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Purification and characterization of mycoferritin from *Fusarium verticillioides* MRC 826

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Abstract The fungus *Fusarium verticillioides* MRC 826 (ascomycetes species), a toxigenic isolate is capable of synthesizing mycoferritin only upon induction with iron in yeast extract sucrose medium. The molecular mass, yield, iron and carbohydrate contents of the purified mycoferritin were 460 kDa, 0.010 mg/g of wet mycelia, 1.0 and 40.2%, respectively. Native gel electrophoresis of the mycoferritin revealed two bands possibly representing isoforms of ferritin. Subunit analysis by SDS–PAGE showed a single protein subunit of ~24 kDa suggesting similar sized subunits in the structure of apoferritin shell. Immunological cross reactivity was observed with the anti-fish liver ferritin. Transmission electron microscopy revealed an apparent particle size of 100 Å. N-terminal amino acid sequencing of mycoferritin showed identities with other eukaryotic ferritin sequences. The spectral characteristics were similar to equine spleen ferritin. However, circular dichroic spectra revealed a higher degree of helicity. Functionally, induction of mycoferritin minimizes the pro-oxidant role of iron.

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Introduction

Iron is an essential growth factor for virtually all organisms, but poses problems of poor solubility and is toxic in excess, as it catalyses the generation of highly deleterious hydroxyl radicals via Haber–Weiss Fenton reaction (Haber and Weiss 1934; Dunford 1987). Therefore, after uptake, storage of the accessed iron becomes essential in fungal metabolism of the metal to prevent repolymerization (Spiro et al. 1966) and toxicity (Byers and Arceneaux 1998). Ferritin, the iron storage protein (450 kDa) plays a dynamic role in iron metabolism by sequestering toxic free iron (Harrison and Arosio 1996). Structurally, it consists of a central hydrous ferric oxide phosphate core surrounded by an outer protective shell called apoferritin, which accommodates upto 4,500 iron atoms per molecule which exists in mineral phase. The three dimensional structure of ferritin is highly conserved with 24 protein subunits arranged in 432 symmetry. The apoferritin is a heteropolymer of H&L types with molecular weight ranging from 18 to 28 kDa and the subunits display different immunological reactivities, spectroscopic characteristics, surface charge and iron incorporation (Arosio et al.1978).

Ferritin is ubiquitious and has been extensively characterized from bacterial, plant, animal and human species (Theil 1987; Laulhere et al. 1988; Harrison and Arosio 1996; Barcello et al. 1997; Geetha and Deshpande 1999; Suryakala and Deshpande 1999; Shashidhar et al. 2005a; Lakshmi Deepa et al. 2008). However, the understanding of higher molecular iron storage forms in fungal systems is incomplete. In zygomycetes, in addition to polycarboxylate type siderophores, mycoferritin in *Mortierella alpina* (Bozarth and Goenaga 1972), bacterioferritin in *Absidia spinosa* (Carrano et al. 1996) and zygoferritin in *Phycomyces* (David and Easterbrook 1971) were reported. In ascomycetes, hydroxymate type of siderophores (Winkelmann 1992) such as rhodotorulic acid (Atkin and Neilands 1968), coprogen (Hesseltine et al. 1952), fusarinines (Sayer and Emery 1968) and ferrichromes (Emery and Neilands 1961) have been identified. To date, mycoferritin in ascomycetes has been reported only in *Aspergillus parasiticus* 255 (Shashidhar et al. 2005a). The *Fusarium* group of fungi is known to produce toxic secondary metabolites, which include trichothecenes, zearolenome, fusarins, moniliformin, butenolide and fumonisins (FB_1, FB_2, FB_3) associated with deleterious effects on animals and humans (Bennett and Klich 2003; Brown et al. 2005; Ellis et al. 1991; Pirttilä et al. 2004). The formation of these secondary metabolites is complex involving primary and secondary metabolism (Campbell 1984; Feng and Leonard 1995). The biochemical correlation between aflatoxin production and oxidative stress is well documented, suggesting oxidative stress as a prerequisite for aflatoxin synthesis (Jayashree and Subramanyam 2000; Shashidhar et al. 2005b). The pro-oxidant role of iron in *A. parasiticus* 255 is minimized by the induction of mycoferritin only in the presence of iron in YES media (Shashidhar et al. 2005b). This communication reports on the purification and characterization of mycoferritin (MF) from *Fusarium verticillioides* MRC 826 (Ascomycetes species) and its role on fumonisin B_1 production in YES media containing iron.

Materials and methods

Culturing of *Fusarium verticillioides*

A toxigenic isolate of *F. verticillioides* MRC 826 Tygerberg, South Africa was employed for the study. The cultures were maintained on potato dextrose agar media and were grown in stationary liquid culture yeast extract sucrose (YES) medium (yeast extract 2%, sucrose 15%), pH 6.0, at 28°C for 21 days both in the absence and presence of iron (ammonium ferric citrate, 30 μg of Fe^{3+}/ml of the medium). Mycelium of the 21-day-old cultures was used for the study. Production of fumonisin B_1 as a secondary metabolite was assessed by culturing the fungus in the absence and presence of iron (30 μg of Fe^{3+}/ml of the medium) in YES media over a period of 1–30 days.

Isolation of mycoferritin

A method standardized for MF isolation from *A. parasiticus* 255 (Shashidhar et al. 2005a) was adopted in the present study. The protein obtained by ammonium sulphate fractionation (25–60% saturation) was designated as crude protein.

