* life cycle. Very little is known concerning the molecular and biochemical processes involved in the fructification phase. 2-DE maps of unripe and ripe ascocarps revealed different protein expression levels and the comparison of the electropherograms led to the identification of specific proteins for each developmental phase. Associating micropreparative 2-DE to microchemical approaches, such as N-terminal sequencing and 2-D gel-electrophoresis mass-spectrometry, proteins playing pivotal roles in truffle physiology were identified.
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Keywords: *Tuber borchii*; Ectomycorrhizal fungi; Fruit body maturation; Edman degradation; Mass spectrometry

1. Introduction

Truffles are hypogeous ascomycetous fungi belonging to the genus *Tuber* which give origin to highly specialised symbiotic associations with the fine roots of gymnosperms and angiosperms, named ectomycorrhizae (Trappe, 1979). They are in great demand due to the organoleptic properties of their fruit bodies (white truffles), having a distinct flavour and aroma, while also of considerable interest in the fields of forestry and agronomy. The ontogenetic cycle of these fungi consists in a limited initial phase of mycelial growth during which hyphae proliferate; successively, they come into contact with the roots of host plants leading to the development of the ectomycorrhiza. In the final developmental phase the fungus organises a fruit body the role of which is to produce sexual spores that are dispersed in the soil when the fruit body ripens. From these spores, vegetative mycelia develop giving rise to a new extraradical phase thus closing the truffle life cycle.

Tuber hypogeous fruit bodies have a globular structure and their development begins with the aggregation of different types of hyphae which give origin to an external tissue, the peridium, and an internal tissue, named gleba. Within this last tissue the ascogonium forms and from this the reproductive hyphal element, the ascus containing the ascospores, originates (Pegler, 1993; Read and Beckett, 1996). Although the succession of events leading to fructification has been investigated, little is known about the molecular and biochemical processes on which hyphal differentiation, fruit body development and the subsequent maturation, are based (Balestrini et al., 1996). Studies on truffle biology are difficult because, unlike other filamentous fungi such as *Pisolithus tinctorius* (Malajezuk et al., 1990) or *Hebeloma cylindrosporum* (Debaud and Gay, 1987), these mycelia grow very slowly in vitro and it is difficult to obtain mycorrhizae or fruit bodies under axenic and controlled conditions. Using an untargeted technique such as the mRNA differential display we recently identified several cDNAs differentially expressed during

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the fructification phase of *T. borchii* (Zeppa et al., 2002). By this approach a global vision of the gene expression profile related to the maturation of fruit bodies, was achieved. The identification of several putative genes involved in the development of *T. borchii* ascocarps represents a starting point in understanding the molecular mechanisms of cellular differentiation leading to the fructification process. In the present study we performed the analysis of the ascocarp's proteome using two-dimensional electrophoresis (2-DE) in order to obtain more information about events occurring during this phase of the truffle life cycle. This electrophoretic technique possesses high potentiality since it permits the separation of most of the proteins of an organism or tissue on a single gel and reveals possible modifications in protein expression related to disease, stress conditions or ambiental factors. 2-DE maps of both *T. borchii* ripe and unripe fruit bodies were produced and analysed in this study in order to verify the presence of proteins specifically expressed in the two developmental stages. Successively, in order to unequivocally identify some of the ascocarp's proteins micropreparative 2-DE was associated with microchemical approaches, such as N-terminal sequencing and mass spectrometry, and the data obtained were subsequently utilised to perform cross-species matching (Wilkins and Williams, 1997).

2. Results and discussion

Three analytical two dimensional electrophoresis runs on both unripe (stage 0) and ripe (stage 5) *T. borchii* fruit bodies were performed. As shown in Fig. 1a and b the electropherograms evidenced an appreciably different protein expression level related to the maturation stage. In particular, the careful comparison of the 2-DE maps and the evaluation of different parameters carried out using the Melanie 3 software, revealed that, in the ripe fruit body, a decrease in the expression level of many 30–150 kDa proteins occurs. Concurrently other proteins resulted to be more expressed in the mature tissues (Table 1). Moreover, the overlapping and the further matching of the electropherograms revealed specific spots for each developmental stage (circled white or black in the two electropherograms). All these results might be explained by an involvement of these proteins in different phases of the fructification process and with a higher metabolic activity in the unripe ascocarps. It stands to reason that in the initial phase of fructification a higher energy supply is necessary to meet the cell demand since biosynthetic pathways are strongly induced. In fact, for the differentiation of the specialised hyphae in asci and the subsequent formation of the ascospores, new membranes and cell walls are required (Kues, 2000). Moreover, meiotic and mitotic processes are particularly active during spore maturation (Balestrini et al., 2000). Therefore, a large quantity of carbohydrates, polypeptides, lipids and nucleotides are necessary and most metabolic pathways must be stimulated for their production.

