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Antifungal effect of high- and low-molecular-weight chitosan hydrochloride, carboxymethyl chitosan, chitosan oligosaccharide and *N*-acetyl-D-glucosamine against *Candida albicans*, *Candida krusei* and *Candida glabrata*

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Abstract

Objectives: Generally, chitosan is a water-insoluble polyaminosaccharide with antimicrobial activity. The antifungal activity of water-soluble low- and high-molecular-weight chitosan hydrochloride, carboxymethyl chitosan, chitosan oligosaccharide and *N*-acetyl-D-glucosamine against *Candida albicans*, *Candida krusei* and *Candida glabrata* was investigated.

Methods: Solutions of the tested substances in different concentrations (1, 0.5, 0.25, 0.1, 0.05, 0.025, 0.01, 0.005, and 0.0025%) were prepared and the influence on *C. albicans* DSM 11225, *C. krusei* ATCC 6258 and *C. glabrata* DSM 11226 was investigated. Yeasts (3×10^5 cells/mL) were incubated with Sabouraud liquid medium at 30 °C. Measurements were done with a microplate nephelometer (NEPHELOstar Galaxy, BMG LABTECH Ltd.) for 24 h. High values of light scattering correlate with strong cultural growth. Results were shown as growth curves and histograms displaying 24 h end points. These were compared with control by Mann–Whitney test. Furthermore, $MIC_{50\%}$, $MIC_{80\%}$ and Spearman correlation coefficients were calculated.

Results: *C. albicans* and *C. krusei* were the most sensitive species. *C. glabrata* was also inhibited, whereas 1% of tested substances could not prevent its growth completely. However, only both chitosan hydrochlorides showed a definite antifungal effect with high correlation between inhibition and test concentration. Carboxymethyl chitosan, chitosan oligosaccharide and *N*-acetyl-D-glucosamine showed only a weak or no antifungal activity, respectively.

Conclusions: Antifungal activity decreases with declining molecular mass (chitosan oligosaccharide and *N*-acetyl-D-glucosamine) and increasing masking of the protonated amino groups with functional groups (carboxymethyl chitosan).

Keywords: Chitosan; Polycationic; Water soluble; Candida; Antifungal

1. Introduction

Candida albicans is able to cause infections of the human skin, the mucosa and the viscera (Seebacher and Renate, 1990). Other clinical relevant *Candida* species are *Candida dubliensis*, *Candida tropicalis*, *Candida parapsilosis* and the less virulent yeasts *Candida glabrata*, *Candida krusei* and *Candida lusitaniae* (Moran et al., 2002). *C. krusei* causes nosokomial infections and is to be found in 2–4% of all candidaemia (Pfaller et al., 1998; Samaranayake and Samaranayake, 1994). Affected patients are mostly suffering from haematological neoplasm with neutropenia as underlying disease (Abbas et al., 2000; Pfaller, 1996). *C. krusei* is generally fluconazole-resistant (Hof, 2003).

C. glabrata is the most common non-*C. albicans* yeast in oral canidasis of AIDS patients (Maenza et al., 1997; Sangeorzan et al., 1994; Schoofs et al., 1998) and the most frequent fungus in *Candida* septicaemia and candiduria in the USA (Occhipinti et al., 1994; Pfaller et al., 1999). A rapid development of resistance against fluconazole and itraconazole has been reported (Barchiesi et al., 2001; Price et al., 1994). Next to nosokomial infections *C. glabrata* is the agent of a persistent form of vaginal candidosis (Mendling, 2005).

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There is an increasing incidence of candidosis since the 1970s because of frequent use of plastic permanent catheters, antibiotics and immune-suppressive drugs or the appearance of AIDS (Hof, 2003). This requires a lot of systemic and topical therapeutic approaches as well as investigation of new antifungal substances like the poly-cationic antimycotic peptide dermaseptine from amphibian skin (Coote et al., 1998). The polysaccharide chitosan is also a poly-cationic substance with antifungal properties against yeasts, moulds and dermatophytes (Allan and Hadwiger, 1979; Gil et al., 2004; Guo et al., 2006; Muzzarelli et al., 2001; Rhoades and Roller, 2000; Roller and Covill, 1999; Zakrzewska et al., 2005).

Next to antifungal properties, chitosan shows a lot of other medical interesting characteristics. In particular, antibacterial effects (Chung et al., 2004; Helander et al., 2001; Liu et al., 2004; Liu et al., 2001; Vishu Kumar et al., 2005), enhancement of granulation and epithelisation (Shi et al., 2006) and activation of fibroblasts in wounds (Minami et al., 1999) have to be pointed out. Thus, chitosan is used as component of wound dressings like hydrocolloid patches with good wound healing properties (Banks et al., 1999; Baxter, 2000; Niekraszewicz, 2005; Williams, 1996; Williams, 2000). Next to wound therapy, chitosan may be used as supporting medium for stem cells for tissue engineering (Shi et al., 2006).

