Fungal Genetics and Biology 97 (2016) 10–17

Contents lists available at ScienceDirect

Fungal Genetics and Biology

journal homepage: www.elsevier.com/locate/yfgbi

Regular Articles

Heterologous expression of an active chitin synthase from *Rhizopus oryzae*

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ARTICLE INFO

Article history:
Received 11 January 2016
Revised 13 October 2016
Accepted 20 October 2016
Available online 21 October 2016

Keywords:
Chitin synthase
Rhizopus oryzae
Enzyme expression
Enzyme purification
Inclusion bodies

ABSTRACT

Chitin synthases are highly important enzymes in nature, where they synthesize structural components in species belonging to different eukaryotic kingdoms, including kingdom Fungi. Unfortunately, their structure and the molecular mechanism of synthesis of their microfibrilar product remain largely unknown, probably because no fungal active chitin synthases have been isolated, possibly due to their extreme hydrophobicity. In this study we have turned to the heterologous expression of the transcript from a small chitin synthase of *Rhizopus oryzae* (RO3G_00942, Chs1) in *Escherichia coli*. The enzyme was active, but accumulated mostly in inclusion bodies. High concentrations of arginine or urea solubilized the enzyme, but their dilution led to its denaturation and precipitation. Nevertheless, use of urea permitted the purification of small amounts of the enzyme. The properties of Chs1 (Km, optimum temperature and pH, effect of GlcNAc) were abnormal, probably because it lacks the hydrophobic transmembrane regions characteristic of chitin synthases. The product of the enzyme showed that, contrasting with chitin made by membrane-bound Chs's and chitosomes, was only partially in the form of short microfibrils of low crystallinity. This approach may lead to future developments to obtain active chitin synthases that permit understanding their molecular mechanism of activity, and microfibril assembly.

1. Introduction

Chitin, a lineal polymer of 2-acetamido-2-deoxy-D-glucose (N-acetylglucosamine, GlcNAc) units linked through β-(1,4) glycosidic bonds, is an essential component of the cell wall of fungi, where it behaves as an skeletal component, although its amounts in yeast is lower than in filamentous fungi, varying from species to species (Ruiz-Herrera, 1992, 2012; Ruiz-Herrera et al., 1992). Besides fungi, this polysaccharide is widely distributed in other groups of eukaryotic organisms: Protista (Mulisch, 1993) including the cysts of amoeba (Arroyo-Begovich and Ruiz-Herrera, 1979; Campos-Gongora et al., 2004), some algae such as chromophytes, and diatoms (Blackwell et al., 1967; Smucker and Dawson, 1986; Round et al., 1990), prymnesiophytes (Chrétiennot-Dinet et al., 1997), some Chromista (Stramenopiles) (Dietrich, 1975), and Chordates (Wagner et al., 1993; Tang et al., 2015).

All chitin synthases (Chs; EC 2.4.1.16) utilize uridine diphosphate N-acetylglucosamine (UDP-GlcNAc) as the only sugar donor. Divalent metallic ions: Mg^{++}, Mn^{++} or Co^{++} (depending on enzyme species) act as cofactors for chitin synthase activity (Ruiz-Herrera, 1992; Ruiz-Herrera et al., 1992), and in most Chs's, GlcNAc has been described as an allosteric activator (Glaser and Brown, 1957).

The synthesis of chitin occurs by a simple transglycosylation reaction, using as substrate UDP-GlcNAc as described above, but at the cellular level it constitutes a very complex process that is yet not very well understood. It is known that chitin synthases exist within the cell in microvesicles denominated chitosomes (Bracker et al., 1976) that are accumulated in the Spitzenkörper, before reaching the cell surface where chitin synthesis takes place (Riquelme et al., 2007), and it was shown that chitin synthases from *Neurospora crassa* are synthesized in the ER, from where they migrate to the Spitzenkörper by a mechanism different to the classical secretory pathway (Verdín et al., 2009).