Purification of mycoferritin

As purification of the crude protein (25–60%) by Sephacryl-S-300 gel filtration chromatography and DEAE-cellulose chromatography were unsuccessful, native gel electrophoresis of the crude protein followed by electroelution of protein bands stained for iron by potassium ferricyanide method (specific for non-heme iron) was used for the analysis. Electroelution of the protein was carried out in an electroelution apparatus (Hoefer Scientific Instruments, GE 200, CA, USA) in tris–glycine buffer, pH 8.3, at a constant voltage of 50 V, overnight. The homogeneity of the protein recovered by electroelution was assessed by native gel electrophoresis.

Analytical methods

Protein content at different stages of purification was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard. Iron content of the protein at different stages of purification was estimated by phenanthroline method (ICSH 1978) using ammonium ferrous sulphate as standard. The total neutral sugar content of the intact protein was estimated by phenol sulphuric acid method of Dubois et al. (1956) using galactose as standard.

Electrophoresis

Purity of the preparations was evaluated by native gel electrophoresis in 6% slab gels with tris–glycine buffer, pH 8.3 at a constant current of 15 mA (Davis 1964). The protein bands were visualized either by coomassie brilliant blue R-250 or silver staining of Wray et al. (1981). Non-heme iron on gels was detected by ferricyanide method of Leong et al. (1992). SDS–PAGE of the purified MF was performed under reducing conditions in 12% gels along with equine spleen ferritin (ESF) and commercial low molecular weight markers according to the method of Laemmli (1970), at a constant current strength of 15 mA. Protein bands were located by coomassie brilliant blue R-250 staining. Native gel electrophoresis of the crude MF (25–60% pellet) obtained at different time periods (6, 12, 18, 24 and 30 days) along with commercial ESF was performed in 6% gels with tris–glycine buffer, pH 8.3 at a constant current of 15 mA (Davis 1964). Protein bands were located by coomassie brilliant blue R-250 staining.

Immunoblotting

The purified MF (15 μg) separated on 12% SDS–PAGE were electrotransferred to polyvinylidene difluoride transfer membrane by the method of Towbin et al. (1979). Incubation of the membrane with each reagent was followed by extensive washing with 25 mM sodium phosphate-buffered saline (PBS) and Towbin's phosphate buffered saline (TPBS), containing 0.05% Tween-20, pH 7.4.

The non-specific binding sites on the membrane were blocked with blocking buffer (PBS containing 3% BSA) for 1 h at room temperature followed by treatment with primary antibody [1:500 diluted anti-*Clarius batracus* (fish) liver ferritin raised in rabbit] for 2 h at room temperature. Later, the membrane was washed thoroughly and incubated with 1:4000 diluted secondary antibodies i.e., goat anti-rabbit IgG conjugated to alkaline phosphatase for 1 h at room temperature. After incubation, the membrane was washed extensively and treated for 10 min with the developing solution [0.5 mg *p*-nitro blue tetrazolium chloride (NBT) and 7.5 mg 5-bromo-4-chloro-3-indolyl phosphate toluidine (BCIP)] in 50 ml of 0.1 M $NaHCO_3$–NaOH containing 1.0 mM $MgCl_2$, pH 9.8.

Molecular weight determination

The molecular weight of purified MF was determined by Sephacryl-S-300 (1.0 × 80 cm) gel filtration chromatography using 50 mM sodium phosphate buffered saline, pH 7.4. The purified protein (200 μg) in 500 μl of elution buffer was applied to the column. Fractions of 1.0 ml were collected at a flow rate of 10 ml/h and monitored for protein at 280 nm. Prior to the run, the column was calibrated with Sigma high molecular weight markers (Bovine thyroglobulin 669 kDa, apoferritin 443 kDa, *β*-amylase 200 kDa, BSA 66 kDa) under similar conditions.

Thymol-sulphuric acid staining for glycoproteins

For staining glycoproteins, the method described by Racusen (1979) was adopted. After native gel electrophoresis of the purified MF, the gel was washed twice in a mixture of 25% isopropyl alcohol, 10% acetic acid with atleast 2 h for each wash. Later the gel was transferred to a solvent mixture containing 0.2% thymol for 1 h, 20 min and was developed in sulphuric acid–ethanol (80:20 v/v) until the opalescent core disappeared and the red colored zones produced against a pale yellow background became prominent.

Immuno-cross-reactivity assessed by antibody capture assay

Cross-reactivity of the purified MF was assessed by antibody capture assay, using anti-*C. batracus* (fish) liver ferritin raised in rabbit. In this assay, each incubation step was followed by washing the plate four times with washing buffer i.e., 10 mM sodium phosphate buffered saline, pH 7.4 containing 0.02% sodium azide and 0.05% Tween-20. The polystyrene wells were coated with 400 ng of the purified MF/*C. batracus* liver ferritin per well in 50 μl of 100 mM carbonate bicarbonate buffer, pH 9.6 and incubated at 37°C overnight. This was followed by blocking with 0.1% BSA in 10 mM sodium phosphate buffer (100 μl) pH 7.4 for 1 h at 37°C. Later, anti-*C. batracus* liver ferritin (50 μl) diluted (1:5000) in 10 mM sodium phosphate buffered saline, pH 7.4 containing 0.3% BSA was loaded in the wells and incubated for 2 h. Later the plates were incubated with 50 μl of 1 in 4,000 diluted secondary antibody i.e., goat anti-rabbit IgG conjugated to alkaline

phosphatase at 37°C for 1 h. The colour development was achieved by addition of *para*-nitrophenyl phosphate (1.25 mg/ml) in substrate buffer i.e., 10% diethanolamine, pH 9.6 containing 0.5 mM $MgCl_2$ at 37°C for 20 min, followed by termination of the reaction with 150 µl of 5 N NaOH. The yellow colour developed was read in ELISA reader (Microscan MS5608A, Electronics Corporation of India Limited, Hyderabad, India) at 405 nm.