Chitosan is produced via deacetylation of chitin, which is won from shells of krill or other crustacean. It consists of 2-acet-amido-2-desoxy-β-D-glucopyranose and de-acetylated 2-amino-2-desoxy-β-D-glucopyranose monomers, whereas the amount of deacetylated monomers exceeds the acetylated ones. The chemical, physical and biological properties of chitosan depend on degree of deacetylation and molecular weight. Commercial chitosan is characterized by a degree of deacetylation between 70 and 95% and a molecular weight between 50,000 and 2,000,000 Da. Because of the free nucleophilic duplet of the amine group and the low amount of hydrogen bonds, chitosan is soluble in nearly all aqueous acids. This solubility is linked to a pH 2–6.5 (Krohn, 2003; Shi et al., 2006; Tharanathan and Kittur, 2003). In contrast, low degrees of deacetylation allow solubility in neutral and alkaline media (Krohn, 2003). To produce water-soluble chitosan hydrochloride, the substance is dissolved in hydrochloric acid and boiled up. After cooling, the water-soluble chitosan hydrochloride precipitates (Finger, 2000). Other methods are the homogeneous phase reaction (Sannan et al., 1976), the integration of hydrophilic groups like carboxymethyl groups in carboxymethyl chitosan, the reduction of molecular weight and the reaction between amine groups of chitosan and monosaccharides (Maillard reaction) (Chung et al., 2004; Finger, 2000; Jia et al., 2001; Kotze et al., 1998).

Chitosans with a molecular weight less than 50 kDa are subsumed as water-soluble chitosan oligosaccharides (No et al., 2002). These oligosaccharides are produced via chemical scission (acid and alkaline hydrolyis), physical scission (high temperature) or enzymatic scission (chitosanase, chitinase, chitobiase, lysozyme, cellulase, pectinase, pepsine, papaine, lipase, pronase) (Krohn, 2003; Tharanathan and Kittur, 2003; Vishu Kumar et al., 2005).

There is a wide incidence for antimycotic activity of water-soluble and insoluble chitosans against yeasts and moulds mainly affecting agriculture (Allan and Hadwiger, 1979; Gil et al., 2004; Guo et al., 2006; Muzzarelli et al., 2001; Rhoades and Roller, 2000; Roller and Covill, 1999; Zakrzewska et al., 2005). In contrast, only few reports discuss the effect on medical important yeasts like *C. albicans* or *C. glabrata* (Allan and Hadwiger, 1979; Badawy et al., 2004; Eweis et al., 2006; Gil et al., 2004; Guo et al., 2006; Kendra and Hadwiger, 1984; Muzzarelli et al., 1990; Muzzarelli et al., 2001; Peng et al., 2005; Rhoades and Roller, 2000; Roller and Covill, 1999; Zakrzewska et al., 2005).

There are several methods for testing the efficacy of possible antifungal substances. Microbial growth can be described by growth curves, which display the different phases of increase of a yeast population (lag-Phase, log-Phase, stationary phase). Next to counting cells under the microscope, measurement of culture medium opacity displays an excellent parameter for monitoring microbial growth, because it can easily automated using photometric or nephelometric instruments. While photometry measures the decrease in light intensity through a medium, nephelometry uses light scattering as parameter. A high amount of yeast cells in the medium generates a high value of scattered light and, thus, would be a sign for a lack of antifungal efficacy of the tested drug in this case (Hipler et al., 2003).

The presented work compares the antifungal activity of water-soluble high molecular and low molecular weight chitosan hydrochloride, carboxymethyl chitosan, chitosan oligosaccharide and the acetylated monomer *N*-acetyl-D-glucosamin. Inhibition is demonstrated by nephelometric measurements in acidic medium, according to other works investigating antimicrobial efficiency of chitosan (Gil et al., 2004; Roller and Covill, 1999). After 24 h incubation, yeast cells have been examined microscopically after dyeing with fluorescence stain Fun®-1. Affected cells show a bright green to yellow homogeneous fluorescence, whereas vital cells display intracellular vacuols with movable red-coloured cylindrical intravacuolar structures (CIVS) (Millard et al., 1997).

2. Materials and methods

2.1. Incubation of yeasts

Yeasts (*C. albicans* DSM 11225, *C. krusei* ATCC 6258, *C. glabrata* DSM 11226) were inoculated on Sabouraud glucose agar plates (with chloramphenicole and gentamycine, SGC-agar plates from Biomeriéux, Marcy-l'Etoile) and incubated for 24 h at 30 °C. An inoculation loop with cultural material of each yeast was brought into Sabouraud glucose bouillon and shaken for 24 h with 350 rpm (KM-2 Akku, Edmund Bühler, Hechingen). From this suspension, a probe with 6–8 × 10^5cells/mL was produced using an automatic cell counter (Casy 1 TT, Schärfe-System, Reutlingen). From this probe, 100 μL were put into the wells of a 96-well microplate (PS Microplatte 96 wells non-sterile, Fa. Greiner bio-one, Frickenhausen) and mixed with the respective testing substance. Thus, the final inoculum size was 3 × 10^5 cells/mL.

Table 1
Survey on properties of the investigated chitosans

	Chitosan-hydrochloride (low-molecular weight)	Chitosan-hydrochloride (high-molecular weight)	Carboxymethyl-chitosan
Viscosity (mPas)	5	100–150	60
Degree of de-acetylation (%)	84.9	85	83.5

2.2. Tested chitosans

We tested the antifungal activity of low molecular weight chitosan hydrochloride (Chitosan-HCl, Heppe, Queis) and high molecular weight chitosan hydrochloride (Chitosan Flake 1130, ChiPro, Bremen, 120 kDa), carboxymethyl chitosan (Heppe, Queis), chitosan oligosaccharide (Chitosan oligosaccharide lactate, SIGMA–Aldrich, St. Louis/Steinheim) and *N*-acetyl-D-glucosamine (monomer of chitin, SIGMA, St. Louis). Table 1 gives a survey about chemical properties of the tested chitosans.

