Published in final edited form as:

Prep Biochem Biotechnol. 2009 ; 39(3): 277–288. doi:10.1080/10826060902953244.

Immunoaffinity Purification of the Class V Chitin Synthase of *Wangiella (Exophiala) dermatitidis*

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Abstract

The class V chitin synthase is unique because it has a myosin motor-like domain fused to its catalytic domain. The biochemical properties of this enzyme and its function remain undefined beyond the knowledge that it is the only single chitin synthase required for sustained cell growth at elevated temperatures and, consequently, virulence. This report describes our successful efforts to isolate and purify an active and soluble form of the enzyme from the cell membranes of *Wangiella* by using a specific polyclonal antibody. To our knowledge, this is the first purification of a single chitin synthase of a filamentous fungus.

Keywords

Immunoaffinity purification; Transmembrane protein; Chitin synthase; Black pathogenic fungus

INTRODUCTION

Chitin, a long linear homopolymer of β -1,4-linked *N*-acetylglucosamine (GlcNAc), is produced in abundance by invertebrates, in lesser amounts by fungi, but is absent from plants and vertebrates. Among fungi, those called filamentous molds incorporate significantly higher amounts of the chitin into their cell wall than do yeast species such as *Saccharomyces cerevisiae*.^[1,2] The diverse effects caused by the inhibition of chitin synthesis by drugs or by disruption of genes involved in chitin synthesis on the growth, morphology, and virulence of fungi substantiate that the chitin in fungal cell walls plays many vital roles in maintaining cell wall integrity.^[3] Those findings, in turn, have led to the popular hypothesis that chitin synthesis is an excellent target for the design of antifungal agents.^[4,5] To produce chitin, fungi use chitin synthases (CSs) that synthesize the growing chitin polymer by the transfer of GlcNAc from uridine diphosphoglucose *N*-acetylglucosamine (UDP-GlcNAc) as the sugar donor. Surveys of fungal genomes have identified a conserved domain in all CSs which allowed the placement of the chitin synthases of *S. cerevisiae* and *Candida albicans* into three classes and those of many filamentous fungi into seven or more classes.^[6] Although substantial information was collected early on about the biochemical properties of the CSs of different fungi and the common features of those synthases, such as their zymogenic properties *in vitro* and their plasma membrane localization *in vivo*,^[6–9] enzymatic characterizations of the CSs more recently have been limited. The main current impediments to such studies in fungi, and

particularly in molds, have been the discovery of the multiple chitin synthases in each fungus and the more traditional problems associated with the purification of very large proteins in active states from membranes.^[10] In this report we describe the immunoaffinity chromatographic purification and characterization of WdChs5p, which may be the most intriguing chitin synthase of *Wangiella (Exophiala) dermatitidis*. This polymorphic mold is one among more than 100 other dematiaceous (melanized) fungi associated with human disease.^[11,12] Previous reports have described five chitin synthase structural genes in this fungus, each of which encodes an isozyme of a different class,^[11,12] although, recently, two more genes encoding CSs of two additional classes have been cloned and sequence GenBank accession nos. EU428191 and EU428192. However, biochemical characterization of the CSs of *W. dermatitidis* have been limited to enzyme assay of crude membrane preparations of the wild type and selected CSs mutants.^[11,12] We consider WdChs5p to be unique because it has an N-terminal myosin motor-like domain (MMD) with a P-loop fused to its C-terminal chitin synthase catalytic domain (CSCD), is more highly expressed at elevated temperatures, and is the only single chitin synthase of this fungus known to be required for viability at 37°C and, thus, virulence.^[13,14] The large molecular mass of WdChs5p and its transmembrane association suggest why we have been unable to express it as an active recombinant protein. In this report, we instead provide details for a new and alternative method to isolate and purify enzymatically active native WdChs5p.