Transmission electron microscopy

Mycoferritin (30 µg/ml) was negatively stained using saturated uranyl acetate (2%). Later, the specimen was observed under the transmission electron microscope (Model: Hitachi, H-7500, Tokyo, Japan) at a magnification of 60 K.

N-terminal amino acid sequencing of the mycoferritin

The purified MF (15 µg) separated on 12% SDS–PAGE were electrotransferred to polyvinylidene difluoride transfer membrane by the method of Towbin et al. (1979). The blot was stained with coomassie brilliant blue R-250 (0.25% in 45% methanol and 10% acetic acid), washed (2 × 30 sec) in destainer I (7% methanol and 10% acetic acid), and then rinsed in destainer II (50% ethanol) until the background was clear. The MF subunit band was excised from the polyvinylidene difluoride transfer membrane and analyzed for N-terminal amino acid sequencing on Applied Biosystems Precise 492 dual-column instrument (Fostercity, CA, USA).

UV–visible spectral analysis

The UV/Vis absorption spectra (200–700 nm) of the purified MF and ESF (0.1 mg/ml) in Tris–glycine buffer, pH 8.3 was recorded in Spekol 1200, photodiode array based UV–Vis spectrophotometer (Analytic Jena AG, Jena, Germany).

Fluorescence spectra

Apoferritin samples were prepared by reduction of purified MF and ESF (0.1 mg/ml) by dialysis against 1% thioglycolic acid, pH 4.6 for 18 h (Crichton and Bryce 1973). Later the preparations were dialyzed against 10 mM Tris–HCl buffer, pH 7.0 and their fluorescence spectra (300–400 nm) recorded ($Ex_{280\ nm}$) in Jasco FP-750 (Easton, USA) spectrofluorimeter.

Circular dichroic spectra

The far UV circular dichroic spectrum (195–250 nm) of purified MF and ESF was recorded in Jasco J-810 (Easton, USA) spectrophotometer at a protein concentration of 0.2 mg/ml in 10 mM sodium phosphate buffer, pH 7.4. Measurements were made at 27°C in cells of 0.05 cm path length and the molar ellipticities calculated (θ) using a mean residue weight of 115. The alpha, beta and random conformations were calculated using k2d software.

Analysis for fumonisin B_1

In this context, 20 ml of the cultured filtrate was mixed with 20 ml of ethylacetate. Later, the contents were transferred into a separating funnel and the aqueous layer was recovered into a container. The contents were brought to dryness in an incubator at 45°C. The residue was dissolved in methanol:water (3:1; 2 ml) and examined for the presence of fumonisins by spotting 10 µl on pre-coated polyester silica gel-G TLC plates (thickness: 250 µm; particle size: 2–25 µm), and developed in chloroform:methanol:acetic acid (6:3:1). The chromatogram containing fumonisins was derivatized with *p*-anisaldehyde spraying reagent and kept in a hot air oven (110°C) for 3 min. The presence of fumonisins in the culture extracts was determined by comparing the colour and relative R_f value of sample spots with those of fumonisin reference standard FB_1 (Sigma, St. Louis, USA). The detection and quantitation of FB_1 was based on TLC-densitometric method, as described earlier (Karuna and Sashidhar 1999) with minor modifications. The density of FB_1 was quantitated using Charge Couple Device (CCD) based digital image analyzer, in transmittance mode (UVI Tech, Cambridge, UK). The peak area was calculated using Uvi-tech software supplied along with the instrument.

Results

Culturing studies on *F. verticillioides* in YES media demonstrates that the fungus is capable of synthesizing

MF upon induction with iron in YES media. This observation was similar to that of *A. parasiticus* 255 (Shashidhar et al. 2005a) grown under similar conditions. Mycoferritin from *Fusarium* has been isolated and purified by the application of conventional biochemical methods such as thermal denaturation (75°C, 10 min) of the fungal homogenate followed by ammonium sulphate fractionation (Shashidhar et al. 2005a). Native gel electrophoresis of the crude protein (25–60% pellet) followed by protein and non-heme iron staining (potassium ferricyanide method), revealed protein bands with positive non-heme iron staining (data not shown). However, protein staining revealed extensive heterogeneity. Attempts to further purify the MF by Sephacryl-S-300 gel filtration in 50 mM sodium phosphate buffer pH 7.4, a method standardized for mammalian ferritins was unsuccessful. Hence native gel electrophoresis of the crude protein followed by electroelution was employed for further purification of MF. The native gel electrophoresis of the electroeluted material along with ESF followed by protein and iron staining revealed the absence of contaminants. Two bands were observed, possibly representing isoforms of ferritins (Fig. 1A, B). The yield of purified MF was 0.010 mg/g of wet mycelia. An iron content of 1.0% was observed for the purified MF. Interestingly, a high carbohydrate (neutral) content of 40.2% was observed for the purified MF. Native gel electrophoresis of the MF followed by glycoprotein staining (thymol sulphuric acid) revealed protein bands with positive staining (Fig. 2).