The tested substances were diluted with 0.01 M PBS (SIGMA, St. Louis) into different concentrations. 100 μL of these solutions were mixed with the 100 μL fungus suspension in the wells of the microplate, which resulted in the final test concentrations of 1, 0.5, 0.25, 0.1, 0.05, 0.025, 0.01, 0.005 and 0.0025%. The filled microplates were covered with Breathe-easy™ foil (Diversified Biotech, Boston). 1% carboxymethyl chitosan solution could only be pipetted with extreme difficulties due to high viscosity; thus, results for this concentration were not included in the evaluation.

2.3. Nephelometry

Microplates were put into the nephelometer (NEPHELOstar Galaxy, BMG, Offenburg) and incubated for 24 h at 30 °C. During incubation, microplates were shaken in the apparat (except during measurement). The used nephelometer posses a 635-nm laser as radiating source with a laser beam focus between 1.5 and 3.5 mm. Measurements were done every hour and put into growth curves. Each well was measured for 0.1 s with a laser beam focus of 2.5 mm. Subsequently, the content of wells with 1–0.1% chitosan was inoculated on Sabouraud glucose agar plates and colonies were counted after 24 h. The absence of colonies was taken as sign for total inhibition and maximal antifungal effect. This test was only done, when a strong antifungal effect was observed in the growth curve.

2.4. FUN®-1 staining

After incubation of the *Candida* species in microplates, the fluid was removed from each well and the remaining biofilm was re-suspended in 50 μL GH solution: HEPES buffer (SERVA, Heidelberg) + NaOH (Carl Roth, Karlsruhe) + D(+)-glucose (SIGMA, St. Louis). Ten microliter of this suspension were mixed with 10 μL FUN®-1 and incubated at 30 °C in darkness. For fluorescence microscopy (Olympus BX 40 F-3; excitation 470–490 nm, emission 520 nm) 5 μL of this solution were transferred onto the object slide. Pictures of the stained yeast cells were taken at 100-fold magnification and digitalized (digital camera: Camedia C-5050 Zoom, Olympus; software: analysis, Soft Imaging System GmbH).

2.5. Statistics

Measurements were repeated on average 16 times. SPSS (version 13) was used for statistical analysis. Fungal growth is visualized via growth curves. For statistical evaluation relative nephelometry values after 24 h have been calculated as percent of the control growth, i.e. the growth after 24 h incubation without chitosan. The acquired value was termed "relative growth" of the culture. Results for relative growth and standard deviation after 24 h are given in histograms. Correlation between relative growth and concentration of chitosan was calculated with Spearman's correlation coefficient. The concentrations of chitosan, which led to an inhibition of 50% or 80% (Minimal Inhibition Concentrations $MIC_{50\%}$ and $MIC_{80\%}$) were calculated with SPSS probit analysis. The relative growth of control was compared with relative growth at each concentration of the tested substance with Mann–Whitney test.

3. Results

3.1. Incubation with low-molecular-weight chitosan hydrochloride

Low-molecular-weight chitosan hydrochloride displays a concentration dependent influence on fungal growth of *C. albicans*, *C. krusei* and *C. glabrata*. Control curves fitted typical growth curves with lag-phase, log-phase and stationary phase (see Fig. 1A). Upon incubation with low-molecular-weight chitosan hydrochloride, the time until stationary phase was reached prolongated and maxima of fungal growth decreased with chitosan concentration increase. After incubation with 1% of the tested substance, fungal growth was not detectable except *C. glabrata*, which displayed a slight growth in the course of 24 h.

Fig. 2 shows the relative growth of the yeasts after 24 h. With increasing concentrations, the relative growth declines. This context was proven by calculating Spearman's correlation coefficients of −0.789 (*C. albicans*), −0.543 (*C. krusei*) and −0.874 (*C. glabrata*). Thus, there is a high negative correlation between growth and chitosan concentration, except for *C. krusei*, which shows only a moderate correlation.

For further statistical analysis relative growth was compared with the relative growth of the control (=1) using Mann–Whitney test. All tested concentrations of low molecular weight chitosan hydrochloride inhibited growth of the tested *Candida* strains significantly ($p \leq 0.001$, see Fig. 2A).

Calculated $MIC_{50\%}$ and $MIC_{80\%}$ values are shown in Table 2. *C. krusei* possesses the lowest MICs and *C. albicans* the highest.