EXPERIMENTAL

Strains, Culture Media, and General Molecular Methods

In this work, we used the following *Wangiella (Exophiala) dermatitidis* strains: the wild-type Wd8656 (ATCC 34100); the chitin synthase mutants *wdchs5Δ11*^[13] and *wdchs2Δwdchs3Δ*.^[15] Cell culture was in the rich YPD broth medium (YPDB) as reported previously^[16] at 25°C and for 2 days after shift to 37°C to induce the elevated expression of WdChs5p.^[13,14]

The polyclonal anti-Myo12p antibody preparation and purification were performed as follows: the WdMyo12p recombinant protein (unpublished), which is the HIS-tagged version of a conserved region in the MMD of WdChs5p expressed in *E. coli* strain BL21(DE3)pLys, was used for the immunization of rabbits (Pacific Immunology). The anti-Myo12p antibody was purified from serum by an affinity method using antigen blotted to nitrocellulose (NC) membrane.^[17]

Membrane proteins were separated by 8% SDS/PAGE under reducing conditions according to the method of Laemmle and then visualized by Commassie Brilliant Blue R-250.^[17] Proteins were electrotransferred to NC membranes and immunodetected as described by Laemmle^[17]. Briefly, solubilized membrane-protein extract was loaded per lane and blotted onto a nitrocellulose membrane (Millipore) by overnight wet-dry transfer (Bio-Rad). After incubation in blocking buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Tween-20, 5% nonfat dried milk), the membrane was then incubated with anti-Myo12p antibody at a 1:5,000 dilution. After primary antibody incubation, the membranes were three times washed in TBST buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Tween-20) and incubated with goat anti-rabbit IgG conjugated with alkaline phosphatase antibody or horseradish peroxidase (Sigma) at a dilution of 1:8,000 in blocking buffer and the proteins on the membranes were then visualized with NBT/BCIP chromagenic reagents (Sigma) or by the ECL Advanced Western Blotting Detection Kit (GE Healthcare).

Chitin synthase activity assays were performed as follows:

1. membrane and membrane protein samples were treated with 10 ng/mL trypsin (Sigma);

2. incubated for 10 min at 30°C;
3. incubated with 15 ng/mL of soybean trypsin inhibitor (Sigma) to terminate trypsin activity;
4. incubated with 50 mM Tris-HCl, 4 mM magnesium acetate, 0.3 mg/mL phosphatidylserine, 16 mM N-acetylglucosamine (Sigma), 1 mM UDP-N-acetyl[¹⁴C]glucosamine (4×10^5 cpm/ μ mol) (Amersham) in 75- μ L final volume.

After the reactions were terminated by addition of 200 μ L 10% TCA, insoluble material was collected on glass-fiber filter disks (Whatman), washed with 10% TCA, and radioactivity was measured in a liquid scintillation counter (Beckman Coulter LS6500).

Purification of WdChs5p from Cell Membranes

Cell membranes were isolated from shaken YPDB yeast of *W. dermatitidis*. Harvested cells (10–15 g wet weight) were disrupted at 4°C with 0.5 mm glass beads in a Bead Beater (Biospec Products) in the presence of lysis (CW) buffer (0.5% EDTA, 1mM PMSF, protease inhibitor cocktail (Sigma) for use with fungal and yeast extracts). After cell wall particulate matter was removed by centrifugation at $500 \times g$ for 10 min, the resulting supernatant fluid was collected and centrifuged at $50,000 \times g$ for 45 min. The membrane pellet was then homogenized three times in DIG buffer (0.5% digitonin, 50 mM Tris-HCl pH 7.5, 10% glycerol, 5 mM magnesium acetate) using a glass homogenizer (Pyrex), after which the resulting extract was clarified by centrifugation at $100,000 \times g$ for 45 min to provide solubilized membrane-proteins for use in immunoaffinity chromatography or for analysis.