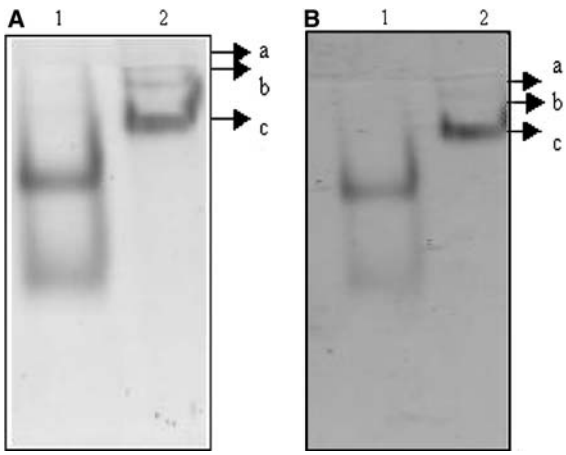

Fig. 1 Native gel-electrophoretic pattern of purified mycoferritin along with equine spleen ferritin (*ESF*). **A** Protein stain. **B** Iron stain. *Lane 1* purified mycoferritin (1 μg in **A** and 20 μg in **B**). *Lane 2* ESF (reference standard, *a* trimer; *b* dimer; *c* monomer; 1 μg in **A** and 10 μg in **B**)

Sephacryl-S-300 gel filtration analysis revealed an apparent molecular mass of 460 kDa for purified MF. Subunit analysis of MF by SDS–PAGE under reducing conditions revealed a single protein band with an apparent molecular weight of 24 kDa (Fig. 3A). Immunological cross-reactivity studies of MF by immunoblotting and antibody capture assay using *C. batracus* (fish) liver ferritin antibodies revealed cross-reactivity. ESF also showed cross-reactivity with anti-*C. batracus* liver ferritin (Fig. 3B). MF showed 4% cross-reactivity by antibody capture assay. Electron micrograph of negatively stained MF is shown in Fig. 4. The study revealed spherical particles with an apparent particle size of 100 Å. The N-terminal amino acid sequence of MF from *F. verticillioides* in comparison with other species is depicted in Table 1. The analysis revealed a sequence of SLPALDYA.

The UV–Vis absorption spectrum of MF was similar to that of standard ESF (data not shown). The fluorescence emission spectrum of the purified MF in comparison with standard ESF is similar (data not shown). The emission maxima for ESF and MF were 334 and 339 nm, respectively. The circular dichroic spectra of the purified MF in comparison with ESF are shown in Fig. 5. The analysis revealed alpha, beta, random conformations of 27, 22, 51% for ESF and 53, 9, 37% for MF.

Thin layer chromatography of fumonisins isolated from the culture extracts of *F. verticillioides* grown at different time periods both in the presence and

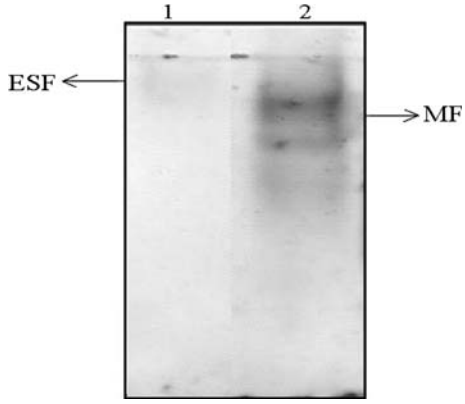

Fig. 2 Thymol-sulphuric acid staining of purified MF along with ESF. *Lane 1* ESF (10 μg). *Lane 2* MF (20 μg)

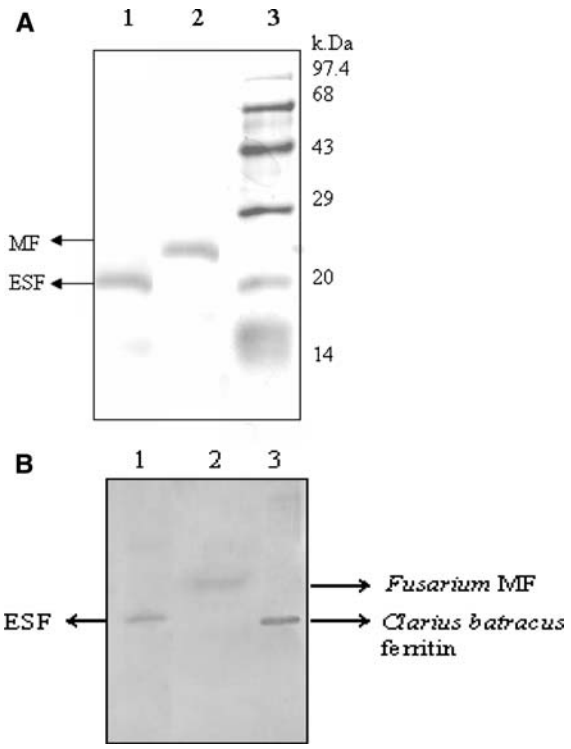

Fig. 3 **A** SDS–gel electrophoretic pattern of purified mycoferritin and ESF along with low molecular weight markers. *Lane 1* equine spleen ferritin (10 μg). *Lane 2* purified MF (10 μg). *Lane 3* low molecular weight markers (7.5 μg). **B** Immunoblot of ferritins. *Lane1* equine spleen ferritin (10 μg). *Lane 2* purified MF (10 μg). *Lane 3 Clarius batracus* liver ferritin (10 μg)