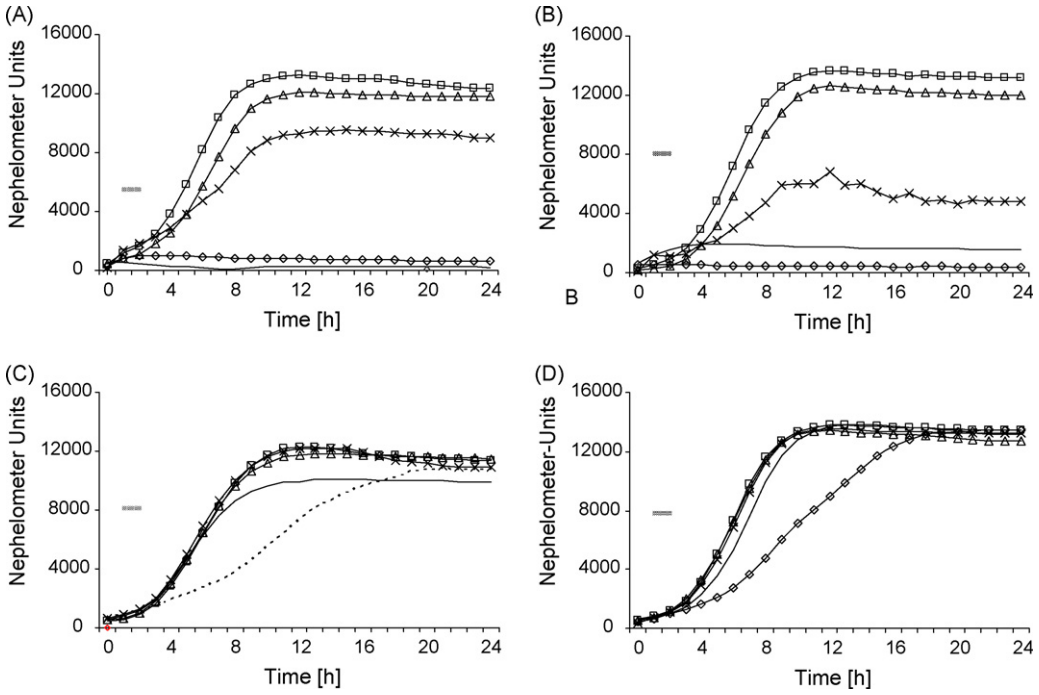

Fig. 1. Growth curves describing the effect of low-molecular-weight chitosan hydrochloride (A), high-molecular-weight chitosan hydrochloride (B), carboxymethyl chitosan (C) and chitosan oligosaccharide (D) on *Candida albicans*. Concentrations: (□) 0% = control; (△) 0.0025%; (×) 0.01%; (–) 0.1%; (◊) 1%. McFarland standard (McF 5) at the beginning of measurement is symbolized as hatched bar graph.

After incubation, the content of wells with 1–0.1% low molecular weight chitosan hydrochloride was inoculated on Sabouraud glucose agar plates. 24 h later, no colonies of *C. albicans* could be observed. Thus, these concentrations inhibited growth of *C. albicans* totally. In contrast, lower concentrations than 1% could not prevent colony formation of *C. krusei*. *C. glabrata* is not even deadened by 1% low molecular weight chitosan hydrochloride. Inoculation on Sabouraud glucose agar plates resulted in generation of an innumerable amount of colonies 24 h later.

3.2. Incubation with high-molecular-weight chitosan hydrochloride

The growth curves are very similar to these, which resulted from incubation with low molecular weight chitosan hydrochlo-

Table 2
Minimal inhibition concentrations (MICs) and Spearman correlation coefficients of the different test courses

		Spearman correlation coefficient	$MIC_{50\%}$ (%)	Confidence interval (%)		$MIC_{80\%}$ (%)	Confidence interval (%)	
				From	To		From	To
Low-molecular-weight chitosan hydrochloride	*Candida albicans*	−0.789	**0.02616**	0.00915	0.07246	**0.69732**	0.2481	2.12742
	Candida krusei	−0.543	**0.01313**	0.00248	0.06568	**0.35008**	0.07	1.85159
	Candida glabrata	−0.874	**0.0184**	0.00714	0.04557	**0.49045**	0.19548	1.32473
High-molecular-weight chitosan hydrochloride	*C. albicans*	−0.857	**0.00699**	0.00271	0.01701	**0.18638**	0.07682	0.47727
	C. krusei	−0.828	**0.01218**	0.00559	0.02539	**0.32469**	0.31962	1.6599
	C. glabrata	−0.919	**0.01815**	0.00876	0.03627	**0.48387**	0.23583	1.07299
Carboxymethyl chitosan	*C. albicans*	−0.644	n.c.	–	–	n.c.	–	–
	C. krusei	−0.418	n.c.	–	–	n.c.	–	–
	C. glabrata	−0.360	n.c.	–	–	n.c.	–	–
Chitosan oligosaccharide	*C. albicans*	−0.323	n.c.	–	–	n.c.	–	–
	C. krusei	−0.502	n.c.	–	–	n.c.	–	–
	C. glabrata	−0.639	n.c.	–	–	n.c.	–	–
N-acetyl-D-glucosamin	*C. albicans*	−0.120	n.c.	–	–	n.c.	–	–
	C. krusei	−0.180	n.c.	–	–	n.c.	–	–
	C. glabrata	+0.331	n.c.	–	–	n.c.	–	–

MIC values are marked bold; n.c.—not calculated.