It is an interesting observation that all fungal species contain more than one gene encoding chitin synthases. Early studies of Chs's sequence fragments obtained by PCR suggested that these enzymes were divided into three classes (Bowen et al., 1992), but more recent studies suggested the existence of two divisions and five classes of chitin synthases (Ruiz-Herrera et al., 2002), although other authors divide them into seven classes (Lenardon et al., 2010; Roncero, 2002).

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http://dx.doi.org/10.1016/j.fgb.2016.10.005
1087-1845/

One peculiar phenomenon is that we have a better knowledge of the genes encoding chitin synthases than the one we have of the enzymes themselves. This lack of knowledge is due to the fact that no fungal chitin synthase has been purified yet in order to determine their kinetic properties that are known mostly from data obtained using crude preparations containing several chitin synthases, and at most from chitosomes, that also contain mixtures of several Chs's.

In this sense, we may indicate that different unsuccessful attempts to purify active Chs's from several fungi have been reported (see Duran and Cabib, 1978; Braun and Calderone, 1979; Kang et al., 1984; Montgomery et al., 1984; Lending et al., 1991; Machida and Saito, 1993; Uchida et al., 1996). A relative positive success was the partial purification with low yields of a class V Chs from *Wangiella dermatitidis* (WdChs5) by affinity chromatography with polyclonal antibodies. It was observed that the partially purified preparation was a mixture of proteolytic fragments (Abramczyk and Szaniszlo, 2009).

The absence of purified fungal chitin synthases has also avoided the analysis of their structure by use of X-ray diffraction, or cryo-electron microscopy (see Callaway, 2015). Apparently, the high hydrophobicity of chitin synthases with its multiple transmembrane domains has impaired this feat. On the other hand, the purification of a trimeric complex of a chitin synthase from *Manduca sexta* (Lars et al., 2009) suggests that possibly the structure of some animal Chs's may be different to the ones from fungi.

One possible strategy to solve the above mentioned difficulty would be the heterologous expression of partial fragments of chitin synthases conserving the probable active domains, but lacking their most hydrophobic stretches. In the literature there is a single report of this approach describing an attempt to obtain a purified preparation of a chitin synthase through its heterologous expression in *Escherichia coli*. In such study, two deleted constructs (both containing the catalytic core) from the fungus *Botrytis cinerea* (BcCHS3a) were partially purified, but unfortunately these variants possessed no enzymatic activity (Magellan et al., 2010).

It has been reported that Zygomycota contain a large number of Chs's (*e.g.* Ma et al., 2009). Specifically, *Rhizopus oryzae* contains 24 genes encoding chitin synthases, 22 of which were expressed under four different growth conditions (Ma et al., 2009). Taking into consideration the possible approach mentioned above, from the *R. oryzae* expressed genes we selected a small *CHS* gene encoding an almost hydrophilic protein to be expressed in a heterologous system of *E. coli*. Its characteristics, *in vitro* activity, partial purification, and the nature of the product synthesized by the enzyme are described below.

2. Materials and methods

2.1. Strains and media used

Rhizopus oryzae 2672 obtained from CECT (Colección Española de Cultivos Tipo) was used as a source of gene RO3G_00942. The strain was maintained and propagated on solid YPD medium [2% Bacto peptone (Difco), 1% Bacto yeast extract (Difco), 2% glucose (SIGMA), and 2% Bacteriological agar (Difco)]. To obtain the mycelium, spores were recovered in sterile distilled water (SDW) and 5×10^{-5} spores ml^{-1} were inoculated into liquid YPG and incubated for 24 h under shaking conditions (180 rpm). Mycelium was recovered by centrifugation and washed twice with SDW by centrifugation.

Escherichia coli BL21 (DE3) (Hussain and Ward, 2003) and the strains modified for gene expression were maintained on solid Luria-Bertani (LB) medium [1% NaCl, 5% Bacto yeast extract, 1% casein peptone (Difco), 2% Bacteriological agar, supplemented with 100 μg ml^{-1} ampicillin and 34 μg ml^{-1} chloramphenicol]. Cells were grown at 37 °C until an optical density (OD_{600}) of 0.6 was reached (12–16 h).

