Preparation and antimicrobial activity of some carboxymethyl chitosan acyl thiourea derivatives

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A B S T R A C T

Acetyl, chloroacetyl and benzoyl thiourea derivatives of carboxymethyl chitosan (ATUCMCs, CATUCMCs, and BZTUCMCs) with comparable grafting degree were synthesized and their structures were characterized by FTIR spectroscopy and elemental analyses. The antimicrobial behaviors of CMCS and its derivatives against three types of bacteria [Bacillus subtilis (B. subtilis), Staphylococcus aureus (S. aureus) and Escherichia coli (E. coli)] and three crop-threatening pathogenic fungi [Aspergillus fumigate (A. fumigate), Geotrichum candidum (G. candidum) and Candida albicans (C. albicans)] were investigated. The results indicated that the antibacterial and the antifungal activities of the acyl thiourea derivatives are much higher than that of the parent CMCS. The acyl thiourea derivatives were more potent in case of Gram-positive bacteria than Gram-negative bacteria. This is illustrated for example by the values of minimum inhibitory concentration (MIC) of the ATUCMCs, CATUCMCs and BZTUCMCs against B. subtilis were 3.9, 15.6 and 62.5, respectively, while the MIC values of these derivatives against E. coli were 62.5, 125 and 500. Moreover, the antifungal activity of the CATUCMC is higher than that of the acetyl and benzoyl thiourea derivatives. This may be due to the presence of chlorine atom.

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1. Introduction

Chitosan, a copolymer of glucose amine and N-acetyl-glucosamine units linked by 1–4 glucosidic bonds, is obtained by deacetylation of chitin [1]. Chitosan has a unique set of interesting characteristics such as biocompatibility, biodegradability, bioadhesivity, non-toxicity and antimicrobial activity which are useful in many fields as biotechnology, pharmaceutics, cosmetics, agriculture, food science and textiles [2]. The poor solubility of unmodified chitosan in both water and organic solvents makes its utilization limited in the pharmaceutical field. One of the reasons for the intractability of chitosan lies in its rigid crystalline structure that is related to the acetamido and primary amino groups that induce relevant conformational features through intra- and or inter-molecular hydrogen bonding [3]. Therefore, considerable attention has been directed to chemical modification of chitosan for production of derivatives soluble in water over a wider pH range [4]. Furthermore, antimicrobial activities of chitosan derivatives have received considerable attention in recent years due to problems associated with chemical fungicide agents [5].

It was found that thiourea derivatives have strong antifungal activities that are comparable to the activity observed for the common antifungal antibiotic ketoconazole [6,7]. Moreover, they have antibacterial and insecticidal effects [8,9]. Five thiourea derivative ligands and their Ni²⁺ and Cu²⁺ complexes have been synthesized and were screened for their in vitro antibacterial and anti-yeast activity. In vitro anti-yeast activity of both ligands and their metal complexes is greater than their in vitro anti-bacterial activity [10]. Eweis et al. [11] have prepared benzoyl thiourea derivative of chitosan and showed that its antifungal activity is much better than that of native chitosan. Other authors have prepared three different acyl thiourea derivatives of chitosan (acyetyl, chloroacetyl and benzoyl) and indicated that the antimicrobial activities of these derivatives are much greater than that of native chitosan [12]. The acetyl thiourea chitosan was also used as an eco-friendly corrosion inhibitor for mild steel in sulfuric acid medium [13]. Many water-soluble chitosan derivatives have been prepared by introducing hydrophilic groups like carboxyalkyl groups such as carboxymethyl, carboxymethyl, carboxybutyl or by grafting water-soluble polymers in the macromolecular chain of chitosan [14–18].