Immunoaffinity beads were prepared by coupling about 150 mg of polyclonal anti-Myo12p antibody with 1.5 mL protein A-sepharose CL-4B beads (Amersham) with continuous overnight agitation by rotation. After collecting the coupled beads by centrifugation at $500 \times g$ for 10 min and washing twice with PBS buffer (20 mM Na₂HPO₄, pH 7.2, 150 mM NaCl) and once with 0.2 M borate-NaOH (pH 8.6), the antibody-bead complex was cross-linked by incubation with 20 mM dimethyl pimelimidate-HCl in 0.2 M triethanolamine pH 8.3 for 30 min. Once the reaction was terminated with 0.2 M ethanolamine-HCl (pH 8.2) for 1 h, the beads were loaded into a glass column, washed with PBS buffer, and finally equilibrated with IC buffer (0.1% digitonin, 20 mM Na₂HPO₄, pH 7.2, 100 mM NaCl, 1 mM PMSF, protease inhibitor cocktail) in a cold room. Prior to the column loading, the membrane protein extracts were dialyzed with IC buffer and then clarified by high speed centrifugation to remove detergent precipitates. After the loading, the proteins were bound to the anti-Myo12p antibody by continuous gentle mixing overnight at 4°C. The column was then gravity-washed, first with PBS buffer, next with IC buffer, after which the bound proteins were eluted with 50 mM sodium phosphate, pH 12. The eluate was immediately neutralized with cold 1 M Tris-HCl (pH 6.0) in the volume ratio of 1 to 2, respectively, and finally dialyzed in IC buffer.

RESULTS AND DISCUSSION

Specificity of the Anti-Myo12p Antibody

The 12 kDa Myo12p epitope of WdChs5p, which is located in the MMD, represents a conserved and unique polypeptide site for this class V chitin synthase. A computational analysis of the WdMyo12p sequence showed high homology with the MMD regions of other class V chitin synthases of filamentous fungi (62–70% identity) and very low homology to myosin proteins themselves, suggesting its potential for high specificity (data not shown). The reactivity of the antiserum analyzed by Western blotting of membrane proteins of *W. dermatitidis* showed that the purified antiserum reacted most strongly with a single protein band corresponding to the size estimated for WdChs5p (about 210 kDa)

among the membrane proteins of the wild type shifted from 25°C to 37°C (Figure 1). As expected, a weaker signal for this differentially expressed chitin synthase was detected among the membrane proteins of cells incubated at 25°C and no reactivity was observed among the membrane proteins of the *wdchsΔ511* deletion mutant, consistent with previous findings.^[13,14] These results confirmed the high specificity of anti-Myo12p antibody for the WdChs5p enzyme incorporated in the membranes of *W. dermatitidis*.