These preliminary electrophoretic data are in agreement with the molecular evidence obtained in a previous study according to which ascocarp maturation is strictly associated with differential gene expression, and most of the differentially expressed identified genes show a much higher expression level in the unripe fruit body compared to the ripe one (Zeppa et al., 2002).

In order to gain more information regarding the events leading to ascocarp development, the identification of proteins specific to one or both differentiation phase has been carried out. About 20 spots excised from 2-DE gels were used for protein identification through cross-species matching (Wilkins and Williams, 1997) utilising both amino terminal sequence and mass spectrometry data.

The N-terminal sequencing was performed on Coomassie-blue stained proteins excised from 2-DE gels and then electroeluted as described in the "Experimental"

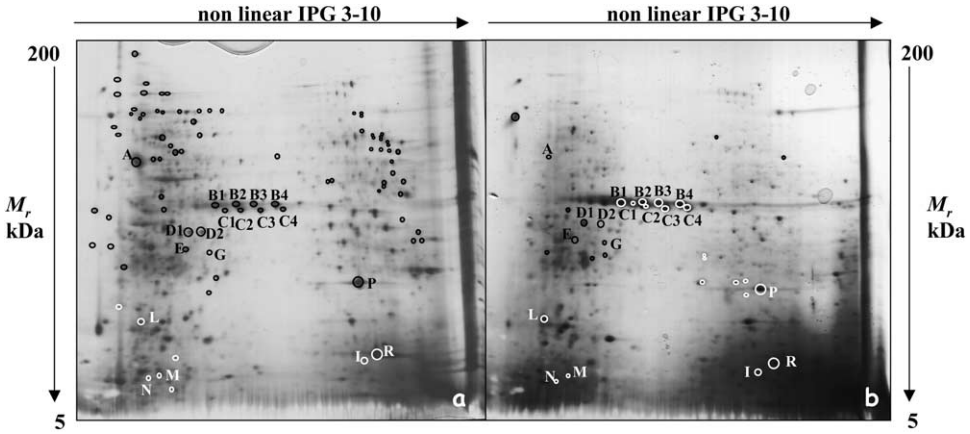

Fig. 1. 2-DE electropherograms of *T. borchii* unripe (a) and ripe (b) fruit bodies. Specific proteins of the two fructification phases are circled; the spots subjected to Edman degradation or mass spectrometry analysis are indicated also by an alphabetical letter.