2.2. Isolation of total RNA and cDNA synthesis

Rhizopus oryzae 2672 mycelium obtained as described above was frozen in liquid nitrogen and stored at −80 °C until used. Total RNA extraction was performed using TRIzol (USB Biochemical, Affymetrix). Briefly, frozen mycelium samples were ground with a mortar and pestle in liquid nitrogen and RNA was extracted using TRIzol reagent following the manufacturer's recommendations. First strand cDNA was synthesized using an oligonucleotide dt20 and Superscript II reverse transcriptase (Invitrogen), according to the manufacturer's instructions.

2.3. Cloning of R. oryzae chitin synthase transcript RO3G_00942 and construction of the expression plasmids

Taking into consideration that the selected gene (RO3G_00942) contains two introns (see Results), we synthesized and cloned the corresponding cDNA, instead of the whole gene in order to express it in *E. coli*. Three primers were designed to amplify the transcript of the RO3G_00942 gene (from here on named *CHS1* for facility): forward (CSF1, 5′-CTCGAGATGTCTGAAGAATCAGGAAGC-3′, *Xho*I site underlined), reverse 1 (CSR1, 5′-TCTAGATTAAGTACAAAGGAAG GATGAG-3′, and reverse 2 (CSR2, 5′-TCTAGATTATTCAATCAACAG CAAGAACG-3′, *Xba*I site underlined) The primers CSF1 and CSR1 were used to synthesize the whole transcript, and CSF1 and CSR2 were used to synthesize an incomplete version of the transcript lacking the transmembrane domain located at the 3′ end of the gene, where the 30 highly hydrophobic amino acids were removed, while preserving the important motifs for chitin synthases (Ruiz-Herrera et al., 2002). The PCR reaction mixture (50 μl) contained *R. oryzae* cDNA, 0.25 μM of each primer, 200 μM of each dNTP, and 1.25 U of Platinum Taq DNA Polymerase High Fidelity (Invitrogen). Thermocycling consisted of 30 cycles at 94 °C for 30 s, 57 °C for 30 s, and 68 °C for 90 s. The resulting ca. 1.5-kb DNA fragments were cloned into the TOPO cloning vector (Invitrogen) according to the manufacturer's instructions. The TOPO cloning vectors containing the DNA fragments were treated with the restrictions enzymes *XhoI* and *XbaI*, and the DNA fragments were purified by agarose gel electrophoresis. The DNA fragments and pCold I vector containing a fragment encoding a tag of six histidine residues to the amino terminal (Takara Bio) were mixed and ligated with T4 DNA ligase (Sigma-Aldrich). The expression plasmid encoding the whole transcript was named pCold-chs942 (530 aa), and the truncated plasmid was named pCold-chs942TD (500 aa).

2.4. Heterologous expression of CHS1 gene in E. coli

pCold-chs942 or pCold-chs942TD expression vectors were transformed into competent *E. coli* BL21 Star (DE3) pRARE cells (Hussain and Ward, 2003). An overnight starter culture was established in LB at 37 °C until an optical density at 600 nm (OD_{600}) of 0.6 was reached. At this time, the cultures were transferred to an ice bath for 30 min, and 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added, and incubation was continued for further 16 h at 18 °C. The cells were then harvested by centrifugation and stored at −80 °C for further use. When required, *E. coli* cells were suspended in lysis buffer (50 mM Na_2HPO_4/NaH_2PO_4 pH 6.5, 0.05% LMNG) containing different additions and 1 mM PMSF, and were mechanically broken by sonication using a pulse sequence of 10 s ON and 10 s OFF for 5 min, and centrifuged at 750*g* for 30 min. Samples of extracts from cells transformed with the empty plasmid, or