Immunoaffinity Purification of WdChs5p

The goal of this research was to solubilize and purify an enzymatically active form of WdChs5p using the polyclonal anti-Myo12p antibody as a tool in an immunoaffinity purification step. Because previous comparisons of relative chitin synthase activities in several chitin synthase mutants revealed that the least total activity is associated with the membranes of a double *wdchs2Δwdchs3Δ* mutant (defective in both class I and class III Chsps), and that the most activity appeared to be associated with WdChs2p (class I) in membranes of cells grown at 25°C or 37°C and with WdChs3p (class III) in membranes of cells grown at 37°C,^[16] we hypothesized that this strain, which has the wild-type *WdCHS5* gene and grows well at both 25°C and 37°C, might be the best of the strains available for purifying WdChs5p. Therefore, membranes of the *wdchs2Δwdchs3Δ* mutant and the wild-type strain cultured at 37°C for 2 days were isolated, solubilized with digitonin buffer, and the resulting solubilized proteins subjected to immunoaffinity chromatography, prior to Western blotting analysis and enzyme assay of eluted proteins. The Western blotting showed that significant degradation of WdChs5p occurred during purification, as suggested by the considerable loss of the 210 kDa protein and the appearance of proteins having sizes around of 120 and 90 kDa (Figures 2A and B). Attempts to protect WdChs5p against this degradation and an associated loss of activity by using protease inhibitors, various pH conditions, or another elution buffer (0.1 M glycine-HCl pH 2.5) were unsuccessful (data not shown). The breakdown of WdChs5p also continued during storage of preparations at -20°C or -70°C, although, generally, the 120-kDa band could be detected at all times. We suggest that the shortened WdChs5p was rendered devoid of its CSCD (Figures 2B and C). This speculation is based on results from combinations of Chsp activity assays and Western blot analyses that showed protein extracts with 120 and 90 kDa bands detected by anti-Myo12p had no Chsp activity, in contrast to protein fractions that had the 210 kDa sized band. Our attempts to purify WdChs5p from cells shifted from 25°C to 37°C and incubated in elevated temperature for 3 and 5 days produced a similar result, suggestive of temporal protein degradation (data not shown). Taken together, these data suggest that WdChs5p is very sensitive to degradation. The sizes of the shorter proteins and the complete lack of any associated Chsp activity also indicate that the cleavage had taken place somewhere in the middle of the protein's structure (Figure 3C). However, it is important to note that a classic degradation resulting in a protein smear was not observed (Figure 3B), indicating that one or more specific cleavage points may exist in a weak polypeptide sequence, possibly resulting from a loss of tertiary protein structure. Similar results were observed for the chitin synthase class V of *A. nidulans* (CsmA), which shows sensitivity for cleavage in a polypeptide region localized between its myosin-motor like and catalytic domains.^[18] Although the nature of instability of the WdChs5p remains unknown, it is not unusual for bioactive materials to be sensitive to degradation and loss of activity in aqueous solutions, particularly when materials often require refrigeration or have short shelf lives under ambient conditions. Furthermore, many membrane proteins have only limited solubility in aqueous solutions. Even when soluble at high concentrations, membrane proteins are prone to aggregation and precipitation. Interestingly, after purification, WdChs5p had more proteolytic stability in extracts from the double mutant than from the wild type strain (Figure 2A). Based on this observation, we speculate that WdChs1p (class II) and WdChs4p (class IV), or possibly also

newly discovered WdChs6p (class VI) and WdChs7p (class VII) may provide protection to WdChs5p structure in cell membranes at standard culture temperatures.

Among all preparations, a chitin synthase activity was detected only in eluted fractions obtained after the immunoaffinity purification of WdChs5p from the *wdchs2Δwdchs3Δ* mutant (Table 1). Starting with 15 g wet mass of cells, which provided 910 mg membrane protein material, a 9.0-fold purification of WdChs5p activity (0.18 mg protein with 0.18% recovery of total chitin synthase activity) was estimated. Trypsin titration showed that the protein required protease treatment to give rise to enzyme activity (Figure 3) which means that, even after solubilization and affinity purification, WdChs5p retained its zymogenic character. Nonetheless, the optimum protease concentration in the purified protein sample, compared to that of the solubilized crude membrane extract, was about 1,000 times lower,^[15,16] thus demonstrating the high proteolytic sensitivity of purified protein. Although the final total detected activity is low, it is not unreasonable. For example, a similar attempt with the *S. cerevisiae* enzymes was only about 20-fold higher, which might not be unexpected because it has only three chitin synthases instead of the seven found in *W. dermatitidis*.^[19] Furthermore, it is most likely that, in the yeast study, the main purified activity was that of Chs1, the predominant activity associated with its membrane: that activity is thought to be 20-fold higher than the other two Chs2 and Chs3 enzymes combined.^[10] In contrast, WdChs5p is thought to provide only a relatively minor amount of the total activity associated with its seven known chitin synthases, with the majority being contributed, as in *S. cerevisiae*, by its class I enzyme which, at 37°C, is augmented significantly by the elevated expression of its class III isozyme, WdChs3p, and to a lesser extent by its class V isozyme WdChs5p.^[15]

More detailed characterization of the properties of the WdChs5p will require additional purification of significantly greater amounts of enzyme. Nonetheless, our findings represent an important first step toward assessing the potential of WdChs5p and the other class V chitin synthases to be useful targets for antifungal drug development.