Table 1
Characteristics of *T. borchii* constitutive protein spots

Spot	Status	Mol. mass (Da)[a]	p*I*[a]	Sample	Normalized volume[b]	Normalized O.D.[b]
A	downregulated	47,000±1320	4.94±0.04	unripe fruit body	1.58±0.12	1.09±0.15
				ripe fruit body	0.06±0.01	0.79±0.05
B1	upregulated		5.50±0.01	unripe fruit body	0.35±0.08	1.05±0.2
				ripe fruit body	1.14±0.21	1.77±0.5
B2	upregulated		5.65±0.03	unripe fruit body	0.57±0.08	1.07±0.25
		37,500±1560		ripe fruit body	1.4±0.32	1.75±0.41
B3	upregulated		5.80±0.02	unripe fruit body	0.37±0.07	1.04±0.24
				ripe fruit body	0.66±0.09	1.67±0.33
B4	upregulated		5.95±0.04	unripe fruit body	0.37±0.07	1.08±0.18
				ripe fruit body	0.84±0.06	1.77±0.23
C1	upregulated		5.55±0.01	unripe fruit body	0.15±0.01	0.85±0.03
				ripe fruit body	0.32±0.05	1.48±0.36
C2	no change		5.70±0.03	unripe fruit body	0.16±0.02	0.96±0.11
		35,000±2450		ripe fruit body	0.12±0.01	1.6±0.24
C3	upregulated		5.85±0.06	unripe fruit body	0.3±0.07	1.06±0.11
				ripe fruit body	0.78±0.09	1.72±0.3
C4	upregulated		5.98±0.01	unripe fruit body	0.18±0.03	0.98±0.1
				ripe fruit body	0.71±0.08	1.72±0.22
D1	no change		5.30±0.04	unripe fruit body	0.16±0.02	1.01±0.18
		30,000±2980		ripe fruit body	0.14±0.05	0.82±0.13
D2	no change		5.45±0.02	unripe fruit body	0.21±0.04	0.99±0.24
				ripe fruit body	0.2±0.03	0.93±0.11
E	downregulated	26,600±2100	5.35±0.02	unripe fruit body	0.24±0.08	0.98±0.13
				ripe fruit body	0.06±0.01	1.17±0.31
G	upregulated	25,500±1540	5.45±0.03	unripe fruit body	0.04±0.009	0.55±0.02
				ripe fruit body	0.11±0.02	0.93±0.1
I	upregulated	12,500±1100	6.50±0.01	unripe fruit body	0.07±0.002	0.92±0.15
				ripe fruit body	0.14±0.02	1.59±0.13
L	no change	15,500±1340	5.00±0.05	unripe fruit body	0.31±0.08	1.01±0.17
				ripe fruit body	0.4±0.07	1.57±0.2
M	no change	11,000±1120	5.10±0.04	unripe fruit body	0.19±0.06	1.08±0.13
				ripe fruit body	0.23±0.05	1.5±0.1
N	no change	10,800±1090	5.05±0.01	unripe fruit body	0.13±0.05	0.94±0.11
				ripe fruit body	0.12±0.03	1.51±0.23
P	downregulated	20,660±1560	6.50±0.03	unripe fruit body	0.54±0.09	1.12±0.24
				ripe fruit body	0.26±0.03	1.8±0.31
R	no change	10,270±1250	6.52±0.09	unripe fruit body	0.65±0.1	1.12±0.17
				ripe fruit body	0.78±0.05	1.8±0.27

[a] Molecular masses and isoelectric points were calculated with Melanie 3 (GeneBio) using human plasma proteins as internal standards; the reported values are the mean of three independent experiments±S.E.
[b] Spot normalised volumes and optical density (O.D.) were calculated with Melanie 3 (GeneBio).

section. The possible interference due to the presence of glycine in the electroelution buffer was avoided by extensive dialysis against HPLC-grade water.

Unfortunately, none of the phase-specific spots analysed provided information when subjected to Edman degradation, thus suggesting that their N-terminals were blocked. In the case of the immature ascocarp specific spots, this could be due to the scarce quantity of material available. In fact, it is very difficult to find unripe fruit bodies and a large quantity of material is required for the micropreparative electrophoretic runs and the following analyses. Therefore, in order to avoid the possible problems due to sensitivity our attention was focused on proteins expressed in both maturation stages. Only in few cases the N-terminal sequence analysis was successful (Table 2). The N-terminal tags and the apparent p*I* and M_r values obtained from the 2-DE gel's calibration were used for matching against SWISS-PROT (http://www.expasy.org), FASTA (http://www.ebi.ac.uk) and NCBI (http://www.ncbi.nlm.nih.gov, using BLASTP algorithm) database entries to find homology with known proteins. Significant similarity to known proteins of interest possibly playing an important role in the *T. borchii* life cycle was found.

For instance, the N-terminal tag of the spot named R (Table 2) showed significant homology with metallothioneins obtained from different sources including fungi. This family of proteins is responsible for metal ion chelation through the formation of tetrahedrically coordinated metal-thiolate clusters, and is therefore of primary importance in buffering the intracellular concentration of free thiophilic metal ions (Clemens, 2001;

Table 2
Edman degradation sequences analysis of *T. borchii* constitutive proteins

