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Insights on the antifungal activity of amphiphilic derivatives of diethylaminoethyl chitosan against *Aspergillus flavus*

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ABSTRACT

In this study, the antifungal activity of chitosan derivatives against *A. flavus* was studied to understand the contribution of the molecular mass (Mw) and of the hydrophobic and electrostatic forces to the inhibition of fungal growth. The interaction of amphiphilics ranging from 8 to 130 kDa with model membranes of zwitterionic L-α-phosphatidylcholine (PC) and anionic L-α-phosphatidylcholine/L-α-phosphatidyl-DL-glycerol (PC:PG, 80:20 mol%) were exploited to obtain information on the inhibition mechanism. The results indicated that concurrent interactions control the antifungal activity. The decrease in the Mw weakens the self-association favoring the electrostatic and hydrophobic associations with the cell wall and anionic lipids of the lipid bilayer, indicating an increasing association of the amphiphilics with the fungal membrane. Laser confocal scanning microscopy of rhodamine labeled-derivatives and transmission electronic microscopy techniques showed that the amphiphilics affect the cell wall integrity by inducing the aggregation of hydrophobic constituents of the conidia.

1. Introduction

Aspergillus flavus has been considered a threat to human health due to its potential for causing invasive aspergillosis and infections, and due to its easy dispersion in the air leading to the contamination of agricultural crops, such as peanuts, maize and tree nuts, as well as oilseed crops (Hedayati, Pasqualotto, Warn, Bowyer, & Denning, 2007). The pathogenicity is mainly due to the production of carcinogenic aflatoxins, such as the aflatoxin B1, which is considered the most carcinogenic natural agent, capable of causing intoxication in bovine adults, domestic animals and implicated in hepatocellular carcinoma in humans (Chang & Ehrlich, 2010; Park & Troxell, 2002).

In the worldwide market of commercial pesticides the use of chemical fungicides corresponds to 17.5% (De, Borse, Kumar, & Mozundar, 2014). The mechanisms of action are varied, involving, for instance, the inhibition of proteins or the deregulation of ergosterol synthesis that consequently affects cell membranes. Moreover, to ensure greater efficacy, the use of a mix of active ingredients is common. The use of these chemical fungicides has caused great concern since they are classified as systemic and cumulative contaminants that are also potentially carcinogenic and teratogenic (Fakruddin, Chowdhury, Hossain, & Ahmed, 2015). Because of their indiscriminate use, increasingly resistant strains have been identified (Mortensen et al., 2010). Therefore, fungicides based on natural products (Bonilla & Sobral, 2016), essential oils (Nogueira et al., 2010) and plant defensins (Lacerda, Vasconcelos, Pelegrini, & de Sa, 2014) are very interesting options and the search for nontoxic compounds has recently driven the research in this field (Alkan & Yemenicioglu, 2016).

In this regard, chitosan has emerged as a promising antimicrobial agent, whose activity has been mainly attributed to the electrostatic interaction between its polycationic chains and the cell wall and cell membranes of the microorganisms (Kong, Chen, Xing, & Park, 2010; Sahariah & Másson, 2017). However, the efficiency of chitosan as an antimicrobial agent has been shown to depend not only on the target

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microorganism but also on the intrinsic characteristics of the polysaccharide such as charge density, molecular weight (Mw), concentration, amphiphilicity and other physicochemical parameters like ionic strength, pH and temperature. Chitosan exhibits higher antimicrobial activity at low pH due to the protonation of its amino groups (Kong et al., 2010). The importance of positive charge density is supported by studies that used quaternized derivatives, which exhibited higher antimicrobial activity against bacteria (Ignatova, Starbova, Markova, Manolovaa, & Rashkov, 2006) and fungi (Pedro et al., 2013; Sajomsang, Gonil, Saesoo, & Ovatlarnporn, 2012; Souza et al., 2013) than plain chitosan. Besides the positive charge density, molecular weight and the hydrophilic/hydrophobic balance may also play an important role in the antimicrobial properties. The modification of the chitosan backbone through the grafting of alkyl chains (Rúnarsson et al., 2010) and aromatic groups (Tamera et al., 2017) has been shown to improve the antifungal and antibacterial activities of chitosan. It suggests that this improvement may result from the interaction of the amphiphilic chain with cell membrane phospholipids (Huang, Du, Zheng, Liu, & Fan, 2004; Rúnarsson et al., 2010). The antimicrobial activity of chitosan depends either on the type of microorganism or on molecular weight (Li, Chen, Liu, Zhang, & Tang, 2011). For instance, chitosans of low Mw showed higher antimicrobial activity against *Candida species* (Kulikov et al., 2014; Seyfarth et al., 2008) than those of high Mw (Tayel et al., 2010). Nevertheless, a fine-tuning on Mw is needed to achieve more effective molecules (Kulikov et al., 2014).

The Mw effect and the insertion of hydrophobic groups in the chitosan backbone are currently under investigation. However, further efforts in the investigation of the characteristics of the mechanism of action against fungi are required. Among them, the interactions with the cell wall components, the permeabilization of the fungal plasma membrane (Palma-Guerrero et al., 2010) and also the results of interactions of chitosan with cytoplasmic constituents (Goy & Assis, 2009). In particular, the mechanism by which amphiphilic chitosans exert their activity is not completely understood and investigations in this regard are pivotal for the preparation of effective antifungal agents.