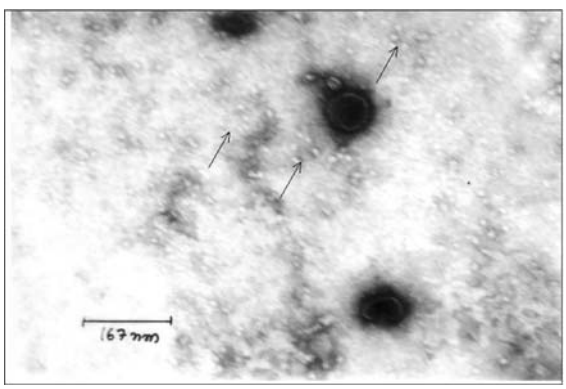

Fig. 4 Electron micrograph of the purified mycoferritin. Magnification 60 K (ferritin molecules indicated by *arrows*)

absence of iron and its quantification by Uvi-tech software is shown in Fig. 6. From the analysis, maximum fumonisin B_1 production was observed on day 24 when the fungus was grown in the absence of iron, whereas on day 12 in the presence of iron. The native gel electrophoretic pattern of the crude MF isolated from *F. verticillioides* grown in YES media containing iron at different growth periods (6, 12, 18, 24, 30 days) along with ESF is shown in Fig. 7. The analysis revealed prominent MF expression from day 18 onwards.

Discussion

In addition to the occurrence of hydroxamate type of siderophores in ascomycetes, MF has been reported only in *A. parasiticus* 255 (Shashidhar et al. 2005a). The present study on *F. verticillioides* demonstrates that this fungus is also capable of synthesizing MF upon induction with iron in YES medium.

MF has been isolated and purified by the application of conventional biochemical methods. The purified protein was confirmed as ferritin by native gel electrophoresis followed by potassium ferricyanide staining. Sephacryl-S-300 gel filtration and DEAE-cellulose ion-exchange chromatography were unsuccessful in purifying the protein. Therefore, native gel electrophoresis of the crude protein followed by electroelution was ideal for the purification of MF. Two isoforms of ferritins were observed in *Fusarium*. Similar observations have been reported for several species (Carrano et al. 1996; Geetha and Deshpande 1999; Shashidhar et al. 2005a; Lakshmi Deepa et al. 2008). The iron content of MF resembles to that of *A. parasiticus* (Shashidhar et al. 2005a). However, Bozarth and Goenaga (1972) reported high iron content of 17% for MF from *M. alpina*. Whether the MF from *F. verticillioides* serves, as a reservoir for iron remains to be an open question. Interestingly, high carbohydrate content was observed for the *F. verticillioides* MF. The presence of carbohydrate was also confirmed by native gel electrophoresis followed by glycoprotein staining (thymol sulphuric acid; Fig. 2). As ferritins of other species, MF from *F. verticillioides* is also a glycoprotein (Geetha and Deshpande 1999; Suryakala and Deshpande 1999; Shashidhar et al. 2005a; Lakshmi Deepa et al. 2008).

Gel filtration has been commonly employed for molecular weight determination of native ferritins by several investigators (Page et al. 1980; Cetinkaya et al. 1985; Lakshmi Deepa et al. 2008). The molecular weight of the MF (460 kDa) is in agreement with the

Table 1 Comparison of N-terminal amino acid sequence of mycoferritin from *Fusarium verticillioides* with other ferritin sequences

Ferritin subunit	N-terminal sequence	Reference
Human L-chain	S**S**QIR**Q**N**Y**ST	Boyd et al. (1985)
Horse L-chain	S**SQIRQ**N**Y**ST	Heusterspreutc and Crichton (1981)
Bullfrog L-chain[a]	**ME**S**Q**VR**Q**NFH	Theil (1987)
Schistosoma mansoni Sch 1	**SLCRQNYH**	Dietzel et al. (1992)
Soybean H-type	**SL**AR**Q**N**YA**	Ragland et al. (1990)
Absidia spinosa H-chain[a]	**MRG**NPE**V**IDY	Carrano et al. 1996)
Absidia spinosa L-chain[a]	**MKG**NRE**V**INQ	Carrano et al. 1996
Fusarium verticillioides	**SL**PALD**YA** **S Q Y**	Present work

[a] Bacterioferritin

values reported for ferritins of other species (Shashidhar et al. 2005a; Lakshmi Deepa et al. 2008; Carrano et al. 1996). Subunit analysis of MF revealed a single protein band with an apparent molecular weight of 24 kDa (Fig. 3A). This value is similar to molecular masses of 20, 25 and 19.3 kDa reported for protein subunits in *A. parasiticus* 255 (Shashidhar et al. 2005a) *Phycomyces* (LaBombardi et al. 1982) and *Mortierella* (Bozarth and Goenaga 1972) mycoferritins, respectively. Further, the presence of similar sized subunits in the native structure of MF has been confirmed by immunoblotting (Fig. 3B) using antibodies raised against *C. batracus* liver ferritin. The subunit pattern of fungal ferritins resembles the studies made on fish ferritins from our laboratory wherein a single protein band of 21 kDa was observed (Geetha and Deshpande 1999). This pattern reflects the structural simplicity of MF, which is consistent with the accepted postulate that the apoferritin shell is comprised of similar sized subunits (Crichton et al. 1973). Immunological cross-reactivity studies of MF by immunoblotting and antibody capture assay using *C. batracus* (fish) liver ferritin antibodies revealed cross-reactivity (Fig. 3B). The low degree of cross-reactivity between anti-fish ferritin and MF is consistent with their distant phylogeny. Treffry et al. (1984) have attributed such immunological variations to differences in amino acid sequences and to post translational modifications. In addition, factors such as subunit heterogeneity and surface charges explain the differential immunological behaviour of ferritins (Otsuka et al. 1981).