Spot	pI [a]	Mol. mass (Da)[a]	Sequence tag[b]	Database hit[c]	Homologies	*S*/*E*/*I* [d]
B_4	5.95±0.04	37500±1560	SFDHKVAGYDCCLLE	P45699	Endoglucanase (*Fusarium oxysporum*)	41/0.0096/44.4
				P46239		21/0.21/66.7
				P46236		32/0.049/50
				CAD58875	Endoglucanase (*Agaricus bisporus*)	20/0.12/60
				AAM77702	Endoglucanase (*Emericella desertorum*)	24/0.084/33.3
				P22669	Endoglucanase (*Apergillus aculeatus*)	20/0.15/40
L	5.00±0.05	15500±1340	EQDCACXSNPLQG	P57661	Ferredoxin, 2Fe-2S (*Buchnera aphidicola*)	45/4.9/62.5
				P25528	Ferredoxin, 2Fe-2S (*Escherichia coli*)	41/18/50
				Q51383	Ferredoxin, 2Fe-2S (*Pseudomonas aeruginosa*)	41/18/50
P	6.50±0.03	20660±1560	RTVIHMAIACLI	P00852	ATP-synthetase A chain (protein 6) (*Emericella nidulans*)	24/0.088/60
				NP039504	ATP-synthetase A chain (protein 6) (*Schizosaccharomyces pombe*)	19/0.18/50
R	6.52±0.09	10270±1250	DPLPQCFTLESCCSQC	P58280	Metallothionein (Bovin)	53/31/54
				P02802	Metallothionein (Mouse)	48/25/46
				CAD13456	Metallothionein (*Gigaspora margarita*)	33/0.0096/25
				AAF78959	Metallothionein (*Candida albicans*)	29/0.014/30
				CAB85689	Metallothionein (*Agaricus bisporus*)	34/0.005/50
				CAA06385	Metallothionein (*Podospora anserina*)	34/0.0005/42
				NP593743	Metallothionein (*Schizosaccharomyces pombe*)	31/0.0033/30

[a] Molecular masses and isoelectric points were calculated with Melanie 3 (GeneBio) using human plasma proteins as internal standards; the reported values are the mean of three independent experiments±S.E.
[b] N-terminal sequences were obtained utilising the automated Edman sequencer LF3000 Protein Sequencer (Beckman).
[c] Database entries.
[d] *S*, score; *E*, expected value; *I*, percent identity.

Prasad and Stzalka, 2002). Several studies have evidenced high levels of mycorrhization in soils containing different kinds of heavy metal contamination, indicating that in mycorrhizal fungi a heavy metal tolerance has evolved. Because of their function metallothioneins may play a crucial role in the fungal process of metal tolerance. Therefore, the presence of these proteins in the tissues of *T. borchii* ascocarps could suggest an involvement of this fungus in the phytoremediation of the soil i.e. use of the plants to remove non-volatile and immisible soil contents (Weissenborn et al., 1995; Schützendübel and Polle, 2002).

Comparing the two-dimensional electropherograms of both ripe and unripe *T. borchii* fruit bodies no significant differences were detected regarding the expression level of spot R, thus suggesting a constitutive role of metallothioneins during the fructification phase of this fungus (Table 1). At present further analyses are in progress to clone the complete gene coding these proteins. The subsequent evaluation of its expression levels during the different phases of truffle maturation as well as in the mycelial and symbiotic tissues will allow a better understanding of the physiological role of metallothioneins in the ontogenetic cycle of our fungus.

The N-terminal sequence of spot B_4 showed the highest similarity with endoglucanases from fungi belonging to *Fusarium*, *Aspergillus*, *Agaricus* and *Emericella* species, respectively, as well as from other organisms (Table 2). Endoglucanases (E.C. 3.2.1.4, *endo*-1,4-β-D-glucanases) belong to the cellulose family which includes all those enzymes able to degrade crystalline cellulose to glucose and are produced by a broad range of organisms. The first step in the colonisation process of plant roots by mychorrizal fungi involves physical contact of the outer layers of the cell walls and the extracellular matrix of the two partners, and some specialised molecules (proteins, carbohydrates etc.) probably play essential roles. Then, hyphae progress to the surface or penetrate the host tissues. In the case of colonisation of tree roots by ectomycorrhizal fungi, such as *T. borchii*, the plants accept a limited penetration of the mycelium (Podila and Douds, 2000), whereas the colonisation of plant roots by arbuscolar mycorrhizal fungi requires the complete penetration of the host cell by the fungus (Garcia-Garrido et al., 1996). It stands to reason that cell wall-hydrolysing enzymes such as cellulases must be involved in the establishment of these symbioses (Garcia-Romera et al., 1990). The capacity of fungi to produce endoglucanases also allows them to grow and fruit on particular substrates, thus conferring to this class of enzymes a pivotal role even in the process producing energetic substrate (Cai et al., 1999; Buswell et al., 1996). Biochemical and molecular investigations carried out with the edible mushrooms *Lentinula edodes* (Berk) and *Agaricus bisporus* showed that the activities of endoglucanases are strongly regulated during fruit body development (Ohga and Royse, 2001; Manning and Wood, 1983). In particular, enzymatic activity, as