Acknowledgments

We thank Tuan Vo for the valuable technical assistance he provided during the course of this work. This research was supported by a grant to P. J. S. from the National Institute of Allergy and Infectious Diseases (AI33049).

ABBREVIATIONS

CS	chitin synthase
CSCD	chitin synthase catalytic domain
DIG	digitonin
EDTA	ethylene diamine tetraacetic acid
GlcNAc	β-1,4-linked <i>N</i> -acetylglucosamine
IgG	Immunoglobulin G
MMD	myosin motor-like domain
NBT/BCIP	Nitro-Blue Tetrazolium Chloride/5-Bromo-4-Chloro-3'-Indolylphosphate p-Toluidine Salt
NC	nitrocellulose membrane
PMSF	phenylmethanesulphonylfluoride

SDS/PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TCA	trichloro acetic acid
UDP-GlcNAc	uridine diphosphoglucose <i>N</i> -acetylglucosamine
WdChs5p	chitin synthase class V of <i>Wangiella dermatitidis</i>
YPDB	yeast peptone dextrose broth

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Figure 1. Western blots of membrane proteins (50 μ g) isolated from the wild-type strain cultured at 25°C (wt25) and at 37°C (wt37) and the mutant strain *wdchs5Δ11* cultured at 25°C (Δ 5). WdChs5p was detected by anti-Myo12p visualized by chemiluminescence detection.

Figure 2.

Western analysis of WdChs5p after purification by immunoaffinity chromatography and temporal degradation of the protein during storage. Panels A and B: Chromatographically purified protein samples analyzed by Western blotting using the anti-Myo12p antibody as a primary antibody and goat anti-rabbit IgG conjugated with alkaline phosphatase as a secondary antibody and developed by reaction with NBT/BCIP. (wt37) - purified protein isolated from membranes of wild type cultured at 37°C; ($\Delta 2\Delta 3$) - purified protein isolated from membranes of the *wdchs2\Delta wdchs3\Delta* mutant strain cultured at 37°C; T0 protein sample analyzed immediately after chromatography; T1 and T2, protein samples analyzed after 1 and 2 days of freezer storage (-70°C), respectively. Detectable (+) or undetectable (–) chitin

synthase activities in the individual protein samples are indicated at the bottoms of each lane. C. Graphic scheme of putative truncated forms of WdChs5p after chromatographic purification. The myosin-motor like domain (MMD) is indicated by the gray box; the catalytic domain (CSCD) by the black box; the shaded box in the MMD represents the 12-kDa WdMyo12p region recognized by anti-Myo12p.

Figure 3.

Trypsin titration assay of the purified WdChs5p protein after immunochromatography. The enzyme assay results include the background result. % activity is the ratio between the best of the Chs activities obtained for a trypsin concentration and the other calculated results.

Table 1

Purification of WdChs5p from the *W. dermatitidis* *wdcHs2ΔwdcHs3Δ* mutant strain grown at 37°C.

Samples	vol (ml)	total CPM	total activity(a) (units [*])	total protein (mg)	specific activity (U/mg) μ M/min/mg	purification fold	yield %
Crude extract	45	2520000	0.367454	910	0.0004037	1.0	100
0.5% digitonin- solubilized membranes	35	595000	0.086762	105	0.0008263	2.0	23.6
Immunoaffinity Chromatography	1.8	4500	0.0006561	0.18	0.0036450	9.0	0.18

* Units are defined as μ mol of ¹⁴C-labeled UDP-GlcNAc incorporated in 1 min into insoluble chitin product.

Data represent the means of at least three independent reactions. Standard error of CPM results $\pm$ 5%. The CPM results represent the activity minus background from assays without protein in reaction mixtures. In all steps, the chitin synthase activity was measured after activation with optimal amounts of trypsin.