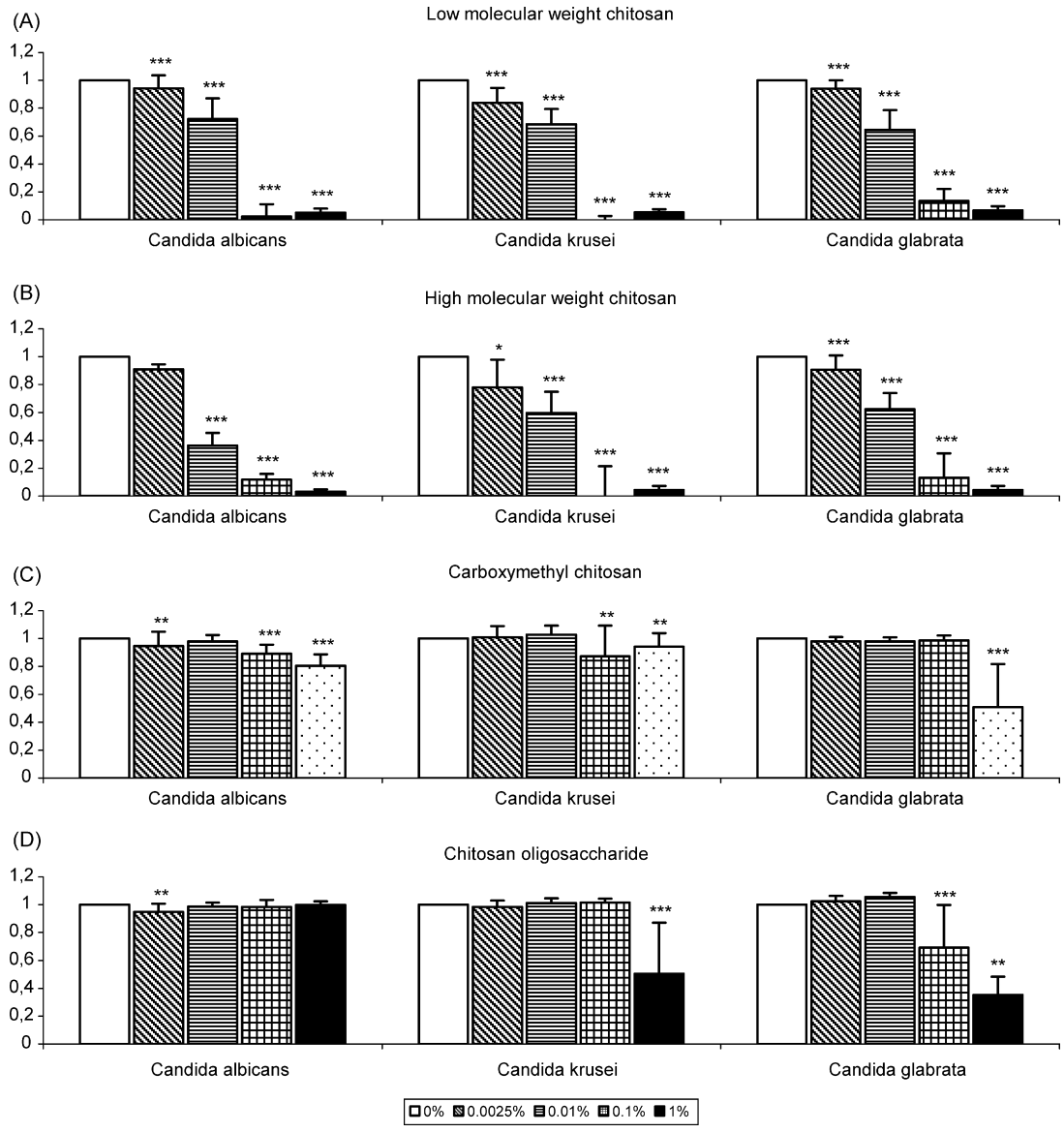

Fig. 2. Relative growth (*y*-axis) of *C. albicans*, *Candida krusei* and *Candida glabrata* based on nephelometry after 24 h incubation with low-molecular-weight chitosan hydrochloride (A), high-molecular-weight chitosan hydrochloride (B), carboxymethyl chitosan (C) and chitosan oligosaccharide (D). Significances of Mann–Whitney test: *$0.05 \geq p \geq 0.01$; **$0.01 \geq p \geq 0.001$; ***$p \leq 0.001$.

ride (see Fig. 1B). High concentrations prolonged growth and decreased growth maxima. This is reflected by a strong negative correlation between chitosan concentration and progeny of yeasts after 24 h (Spearman correlation coefficients: *C. albicans* −0.857, *C. krusei* −0.828, *C. glabrata* −0.919). The difference between relative growth and growth of control is statistical significant in almost all tested concentrations ($p < 0.001$, Mann–Whitney test, see Fig. 2B), which underlines at least a mean antifungal effect of the lowest tested doses. The highest $MIC_{80\%}$ and thus, the lowest sensitivity were found for *C. glabrata* (see Table 2). The most sensitive species with a lower $MIC_{80\%}$ was *C. albicans*. After incubating this yeast on Sabouraud glucose agar plates for 24 h, no colonies were detected, when the fungus had been incubated with 1 and 0.5% high-molecular-weight chitosan hydrochloride before. Similar results were seen in *C. krusei*. Thus, both yeasts are completely inhibited by 1 and 0.5% high-molecular-weight chitosan hydrochloride. In contrast, *C. glabrata* formed colonies even after incubation with 1% of the tested substance.

The photographs taken after fluorescence microscopy with Fun®-1 are shown in Fig. 3. Each *Candida* species possesses a typical lucid morphology. *C. krusei* (see Fig. 3D–F) is characterized by long drawn-out yeast cells. In contrast, *C. glabrata* (see Fig. 3G–I) presents small roundish cells. *C. albicans* displays larger roundish yeast cells (see Fig. 3A–C). The control (Fig. 3A, D and G) displays vital cells with red coloured, fast moving "cylindrical intravacuolar structures" (CIVS, diameter between 0.5 and 0.7 μm). After incubation with 1% test substance, only few cells (*C. albicans*—see Fig. 3C; *C. glabrata*—see Fig. 3I) or no cells (*C. krusei*—see Fig. 3F) are detectable. If apparent, they are characterized by a bright yellow-green fluorescence. This is a sign for avitality of the yeasts. The integrity of cell