Compared with chitosan, the solubility of carboxymethyl chitosan (CMCS) in aqueous solution is improved remarkably because of the introduction of carboxymethyl group. CMCS possess modulated physical and biological properties as chelating, sorption, moisture retention, cell functioning antioxidant, antibacterial, drug delivery, pH responsive drug delivery, etc. CMCS can be further modified with alkylation, acylation and grafting [19]. It was found that the antibacterial activity of CMCS is greater than chitosan due to the dependence of polycations antibacterial action on...
effective number of $-\text{NH}_3^+$ groups, where in CMCS the $-\text{COOH}$ groups may react with the NH$_2$ groups intra or intermolecularly and changed these NH$_2$ groups. So, in the same condition, the number of $-\text{NH}_3^+$ groups of CMCS is more than that of chitosan. Therefore, the antibacterial activity of CMCS increased [20]. CMCS has been modified to introduce non-pH dependent positive charge on CMCS backbone, by introducing of hydrophobic moieties on its backbone which facilitate the binding with the microorganism cell wall. In the present study we will prepare acetyl, chloroacetyl and benzoyl thiourea carboxymethyl chitosan derivatives, with expected much higher antimicrobial activities than that of CMCS due to their higher solubilities in organic and aqueous media, which facilitate the penetration into the cells of the microorganisms and inhibit the growth of cells by preventing the transformation of DNA into RNA. The minimum inhibitory concentration (MIC) values of these derivatives against different microorganisms will also be determined.

2. Experimental

2.1. Materials

Chitosan with a degree of deacetylation of 88% and molecular weight of 2.0 $\times$ $10^5$ was purchased from Acros Organics, NJ, USA. All other chemicals and reagents were of analytical grade, from Aldrich and were used as received. The crop-pathogen threatening fungi (Aspergillus fumigatus, Geotrichum candidum and Candida albicans) and bacteria (Bacillus subtilis, Staphylococcus aureus and Escherichia coli) used for the antimicrobial assay were provided by the Regional center for Mycology and Biotechnology Culture Collection.

2.2. Preparation of carboxymethyl chitosan (CMCS)

Carboxymethyl chitosan (CMCS) was prepared following the method reported previously [21], where chitosan (10 g), sodium hydroxide (13.5 g) and solvent isopropanol (100 ml) were suspended in a flask to swell and alkalize at room temperature for 1 h. The monochloro acetic acid (15 g) was dissolved in isopropanol, and added to the reaction mixture drop-wise within 30 min and reacted for 4 h at 55 °C. Then the reaction was stopped and isopropanol was discarded. Ethyl alcohol (80%) was added and solid product was filtered and rinsed with 80–90% ethyl alcohol to desalt and dewater, and vacuum dried at 50 °C. The degree of substitution of carboxymethyl chitosan was determined by pH-titration [22] and found to be 0.75.

2.3. Preparation of acyl thiourea carboxymethyl chitosan derivatives

Acyli thiocyanate derivatives were prepared according to the method described by Zhong et al. [12]. Dry ammonium thiocyanate (NH$_4$SCN, 0.1 mol) was dissolved in CH$_2$Cl$_2$ (30 ml), then a CH$_2$Cl$_2$ solution containing 0.1 mol of acetyl chloride, chloro-acetyl chloride or benzoyl chloride was added. Polyethylene glycol-400 (0.8 ml) was added drop wise as phase transfer catalyst. After stirring for 2 h at room temperature, the mixture of products was filtered through a Buchner funnel under reduced pressure. The filtrate (acyli thiocyanate) was added to a carboxymethyl chitosan aqueous solution. The reaction mixture was stirred at 100 °C for 5 h, cooled, and filtered. The yellowish white product was washed with methanol and dried at 50 °C. The preparation of these acyl thiourea derivatives can be illustrated by Scheme 1.

2.4. Characterization

FTIR spectra were recorded on Testcan Shimadzu FT-IR Spectrophotometer (Model 8000) using KBr pellets within the wave number range of 4000–400 cm$^{-1}$ at 25 °C.

Elemental analyses were carried out by the micro-analytical unit at the National Research Center, Giza, Egypt.