well as mRNA level, peaked at the veil break stage of fruit body development. All these results support the presence of these proteins in both ripe and unripe *T. borchii* fruit bodies and suggest an involvement of endoglucanases even in truffle formation and maturation processes. Moreover, the comparison of the two electropherograms revealed that this protein is markedly more expressed in the advanced phase of the fructification process than in the initial phase as reported above for other fungi (Table 1). Regarding the N-terminal tags of spots L and P homology was found with two interesting proteins that play a fundamental "energetic" role. In particular, the 13 amino acid N-terminal tag from spot L showed homology with ferredoxin (2Fe–2S) (Table 2). These iron-sulfur proteins transfer electrons in a variety of metabolic reactions and play a crucial function in ATP-synthesis being one of the components of electron transport chain complexes. During truffle formation and maturation processes, most of the biosynthetic metabolisms are strongly induced and a large amount of energy is required for spore formation as well as mitosis/meiosis processes (Balestrini et al., 2000), therefore a deep involvement of ferredoxin in these developmental phases it is very likely. No substantial changes in the expression level of this protein were detected suggesting that it is not influenced by the maturation stage of ascocarps (Table 1).

Finally, the N-terminal sequence obtained for spot P matches with various fungal ATP-synthetase A chains (protein 6) such as those from *Schizosaccharomyces pombe* and *Emericella nidulans* (Table 2). This protein is an integral membrane protein and a key component of the proton channel constituting ATP-synthase the activity of which is of primary importance in this phase of the *T. borchii* ontogenetic cycle. The comparison of 2-DE maps revealed a decrease in the expression level of this protein during truffle maturation (Table 1). These data were in agreement with the higher energy supply required in the initial phase of fructification for the formation of membranes, cell walls and other processes (Balestrini et al., 2000) and supported the molecular data concerning *T. borchii* fruit body maturation previously obtained (Zeppa et al., 2002).

The evidence obtained regarding the expression levels of all the analysed spots supports the hypothesis that the ripe fruit bodies are still metabolically active structures even if to a lesser extent when compared to those which are not yet ripe.

In order to identify some of the N-terminal blocked proteins, mass-spectrometry experiments were carried out, also. Spots from micropreparative 2-DE gels of ripe and unripe fruit bodies were excised, digested overnight with trypsin, and analysed by MALDI/TOF. The peptide mass fingerprinting was limited to search for homologous proteins that might show conserved tryptic peptides in databases of fungi or other similar species, but no entries were found. It should be underlined that this analysis represented just an attempt to find some homologous sequence; in fact it is well known that MALDI MS approach can be successfully used with fully-sequenced genomes or when enough ESTs are available. Therefore, the peptides digested from the most abundant spots were fractionated by HPLC. Peptide sequencing by tandem mass spectrometry was undertaken on all abundant peaks from each spot (1–5 sequences were obtained per spot), leading to sequences ranging from 5 to 10 residues. The variability in the number and length of the sequence fragments from each spot was typical of this kind of analysis (Table 3).

The MALDI analyses performed permitted us to observe the same peptide pattern in the mass spectra for spot C1, C2, C3 and C4, thus we combined the peptide mixtures before LC/MS/MS analyses (named spot C in Table 3); a similar procedure was applied for D1 and D2 samples (named spot D). The occurrence of very similar proteolytic patterns for different spots sharing the same molecular mass but differing in the isoelectric point is probably due to post translational modifications such as glycosylation or phosphorylation. Spots C and D gave us the best results, in spite of this it was rarely possible to obtain a complete sequence for the ions isolated in the ion trap and fragmented. Some interesting data were also obtained for the spots A, I, M and N. The peptide sequences obtained from each different spots were submitted to the FASTA database but no certain identification was possible. Due to the limited amount of proteins, the intensity of the peaks in the mass spectra of the spots E, G, H and L was not sufficient to furnish relevant MS/MS results.