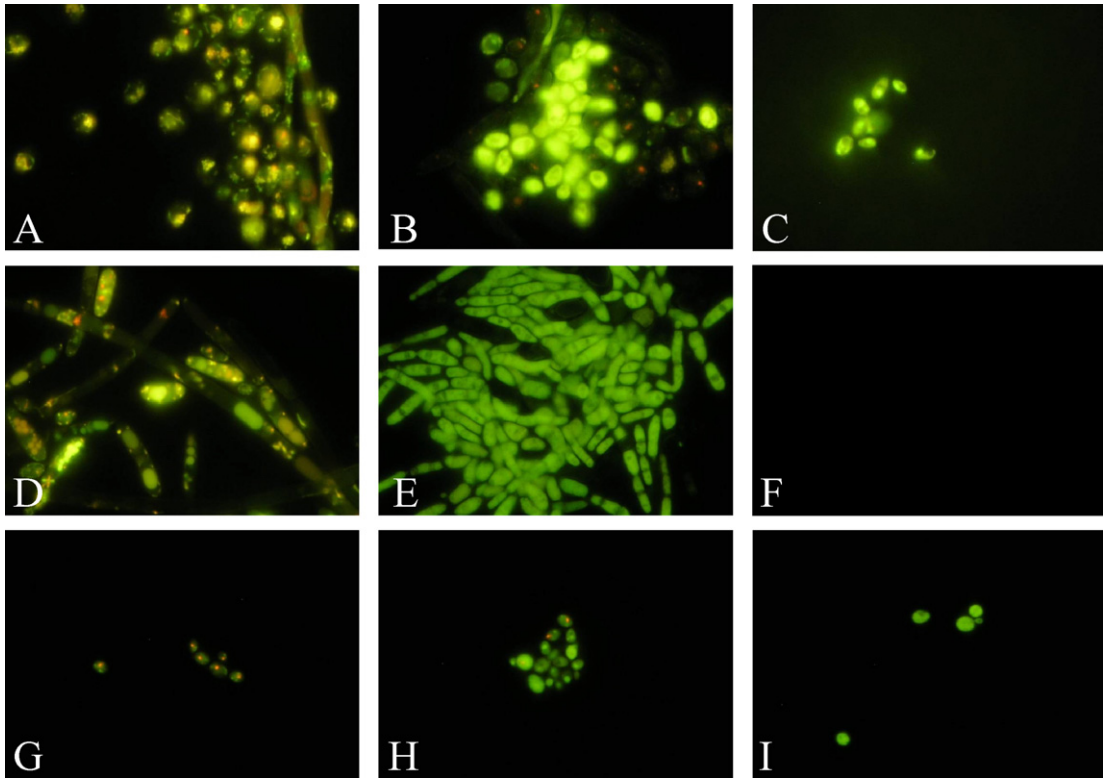

Fig. 3. Fun®-1 stained *C. albicans* (A–C), *C. krusei* (D–F) and *C. glabrata* (G–I) after 24 h incubation with different concentrations of high-molecular-weight chitosan hydrochloride. Healthy cells show red vacuols, inhibited cells appear homogeneous green. Control: A, D and G; 0.025%: B, E and H; 1%: C, F and I. F: No cells detectable.

surface is disturbed and more Fun®-1 can be transferred into the cytoplasm. 0.025% chitosan hydrochloride does not affect all cells; some of them are viable (see Fig. 3B, E and H).

3.3. Incubation with carboxymethyl chitosan

All investigated concentrations of carboxymethyl chitosan (max. 0.5%) allow a definite proliferation of *C. albicans*, *C. krusei* and *C. glabrata* (see Figs. 1C and 2C). The beginning of different growth phases is delayed with increasing carboxymethyl chitosan concentrations and differences between control and the decreased relative growth after incubation with higher concentrations of the tested substance (*C. albicans*: concentrations higher than 0.025%, *C. krusei*: concentrations higher than 0.025%, *C. glabrata*: 0.5% carboxymethyl chitosan) are significant. Thus, the inhibitory effect is quite lower than the antifungal activity of low and high molecular weight chitosan hydrochloride. This is reflected by a low to moderate negative correlation between relative growth after 24 h and concentration of carboxymethyl chitosan (Spearman correlation coefficient: *C. albicans* −0.644, *C. krusei* −0.418, *C. glabrata* −0.360).

3.4. Incubation with chitosan oligosaccharide

The tested chitosan oligosaccharide has a very low antifungal effect. Growth of *C. krusei* and *C. glabrata* is delayed by concentrations higher than 0.025 and 0.01%, respectively. Additionally, there is a decrease of growth maxima after 24 h caused by doses higher than 0.1% (*C. krusei*) and 0.025% (*C. glabrata*). In contrast, growth of *C. albicans* is not decreased even by a test concentration of 1% (see Fig. 1C). Consequently, the negative correlation between growth and concentration of chitosan-oligosaccharide is low to moderate (Spearman correlation coefficients: *C. albicans* −0.323, *C. krusei* −0.502, *C. glabrata* −0.639). Mann–Whitney test results in significant inhibition of *C. krusei* by 0.25% (bar graph not published), 0.5% (bar graph not published) and 1% chitosan oligosaccharide and *C. glabrata* by concentrations higher than 0.05%. *C. albicans* is not inhibited significantly even by highest tested concentrations (see Fig. 1D).

3.5. Incubation with N-acetyl-D-glucosamine

All *C. albicans*, *C. krusei* and *C. glabrata* show very similar growth curves, meeting almost the control curve. There is no negative correlation between relative growth after 24 h and concentration of *N*-acetyl-D-glucosamine (Spearman correlation coefficients: *C. albicans* −0.120, *C. krusei* −0.180, *C. glabrata* +0.331!). Because of the positive correlation, a small positive influence on growth of *C. glabrata* can be expected. This hypothesis is supported by a significant difference between the higher relative growth after 24 h incubation with 1% *N*-acetyl-D-glucosamine and the lower growth of control (Mann–Whitney test).