2.5. Antibacterial activity

Antibacterial activities were investigated using agar well diffusion method. The activity of tested samples was studied against the S. aureus (RCMB 000106) and B. subtilis (RCMB 000107) (as Gram-positive bacteria) and E. coli (RCMB 000103) (as Gram-negative bacteria). Centrifuged pellets of bacteria from a 24 h old culture containing approximately 104–106 CFU (colony forming unit) per ml spread on the surface of nutrient agar (tryptone 1%, yeast extract 0.5%, NaCl 0.5%, agar 1%, 1000 ml of distilled water, PH 7.0) which was autoclaved under 121 °C for at least 20 min. Wells were created in medium with the help of a sterile metallic bores and then cooled down to 45 °C. The activity was determined by measuring the diameter of the inhibition zone (in mm). 100 ml of the tested samples (10 mg/ml) were loaded into the wells of the plates. All compounds were prepared in dimethyl sulfoxide (DMSO), DMSO was loaded as control. The plates were kept for incubation at 37 °C for 24 h and then the plates were examined for the formation of zone of inhibition. Each inhibition zone was measured three times by caliper to get an average value. The test was performed three times for each bacterium culture: penicillin and streptomycin were used as antibacterial standard drugs [23].

2.6. Antifungal activity

Tested samples were screened separately in vitro for their antifungal activity against various fungi [A. fumigates (RCMB 002003), G. candidum (RCMB 052006) and C. albicans (RCMB 050502)], on sabourad dextrose agar plates. The culture of fungi was purified by single spore isolation technique. The antifungal activity was by agar well diffusion method [24] as follows.

Sabourad dextrose agar plates: a homogenous mixture of glucose–pepton–agar (40:10:15) was sterilized by auto claving at 121 °C for 20 min. The sterilized solution (25 ml) was poured in each sterilized petridish in laminar flow and left for 20 min to form the solidified sabourad dextrose agar plate. These plates were inverted and kept at 30 °C in incubator to remove the moisture and to check for any contamination.

Antifungal assay: fungal strain was grown in 5 ml sabourad dextrose broth (glucose:peptone; 40:10) for 3–4 days to achieve 105 CFU/ml cells. The fungal culture (0.1 ml) was spread out uniformly on the sabourad dextrose agar plates by sterilized triangular
folded glass rod. Plates were left for 5–10 min so that culture is properly adsorbed on the surface of sabourad dextrose agar plates.

Now small wells of size (4 mm × 2 mm) were cut into the plates with the help of well cutter and bottom of the wells were sealed with 0.8% soft agar to prevent the flow of test sample at the bottom of the well. 100 μl of the tested samples (10 mg/ml) were loaded into the wells of the plates. All compounds were prepared in DMSO, DMSO was loaded as control. The plates were examined for the formation of zone of inhibition. Each inhibition zone was performed three times for each fungus. Clotrimazole and itraconazole were used as antifungal standard drugs.

2.7. Minimum inhibitory concentration (MIC)

The agar plate method was used to determine the minimum inhibition concentration (MIC) of tested samples, two-fold serial dilutions of each sample were added to nutrient broth for bacteria (beef extract 5 g, peptone 10 g added to 1000 ml distilled water, pH 7.0) and to sabourad dextrose broth for fungi, DMSO was used as the control. Then they were heated in autoclave at 121 °C for 25 min. The culture of each organism was diluted by sterile distilled water to 105–106 CFU/ml, a loop of each suspension was inoculation, the plates were incubated at 37 °C for 24 h for bacteria, and at 30 °C for 3–4 days for fungi. The colonies were counted and the MIC values were obtained. The MIC was considered to be the lowest concentration that completely inhibits against inoculums comparing with the control, disregarding a single colony or a faint haze caused by the inoculums [25].