Electron microscopic studies of the MF revealed spherical particles of 100 Å size which is in agreement with the MF particle size of 100 Å in *M. alpina* (Bozarth and Goenaga 1972) and zygoferritin particle size of 105 Å in *Phycomyces blakesleeanus* (David and Easterbrook 1971).

N-terminal amino acid sequence of MF revealed similarities with other eukaryotic ferritin sequences.

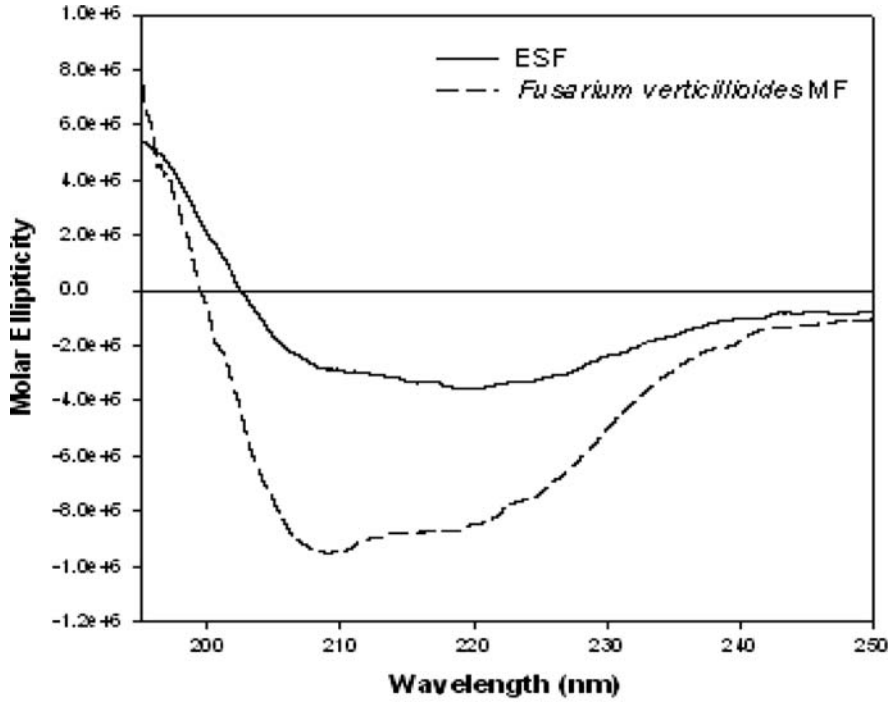

Fig. 5 Far UV circular dichroic spectra of mycoferritin in comparison with equine spleen ferritin

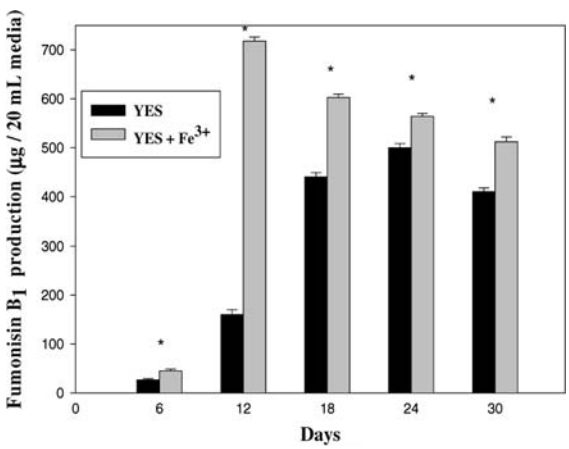

Fig. 6 Quantification of fumonisin B_1 from *Fusarium verticillioides* separated by silica gel TLC using Uvi tech software. Values are Mean ± SD for triplicate analysis at a given time point (* $P < 0.001$)

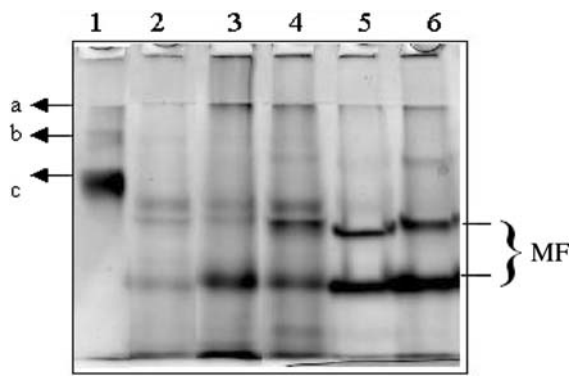

Fig. 7 Native (6%) gel electrophoresis of the crude protein from *Fusarium verticillioides* obtained at 6, 12, 18, 24 and 30 days when grown in YES media containing iron (30 µg of Fe^{3+}/ml of the media). *Lane 1* std ESF (5 µg, *a* trimer; *b* dimer; *c* monomer), *Lane 2* 6th day (20 µg), *Lane 3* 12th day (20 µg), *Lane 4* 18th day (20 µg), *Lane 5* 24th day (20 µg), *Lane 6* 30th day (20 µg)

Serine, glutamine and tyrosine are the amino acid residues conserved among ferritin sequences. The sequence analysis also indicate that the MF isolated from *Fusarium* is not a bacterioferritin as the amino acid methionine is absolutely conserved as an N-terminal amino acid, as in the case of *A. spinosa* (Carrano et al. 1996).