4. Discussion

4.1. Mode of action

There are different hypotheses concerning the mechanism of antifungal action of chitosan. Chitosan can bind calcium and iron and cause a lack of nutrients for fungi (Cuero, 1999; Roller and Covill, 1999) and is able to weaken virulence factors like secreted aspartyl proteinases (SAPs), which participates in adhesion to hosts (Calamari et al., 2004; McMullan-Vogel et al., 1999). Many authors interpret the polycationic character of chitosan as its most important factor of antifungal activity. The cationic amino groups may interact with anionic components of the cell wall (Cuero, 1999; Gil et al., 2004; Roller and Covill, 1999; Zakrzewska et al., 2005). Zakrzewska et al. suppose that the anionic cell wall sphingolipids are "attacked" by chitosan (Zakrzewska et al., 2005). Sphingolipids are the most common anionic surface molecules in *Saccharomyces cerevisiae*. Affection of them is also discussed as mode of action of the polycationic defensins. Their binding capacity to sphingolipids and antifungal activity increases with increasing content of cationic amino acids (Thevissen et al., 2004; Thomma et al., 2002).

Our own investigations with FUN®-1 stain verified the cell surface perturbing action of high-molecular chitosan hydrochloride against *C. albicans*, *C. krusei* and *C. glabrata*. Incubation with 0.025% high molecular weight chitosan hydrochloride resulted in a diffuse yellow-green colour of most yeast cells, which is due to affected, permeable cells (Millard et al., 1997).

4.2. Comparison of test methods

For investigation of antifungal activity of chitosan and its derivatives, some authors counted cells of yeasts and moulds (*S. cerevisiae*, *Rhizoctonia solani*, *Sclerotium rolfsii*, *Fusarium solani*, etc.) before and after incubation with chitosan (Eweis et al., 2006; Gil et al., 2004; Roller and Covill, 1999). Cuero et al. did not count cells, but measured dry weight of *Aspergillus flavus* and *parasiticus* (Cuero et al., 1991). Another method is the radial growth bioassay, in which the cultures diameter is measured and compared with a control (Badawy et al., 2004; Guo et al., 2006; Muzzarelli et al., 2001; Peng et al., 2005). Peng et al. incubated different moulds with hydroxypropyle chitosan and defined MIC as lowest concentration, where no turbidity was observed (with eyes!) after incubation (Peng et al., 2005). A more exact and objective method is photometry at different time points of incubation and generation of growth curves (Rhoades and Roller, 2000; Zakrzewska et al., 2005). Next to this, increase of UV absorption (260 nm) of the fungus supernatant after centrifugation is proof for antifungal activity (Zakrzewska et al., 2005). This increase is caused by nucleotides and co-enzymes released from affected cells (De Nobel et al., 1990; Yphantis et al., 1967; Zakrzewska et al., 2005). Nephelometry was not used as test method for chitosan yet. Fouda et al. investigated the antifungal activity of cyclodextrin complexes with a microplate nephelometer (Fouda et al., 2006).

In common, an inoculum size between 10^4 and 10^6 cells/mL was chosen, which conforms the inoculum size in our work of 3×10^5 cells/mL (Cuero et al., 1991; Gil et al., 2004; Peng et al., 2005; Rhoades and Roller, 2000; Roller and Covill, 1999; Zakrzewska et al., 2005). Methodically a slightly acidic medium and incubation at 20–30 °C is recommended in the literature and we chose culture conditions of pH 6 and 30 °C (Allan and Hadwiger, 1979; Badawy et al., 2004; Cuero et al., 1991; Eweis et al., 2006; Gil et al., 2004; Guo et al., 2006; Kendra and Hadwiger, 1984; Muzzarelli et al., 2001; Peng et al., 2005; Rhoades and Roller, 2000; Zakrzewska et al., 2005). In contrast, there are different opinions concerning the right test media. We used Sabouraud glucose bouillon, which is accordance to the works of Allan and Hadwiger, Kendra and Hadwiger and Peng et al. (Allan and Hadwiger, 1979; Kendra and Hadwiger, 1984; Peng et al., 2005).

4.3. Comparison of results

We could demonstrate an antifungal effect of low and high-molecular-weight chitosan hydrochloride against *C. albicans*, *C. krusei* and *C. glabrata*. We found high negative correlations between growth of *C. albicans*, *C. krusei* and *C. glabrata* and the concentration of low- and high-molecular-weight chitosan hydrochloride (Spearman correlation coefficient, see Table 2). However, concentration of low-molecular-weight chitosan hydrochloride and progeny of *C. krusei* correlated only moderately negative. The inhibitory effect was proven statistically by comparing relative growth of control and relative growth after incubation with different concentrations of the tested substances via Mann–Whitney test. Even lowest concentrations of both chitosan hydrochlorides (0.0025%) inhibited growth of the *Candida* species significantly.

$MIC_{50\%}$ und $MIC_{80\%}$ were calculated via SPSS probit analysis (Badawy et al., 2004). The lowest MIC against low molecular weight chitosan hydrochloride showed *C. krusei*, whereas *C. albicans* featured the lowest MIC against high molecular weight chitosan hydrochloride (see Table 2). To prove complete inhibition, material from the microplates was inoculated after 24 h incubation on Sabouraud glucose agar plates. *C. albicans* did not form any colonies after incubation with low- and high-molecular-weight chitosan hydrochloride in concentrations as low as 0.1%, which speaks for complete inhibition. *C. krusei* showed the same result when incubated with the high-molecular test substance. In contrast, only the 1% low molecular weight chitosan hydrochlorid completely inhibited growth of *C. krusei*.

C. glabrata formed colonies even after incubation with 1% of both substances, which had already been suspected by viewing the slightly rising growth curves at 1% low- and high-molecular-weight chitosan hydrochloride. Interestingly, *C. glabrata* is a hard to treat fungus in clinical practice, which is possibly caused by the haploid genome of the yeast (Fidel et al., 1999).