Recently it has been demonstrated that amphiphilic derivatives of diethylaminoethyl chitosans have a significant antifungal activity against *A. flavus* and *A. parasiticus*. In general, the results showed that the antifungal activity was strongly dependent on Mw, hydrophobic groups content and polymer concentration (Gabriel, Tiera, & Tiera, 2015; Souza et al., 2013). The findings indicated that amphiphilic chitosans are more effective if they have either high Mw and lower hydrophobicity or low Mw and higher hydrophobicity.

In this current work, the main purpose was to gain insights into the mechanism of the antifungal activity of amphiphilic derivatives of chitosan against *A. flavus*, which could be related to the cell wall and cell membrane impairment. By varying the Mw, the role of hydrophobic interactions on the antifungal activity could be better clarified. A series of derivatives of varied Mw having fixed contents of diethylaminoethyl (DEAE) and hydrophobic groups were synthesized and characterized. The antifungal activities of these derivatives were tested against *A. flavus* and their interaction with model membranes made of phosphatidylcholine (PC) and phosphatidylcholine-phosphatidylglycerol (PC:PG) was studied (Lohner & Prenner, 1999; Palma-Guerrero et al., 2010). Additionally, laser scanning confocal microscopy and transmission electron microscopy were employed to evaluate structural modifications in the cell wall.

2. Experimental part

2.1. Materials

Commercial chitosan (CHC) with a degree of deacetylation (DD) of 85% (Polymar, Fortaleza, Brazil) was deacetylated to generate a highly deacetylated sample with DD of 97%. Sodium acetate, acetic acid, and sodium hydroxide were purchased from Synth (Diadema, Brazil). 2-Chloro-*N*,*N*-diethylethylamine hydrochloride (DEAE), deuterium chloride (35%) in deuterium oxide, deuterium oxide, 5,6-carboxyfluorescein (CF), egg L-α-phosphatidylcholine (PC) and egg L-α-phosphatidylglycerol sodium salt (PG) were purchased from Sigma-Aldrich Chemical Co. (São Paulo, Brazil). Potato dextrose agar (PDA) was purchased from Acumedia Manufacturers, Inc. (Lansing, USA). Water was deionized using a Gehaka water purification system. Spectra/Pore membranes (Spectrum) were employed for dialysis. All solvents were of reagent grade and used as received.

2.2. Synthesis of the amphiphilic derivatives of diethylaminoethyl chitosan of varied molecular weight (DEAE-CH_{Dod})

The amphiphilic derivatives were synthesized in a two-step process using the highly deacetylated chitosan (CH, DD 97%) as starting material. First, CH was modified with 2-Chloro-*N*,*N*-diethylethylamine hydrochloride (DEAE) at pH 8.0, as previously described (Gabriel et al., 2015), to generate a derivative with a degree of substitution of about 40%. This first derivative was subsequently degraded by sodium nitrite in acetic acid solution (Huang, Khor, & Lim, 2004; Tømmeraas, Vårum, Christensen, & Smidsrød, 2001). To obtain DEAE-CH derivatives with varied molecular weights (Mw), the molar ratio of $NaNO_2$/glucosamine was varied from 0.02 to 0.38 (Table 1). Next, the samples of varied Mw were recovered by lyophilization and subjected to alkylation with dodecyl aldehyde followed by reduction with sodium borohydride (Desbrieres, Martinez, & Rinaudo, 1996; Souza et al., 2013) to reach a degree of substitution of 0.2 (Fig. 1a).

2.3. Characterization of the amphiphilic derivatives

The DEAE-CH derivatives were characterized by proton nuclear magnetic resonance (^{1}H NMR) and gel permeation chromatography

Table 1
Reaction conditions and physico-chemical properties of chitosan and its amphiphilic derivatives.

Polymer	[a]MR Dodecyl/NH_2	[b]MR DEAE/NH_2	$^+DS_{DEAE}$ (%)	$^{++}DS_{Dod}$ (%)	[c]MR_D $NaNO_2$/CH	Mw	Mw/Mn	CAC (g L^{-1})	$DS_{RHODAMINE}$ (%)
$CH_{130(DD^a = 97\%)}$	–	–	–	–	–	122.4	2.93	–	–
DEAE-CH	–	0.5	40	–	–	136.5	2.97	0.069	0.85
DEAE-CH_{136}-Dod	0.22	0.5	40	19.3	–	136.5	2.97	0.004	0.52
DEAE-CH_{116}-Dod	0.22	0.5	40	19.3	0.02	116.1	2.81	0.006	0.82
DEAE-CH_{70}-Dod	0.22	0.5	40	18.7	0.09	69.0	2.54	0.009	0.78
DEAE-CH_{25}-Dod	0.22	0.5	40	18.3	0.21	25.3	2.92	0.013	0.79
DEAE-CH_8-Dod	0.22	0.5	40	20.3	0.38	8.7	3.08	0.021	2.67

DS_{DEAE} = Degree of substitution by DEAE groups; $++DS_{Dod}$ = Degree of substitution by dodecyl groups; Mw = Molecular weight (kDa); Mw/Mn = Polydispersity; CAC = Critical Aggregation Concentrations (g L^{-1}).

[a] DD = Degree of deacetylation.

[b] MR = Molar ratios of DEAE and dodecyl aldehydes employed for the synthesis.

[c] MR_D = molar ratios of sodium nitrite employed for degradation.