The UV–Vis absorption spectrum of MF from *F. verticillioides* was similar to that of ferritin from *A. parasiticus* (Shashidhar et al. 2005a), *M. alpina* (Bozarth and Goenaga 1972) and ESF (data not shown). Removal of iron from the protein leads to the appearance of intrinsic fluorescence. The fluorescence spectrum of the purified MF was similar to that

of ESF. The fluorescence emission maximum of MF is in agreement with the values reported for other species (Otsuka et al. 1981; Shashidhar et al. 2005a; Lakshmi Deepa et al. 2008). The fluorescence intensity of MF was higher than ESF (data not shown). The circular dichroic spectral analysis revealed a higher degree of helicity (53% alpha helix) for *Fusarium* MF when compared to ESF (Fig. 5).

Thin layer chromatography of fumonisin B_1 (FB_1) isolated from the culture extracts of *F. verticillioides* grown at different time periods (6, 12, 18, 24, 30 days) both in the presence and absence of iron in YES media revealed maximum production on day 24 when the fungus was grown in the absence of iron, whereas on day 12 when the fungus was grown in the presence of iron. A decreasing trend in FB_1 content was observed from day 18 onwards when the fungus was grown in the presence of iron (Fig. 6). There was statistically difference ($P < 0.001$) in the fumonisin B_1 production between the groups (YES and YES + Fe^{3+}).The native gel electrophoretic pattern of the crude MF isolated from *Fusarium* grown in YES media containing iron at different time periods (6, 12, 18, 24, 30 days) revealed prominent MF expression from day 18 onwards (Fig. 7). Therefore, the increased levels of FB_1 on day 12 when the fungus was grown in YES media containing iron might be due to oxidative stress promoted by free iron present in the media, and the decrease in the FB_1 levels from day 18 onwards coincides with the MF expression. Mammalian ferritin, as an antioxidant has the ability to oxidize and sequester intracellular iron into the central mineral core, limiting the levels of catalytically available iron, thus functioning as a cytoprotective protein (Harrison and Arosio 1996). This observation confirms the pro-oxidant role of iron in *Fusarium* and was minimized by the induction of mycoferritin. These observations were similar to the findings on *A. parasiticus* 255 (Shashidhar et al. 2005b). Further, the role of MF in relation to other metabolic activities in fungal system is yet to be studied.

In conclusion, *F. verticillioides* (Ascomycetes species) is capable of synthesizing MF upon induction with iron in YES media. MF has been isolated and purified by conventional biochemical techniques. The iron content of MF was low. Observations made on immunological cross-reactivity studies using antibodies to fish ferritin suggest certain degree of phylogenetic relationship. Comparison of the N-terminal amino acid sequence of MF from *F. verticillioides* with that of other species reveals conservation of specific amino acid residues. The information on amino acid sequence also suggests that the MF from *F. verticillioides* is not a bacterioferritin as the N-terminal amino acid is serine instead of methionine. The pro-oxidant role of iron was minimized by the induction of mycoferritin. This is the first report documenting the presence of MF in *Fusarium* and the study will enhance the understanding of the status of higher molecular iron storage forms in Ascomycetes. In addition the data will be of immense value in the comparative biochemistry of fungal ferritins.

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References

Arosio P, Adelman TG, Drysdale JW (1978) On ferritin heterogeneity. Further evidence for heteropolymers. J Biol Chem 253:4451–4458

Atkin CL, Neilands JB (1968) Rhodotorulic acid, a diketopiperazine dihydroxamic acid with growth-factor activity: isolation and characterization. Biochemistry 7:3734–3739

Barceló F, Miralles F, Otero Areán C (1997) Purification and characterization of ferritin from Alfalfa seeds. J Inorg Biochem 66:23–27

Bennett JW, Klich MA (2003) Mycotoxins. Clin Microbiol Rev 16:497–516

Boyd D, Vecoli C, Belcher DM, Jain SK, Drysdale JW (1985) Structural and functional relationship of human ferritin H and L chains deduced from cDNA clones. J Biol Chem 260:11755–11761

Bozarth RF, Goenaga A (1972) Purification and properties of mycoferritin from *Mortierella alpina*. Can J Microbiol 18:619–622

Brown DW, Cheung F, Proctor RH, Butchko RA, Zheng L, Lee Y, Utterback T, Smith S, Feldblyum T, Glenn AE, Plattner RD, Kendra DF, Town CD, Whitelaw CA (2005) Comparative analysis of 87, 000 expressed sequence tags from the fumonisin-producing fungus *Fusarium verticillioides*. Fungal Genet Biol 42(10):848–861

Byers BR, Arceneaux JEL (1998) Microbial iron transport: iron acquisition by pathogenic microorganisms. Metal Ions Biol Syst 35:37–66

Campbell IM (1984) Secondary metabolism and microbial physiology. Adv Microb Physiol 25:1–60

Carrano CJ, Böhnke R, Matzanke BF (1996) Fungal ferritins: the ferritin from mycelia of *Absidia spinosa* is a bacterioferritin. FEBS Lett 390:261–264

Cetinkaya N, Lengemann FW, Kogan P (1985) Isolation, purification and characterization of bovine spleen ferritin. Comp Biochem Physiol B 80:773–778

Crichton RR, Bryce CFA (1973) Subunit interaction in horse spleen apoferritin. Dissociation by extremes of pH. Biochem J 133:289–299

Crichton RR, Eason R, Barclay A, Bryce CFA (1973) The subunit structure of horse spleen ferritin: the molecular weight of the oligomer and the stability to dissociation by dilution. Biochem J 131:855–857

David N, Easterbrook K (1971) Ferritin in the fungus *Phycomyces*. J Cell Biol 48:15–28

Davis BJ (1964) Disc electrophoresis. II. Method and applications to human serum proteins. Ann New York Acad Sci 121:404–427