There are no publications concerning antifungal activity of chitosan hydrochloride or susceptibility of *C. krusei* against native chitosan and its derivatives. Zakrzewska et al. compared activity of native chitosan against *S. cerevisiae, Zygosaccharomyces bailii, C. albicans* and *C. glabrata* by photometric measurements of growth curves (Zakrzewska et al., 2005). They also observed the lower efficiency of chitosan against *C.*

glabrata. $MIC_{50\%}$ for *C. albicans* was between 0.005 and 0.01%, which is in accordance with our results (see Table 2). However, incubation time was shorter (8 h), the medium was different (YPD bouillon) and chitosan was solved in acetic acid. Other authors reported $MIC_{100\%}$ for *S. cerevisiae* between 0.001 and 0.1% (Allan and Hadwiger, 1979; Gil et al., 2004; Rhoades and Roller, 2000; Roller and Covill, 1999). For comparison, moulds and dermatophytes showed higher MICs (Allan and Hadwiger, 1979; Roller and Covill, 1999).

According to our measurements, carboxymethyl chitosan delays growth of *C. albicans, C. krusei* and mostly *C. glabrata*, which is similar to chitosan hydrochloride. Indeed, growth reaches almost the control values in the course of 24 h. Only higher concentrations show a statistical significant inhibitory effect according to Mann–Whitney test (*C. albicans*: concentrations $\geq$0.025%, *C. krusei*: concentrations $\geq$ 0.05%, *C. glabrata*: only 0.5%). Additionally, there is only a moderate (*C. albicans*) or low (*C. krusei* and *C. glabrata*) negative correlation between growth and concentration of carboxymethyl chitosan (Spearman correlation coefficient, see Table 2). This low antifungal activity was reported by other authors, too. In contrast, dicarboxymethyl chitosan enhances fungal growth (Muzzarelli et al., 2001). However, these data referred to other fungi than yeasts (Guo et al., 2006; Muzzarelli et al., 2001). El-Ghaouth et al. explained the lower activity with the loss of polycationic character by carboxymethylation of the free amino group (El Ghaouth et al., 1992).

There are described different water-soluble derivatives of chitosan with activity against moulds: imines of carboxymethyl chitosan with chlorinated or aminated aldehydes (Guo et al., 2006), 4-chlorobutyl chitosan ($MIC_{50\%}$ = 0.043%) and decanoyl chitosan ($MIC_{50\%}$ = 0.044% compared to a $MIC_{50\%}$ = 0.56% of native chitosan) (Badawy et al., 2004), thiourea chitosan (Eweis et al., 2006) and hydroxypropyl chitosan (Peng et al., 2005).

Next to chitosan hydrochloride and carboxymethyl chitosan, we investigated the antifungal activity of chitosan oligosaccharide ($n > 15$) and *N*-acetyl-D-glucosamine. *C. albicans* was not inhibited, neither by the tested oligosaccharide nor the monomer. There was only a small delay of growth caused by 1% chitosan oligosaccharide. In contrast, there is a slight inhibition of *C. krusei* and *C. glabrata* by high doses of chitosan oligosaccharide. This fact is reflected by the low to moderate negative correlation between growth and concentration of the tested substance (Spearman correlation coefficient, see Table 2). The lowest correlation was observed for *C. albicans*.

According to Kendra and Hadwiger, the heptamer is the smallest antifungal chitosan oligosaccharide against *F. solani* (Kendra and Hadwiger, 1984). Rhoades and Roller compared different oligosaccharides with native chitosan (Rhoades and Roller, 2000). The molecular weight was characterized by measuring viscosity (native chitosan 802s). Molecules featuring values below 11s did not show any activity against *S. cerevisiae, Saccharomyces ludwigii* or *Saccharomycodes bailii*. Interestingly, 96s and 154s chitosan were more effective than the native one.

Our results show that the acetylated monomer *N*-acetyl-D-glucosamine is not active against *C. albicans* and *C. krusei.* In contrast, there is a slight positive correlation between concentration and growth of *C. glabrata.* Thus, *N*-acetyl-D-glucosamine can be expected to be beneficial for progeny of *C. glabrata.* This effect has been reported earlier by Rhoades and Roller, too (Rhoades and Roller, 2000).

Vishu Kumar et al. showed that *N*-acetyl-D-glucosamine lacks antimicrobial activity against *Escherichia coli* and *Bacillus cereus.* In contrast, glucosamin inhibited growth about 10% (Vishu Kumar et al., 2005). The authors explain the low activity of *N*-acetyl-D-glucosamine with the missing of a free, protonable amino group.

Crucial for protonation of amino groups is an acidic medium. Interestingly, most investigations were done in such media. Indeed, Gil et al. conducted comparative measurements in neutral medium (Gil et al., 2004). There the antifungal effect of chitosan against *S. cerevisiae* under neutral conditions was lower than under acidic. This confirms the hypothesis of the essential importance of protonated amino groups in acid medium, which determine the polycationic character of chitosan and allow antimycotic interaction with negative surface molecules of fungi.

5. Conclusions

For the first time, we observed a concentration dependent antifungal activity of low- and high-molecular-weight chitosan hydrochloride against *C. albicans*, *C. krusei* and *C. glabrata* in acid medium. To the best of our knowledge, no studies using nephelometry to investigate the antifungal effect of chitosan have been published yet. Next to this, we observed the influence of molecular weight on the antifungal activity: a low-molecular weight is associated with low antifungal activity. Another interesting detail was the low activity of carboxymethyl chitosan against *C. albicans*, *C. krusei* and *C. glabrata*. These findings support the theory, that the polycationic character of chitosan is crucial for antifungal activity, because this functional group masks the cationic amino groups.

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