Table 3
Tandem mass spectrometry analysis of *T. borchii* constitutive proteins

Spot	Peptide sequence obtained by tandem mass spectrometry[a]	Parent ion
A	**NH_2-FD(L/I)(L/I)(L/I)SR-COOH**	862.7 (MH^+)
	NH_2-**Y(L/I)(L/I)SYPDV(L/I)K**-COOH	1210.8 (MH^+)
C	-(L/I)AGVR-**COOH**	716.9 (MH^+)
	NH_2-(Q/K)P(L/I)(L/I)SDHVPK-**COOH**	1134.4 (MH^+)
	-DS(L/I)(L/I)PK-**COOH**	1167.4 (MH^+)
	-VSV(L/I)PA(Q/K)K-**COOH**	1547.7 (MH^+)
D	**nh_2**-(L/I)EG(L/I)VSGK-**COOH**	802.6 (MH^+)
	-TSAV(L/I)STGR-**COOH**	577.6 (MH_2^{2+})
	-A(L/I)(L/I)SDPP-**COOH**	615.9 (MH_2^{2+})
	-G(L/I)VFGSGWSVG(L/I)PH-**COOH**	822.36 (MH_2^{2+})
I	**NH_2**-WSMTWTVPVPR-**COOH**	1359.8 (MH^+)
M	-EP(L/I)(L/I)SDF-	643.8 (MH_2^{2+})
	-CCGFVTFW-	859.4 (MH_2^{2+})
N	-DNS(L/I)(L/I)-	539.2 (MH_2^{2+})
	-GSGWSVHEK-**COOH**	745.9 (MH_2^{2+})

[a] The sequences obtained by tandem mass spectrometry contain ambiguous residues represented within parentheses.

In light of the data reported above, assuming that enough protein material was available and the obtained amino acid sequences were long enough, the tandem mass-spectrometry approach resulted to be unsuitable to *T. borchii* protein identification certainly due to the limited availability of information concerning its genome and proteome.

3. Conclusions

This study represents a useful step toward the comprehension of events responsible for the formation and maturation of the *T. borchii* fruit body. The comparison of 2-DE maps of ripe and unripe ascocarps evidenced a different protein expression suggesting that the initial phase of the fructification is associated with higher metabolic activity compared to that of more advanced stages of fruit body maturation. Moreover, maturation is characterised by the appearance and disappearance of a certain number of specific proteins. Even though there is a lack of information regarding the *T. borchii* genome, some of the proteins present in both maturation stages were identified by Edman degradation analysis. This provided some new evidence concerning the cellular processes that might take place during this phase of *T. borchii* life cycle. Other proteins were subjected to mass-spectrometry analysis, but no suitable matches were obtained from these data. In order to identify the processes leading to fructification, new sequencing and mass spectrometry experiments are in progress to gain de novo sequences for all those proteins specific to either the ripe or unripe *T. borchii* fruit body.

4. Experimental

4.1. General experimental procedures

All the chemicals and solvents used for Edman degradation analysis were sequence- or HPLC-grade and supplied by Beckman (Beckman Coulter, Fullerton-USA).

Trypsin, dithiothreitol and α-cyano-4-hydroxy-cinnamic acid were purchased from Sigma. HPLC-grade trifluoroacetic acid (TFA) was obtained from Carlo Erba (Italy). All other reagents and solvents for the MS analyses were of the highest purity available from Baker (USA).

4.2. Fruit body collection

T. borchii fruit bodies were collected in an experimental truffle orchard, located near Marina di Ravenna, in Northern Italy (Zambonelli et al., 2000).

The degree of maturation of the ascocarps was defined using the following categorised stages on the basis of the percentage of asci containing mature spores: stage 0 = 0%, stage 1 = 1–5%, stage 2 = 6–25%, stage 3 = 26–50%, stage 4 = 51–75% and stage 5 = 76–100%. The maturation stage of the spores was defined by morphological method: the mature spores presented a reddish-yellow brown colour and a reticulate ornamentation.

The ascocarps were analysed by morphological and molecular methods (Pegler et al., 1993; Bertini et al., 1998) to confirm them as *Tuber* species.