Dietzel J, Hirzmann J, Preis D, Symmons P, Kunz W (1992) Ferritins of *Schistosoma mansoni*: sequence comparison and expression in female and male worms. Mol Biochem Parasitol 50:245–254

Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Colorimetric method for the determination of sugars and related substances. Anal Chem 28:350–356

Dunford HB (1987) Free radicals in iron-containing systems. Free Radic Biol Med 3:405–421

Ellis WO, Smith JP, Simpson BK (1991) Aflatoxins in food: occurrence, biosynthesis, effects on organisms, detection and methods in control. Crit Rev Food Sci Nutr 30:403–439

Emery TF, Neilands JB (1961) Structure of ferrichrome compounds. J Am Chem Soc 83:1626–1628

Feng GH, Leonard TJ (1995) Characterization of the polyketide synthase gene (pksL1) required for aflatoxin biosynthesis in *Aspergillus parasiticus*. J Bacteriol 177:6246–6254

Geetha C, Deshpande V (1999) Purification and characterization of fish liver ferritins. Comp Biochem Physiol Part B Biochem Mol Biol 123(3):285–294

Haber F, Weiss J (1934) The catalytic decomposition of hydrogen peroxide by iron salts. Proc R Soc London Ser A 147:332–351

Harrison PM, Arosio P (1996) The ferritins: molecular properties, iron storage function and cellular regulation. Biochim Biophys Acta 1275:161–203

Hesseltine CW, Pidacks C, Whitehill AR, Bohonos N, Hutchings BL, Williams JH (1952) Coprogen, a new growth factor for coprophilic fungi. J Am Chem Soc 74:1362

Heusterspreute M, Crichton RR (1981) Amino acid sequence of horse spleen apoferritin. FEBS Lett 129:322–327

ICSH Expert Panel on Iron (1978) Recommended methods for measurements of serum iron in human blood. Br J Haematol 38:291–294

Jayashree T, Subramanyam C (2000) Oxidative stress as a prerequisite for aflatoxin production by *Aspergillus parasiticus*. Free Radic Biol Med 29:981–985

Karuna R, Sashidhar RB (1999) Use of ion-exchange chromatography coupled with TLC-laser scanning densitometry for the quantitation of fumonisin B_1. Talanta 50:381–389

LaBombardi VJ, Pisano MA, Klavins JV (1982) Isolation and characterization of *Phycomyces blakesleeanus* ferritin. J Bacteriol 150:671–675

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature (Lond) 227:680–685

Lakshmi Deepa G, Sashidhar RB, Deshphande V (2008) Purification and characterization of phycoferritin from the blue-green alga, *Arthrospira (Spirulina) platensis*. J Appl Phycol 20:359–366

Laulhere JP, Lescure AM, Brait JF (1988) Purification and characterization of ferritins from maize, pea, and soya bean seeds. J Biol Chem 263:10289–10294

Leong LM, Tan BH, Ho KK (1992) A specific stain for the detection of non-heme iron proteins in polyacrylamide gels. Anal Biochem 207:317–320

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with folin phenol reagent. J Biol Chem 193:265–275

Otsuka S, Maruyama H, Listowsky I (1981) Structure, assembly conformation and immunological properties of the two subunit classes of ferritin. Biochemistry 20:5226–5232

Page M, Legueunx J, Gauthier C (1980) A three step purification procedure for human liver ferritin. Can J Biochem 58:494–498

Pirttilä AM, McIntyre LM, Payne GA, Woloshuk CP (2004) Expression profile analysis of wild-type and fcc1 mutant strains of *Fusarium verticillioides* during fumonisin biosynthesis. Fungal Genet Biol 41(6):647–656

Racusen D (1979) Glycoprotein detection in polyacrylamide gel with thymol and sulfuric acid. Anal Biochem 99:474–476

Ragland M, Brait JF, Gagnon J, Laulhere JP, Massenet O, Theil EC (1990) Evidence for conservation of ferritin sequences among plants and animals and for a transit peptide in soybean. J Biol Chem 265:18339–18344

Sayer JM, Emery T (1968) Structure of naturally occurring hydroxamic acids, fusarinines A and B. Biochemistry 7:184–190

Shashidhar J, Sashidhar RB, Deshpande V (2005a) Purification and characterization of mycoferritin from *Aspergillus parasiticus* (255). FEMS Microbiol Lett 245(3):285–294

Shashidhar J, Sashidhar RB, Deshpande V (2005b) Role of mycoferritin from *Aspergillus parasiticus* (255) in secondary metabolism (aflatoxins production). FEMS Microbiol Lett 251:113–117

Spiro TG, Allerton SF, Renner J, Terzis A, Bils R, Saltman P (1966) The hydrolytic polymerization of iron (III). J Am Chem Soc 88:2721–2725

Suryakala S, Deshpande V (1999) Purification and characterization of liver ferritins from different animal species. Vet Res Commun 23:165–181

Theil EC (1987) Ferritin: structure, gene regulation and cellular function in animals, plants and microorganisms. Ann Rev Biochem 56:289–315

Towbin H, Stachelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci 76:4350–4354

Treffry A, Lee PJ, Harrison PM (1984) Iron induced changes in rat liver isoferritins. Biochem J 220:717–722

Winkelmann G (1992) Structure and functions of fungal siderophores containing hydroxamate and complexone type iron binding ligands. Mycol Res 96:529–534

Wray W, Boulikas T, Wray VP, Hancock R (1981) Silver staining of proteins in polyacrylamide gels. Anal Biochem 118:197–203