4.3. Two-dimensional electrophoresis

For two-dimensional analyses unripe (stage 0) and ripe (stage 5) *T. borchii* ascocarps were homogenised using a Potter homogenizer with a lysis buffer containing 8 M urea, 4% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 40 mM Tris-base and 65 mM dithiothreitol (DTT). To prevent protease and phosphatase interference 1 mM orthovanadate was added to the buffer and pH adjusted to the value of 9.0. The suspension obtained was centrifuged at 14,000 rpm for 10 min and the supernatant used for the electrophoretic runs. For the analytical and micropreparative 2-DEs, 45 μg and 1 mg of total protein respectively, were loaded onto 18 cm nonlinear Immobiline strips, pH range 3–10 (Amersham), previously rehydratated (Vallorani et al., 2000). The protein content was determined according to the Bradford method (1976), using bovine serum albumin as standard. For isoelectrofocusing (IEF) a constant voltage of 200 V was applied for 1–3 hours and increased from 300 to 3500 V over 4 h and stabilised at 5000 V for 22 h. The second-dimensional run was carried out on 9–16% polyacrylamide linear gradient gels at 40 mA/gel constant current at 9 °C. The pI and M_r calibration was performed by co-electrophoresis of fruit body proteins with 40 μg of human serum proteins as internal standard (Bjellqvist et al., 1993). Analytical gels were stained with silver salt as described in Oakley (1981), while micropreparative gels were stained with 0.25% (w/v) Coomassie Brilliant Blue R-250 in 50% methanol/ 20% acetic acid. Silver-stained 2-D gels were scanned with a Hewlett Packard Scanjet 4c scanner and then processed using the Melanie 3 software package (Bio-Rad Laboratories, Hercules, USA).

4.4. Electroelution and N-terminal sequencing

Protein spots were excised from micropreparative gels and electroelution was performed using a Centrilutor Micro-Electroeluter (Amicon, Millipore-USA) with a buffer consisting of 3% (w/v) Tris, 14.4% (w/v) glycine and 1% (w/v) sodium dodecyl sulfate (SDS). A costant voltage of 200 V was applied for 2 h and then the samples were dialysed against HPLC-grade water using suitable cut-off Amicon Centricon (Amicon, Millipore-USA), then concentrated and dried under vacuum.

Protein samples obtained as above were resuspended in 30 μl of 30% acetonitrile (v/v) (ACN) and the N-terminal sequence determined using an automatic LF 3000 Protein Sequencer equipped with an on-line Gold HPLC system (Beckman Instruments, Fullerton-USA) for the detection of the phenylthiohydantoin (PTH) amino acids. The PTH amino acids were separated on an Ultrasphere ODS column (25 cm×2.0 mm, 5 μm particle size).

4.5. In situ digestion

The analysis was performed on the Coomassie-blue stained proteins excised from 2-D gels. The excised spots were washed first with ACN and then with 0.1 M ammonium bicarbonate. Protein samples were reduced by incubation in 10 mM dithiothreitol (DTT) for 45 min at 56 °C. The cysteines were alkylated by incubation in 5 mM iodoacetamide for 15 min at room temperature in the dark. The gel particles were then washed with ammonium bicarbonate and ACN.

Enzymatic digestion was carried out with trypsin (12.5 ng/μl) in 50 mM ammonium bicarbonate pH 8.5 at 4 °C for 4 h. The buffer solution was then removed and a new aliquot of the enzyme/buffer solution was added for 18 h at 37 °C. A minimum reaction volume, enough for the complete rehydratation of the gel was used. Peptides were then extracted washing the gel particles with 20 mM ammonium bicarbonate and 0.1% TFA in 50% ACN at room temperature and then lyophilized.

4.6. MALDI analyses

MALDI-TOF mass spectra were recorded using an Applied Biosystem Voyager DE-PRO reflector instrument on a new MALDI/TOF mass spectrometer. A mixture of analyte solution and alfa-cyano-hydroxycinnamic acid (10 mg/ml in ACN/0.1% TFA, 2.5:1, v/v,) was applied to the metallic sample plate and dried under vacuum. Mass calibration was performed using external standards. Raw data were analysed by using the computer software provided by the manifactures and reported as mono-isotopic masses.

4.7. Liquid chromatography-electrospray tandem mass spectrometry (LC/ES/MS/MS)

Tryptic peptide mixtures obtained as previously described were analysed by LC/ES/MS/MS "on-line" using an LCQ ion trap instrument (Finnigan Corp., San Josè, CA). Proteolytic digest was fractionated on an HP 1100 HPLC apparatus (Hewlett-Packard, Palo Alto, CA) using a narrowbore Phenomenex Jupiter C18 column (250×2.1 mm, 300 Å) (Torrance, CA) using 0.05% TFA, 5% formic acid in H_2O (solvent A) and 0.05% TFA, 5% formic acid in ACN (solvent B) by means of a linear gradient from 5 to 65% solvent B for 60 min at a flow rate of 0.2 ml/min. The effluent was directly inserted into the ion source through the electrospray probe and both ES/MS and ES/MS/MS spectra were acquired throughout the entire analysis by using the software provided by the manufacturers.

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