3. Results and discussion

3.1. FTIR characterization of carboxymethyl chitosan

FTIR spectroscopy was employed to detect the structural changes of CS and CMCS (Fig. 1). The FTIR spectrum of CS showed four strong peaks at 1155, 1073, 1030, and 895 cm⁻¹ which were characteristic peaks of the saccharide structure. The very strong broad peak around 3600–3200 cm⁻¹ should be assigned to the stretching vibration of O–H, the extension vibration of the N–H, and the intermolecular hydrogen bonds of the polysaccharide. Primary amines have two peaks in this region. There were weak absorption peaks at 1653 and 1567 cm⁻¹ corresponded to amide I and amide II, respectively, which indicated that CS had a high deacetylation degree. The FTIR spectrum of CMCS showed, in addition to the above peaks, a strong peak at 1454 cm⁻¹ which could be assigned to the symmetrical stretching vibration of COO⁻ group. The asymmetrical stretching vibration of COO⁻ group (around 1550 cm⁻¹) is overlapped with the deforming vibration of NH₂ at 1604 cm⁻¹ to obtain a very strong peak. The C–O absorption peak of hydroxyl group became stronger and moved to 1097 cm⁻¹. The results, which are in accordance with the work of Xie et al. [26], indicated that substitution occurred at the C₆ position.

3.2. FTIR characterization of acyl thiourea carboxymethyl chitosan derivatives

Fig. 2 shows a comparison of the transmission FTIR spectra for ATUCMCS, CATUCMCS, and BZTUCMCS with CMCS. The FTIR spectra of ATUCMCS and CMCS are concerned, first; the broad band between 3500 and 3200 cm⁻¹ due to the O–H and N–H group stretching vibration is observed. In addition, the characteristic absorbance of NH₂ at 1600 cm⁻¹ disappeared; these results show that –NH₂ group had reacted with acetyl thiocyanate. This is also well illustrated by the disappearance of the doublet peak at 3382 and 3170 cm⁻¹ corresponding to the –NH₂ group and the appearance of a single peak around 3425 cm⁻¹ for –NH group. Second; new peaks at 1714 and 1655 cm⁻¹ in the ATUCMCS spectrum, which were assigned to the characteristic absorbance of N–H and C=S. All the above results support the structure of ATUCMCS. In the spectrum of CATUCMCS new peaks appeared at 1730 (C=O), 1637 (C=O), 1519 (N–H), and 1068 (C=S) cm⁻¹, which showed that CATUCMCS was obtained. For the spectrum of BZTUCMCS, there are new peaks, support its structure, at 1666 (C=O), 1519 (N–H), 1070 (C=S), 1520 (phenyl), and 795 (phenyl) cm⁻¹.
3.3. Elemental analyses of acyl thiourea carboxymethyl chitosan derivatives

An additional proof for the synthesis of acyl thiourea derivatives of CMCS is given by their elemental analysis data as shown in Table 1. The elemental analysis results also indicate that the grafting degree of ATUCMCS, CATUCMCS, and BZTUCMCS are comparable (89.66%, 88.88%, and 88.00%, respectively).

### Table 1

Elemental analyses and grafting degree of acyl thiourea carboxymethyl chitosan derivatives.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Elemental analyses (%)</th>
<th>Grafting degree (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%C</td>
<td>%H</td>
</tr>
<tr>
<td>CMCS</td>
<td>43.82</td>
<td>5.63</td>
</tr>
<tr>
<td>ATUCMCS</td>
<td>41.69</td>
<td>4.94</td>
</tr>
<tr>
<td>CATUCMCS</td>
<td>38.29</td>
<td>4.30</td>
</tr>
<tr>
<td>BZTUCMCS</td>
<td>49.27</td>
<td>4.70</td>
</tr>
</tbody>
</table>

#### 3.4. The antimicrobial activity of acyl thiourea carboxymethyl chitosan derivatives

Table 2 shows the antibacterial activity of the CMCS and its acyl thiourea derivatives, ATUCMCS, CATUCMCS and BZTUCMCS using inhibition zone method. Compared with CMCS, its acyl thiourea derivatives have a higher antibacterial activity. Several mechanisms elucidating the antimicrobial activity of chitosan have been postulated. The most acceptable mechanism is the interaction between positively charged chitosan molecules and negatively charged microbial cell membrane. The interaction is mediated by the electrostatic forces between the protonated NH₃⁺ groups of chitosan and the electronegative charges on the microbial cell surfaces. This electrostatic interaction results in twofold interference: (i) by promoting changes in the properties of membrane wall permeability, thus provoking internal osmotic imbalances and consequently inhibit the growth of the microorganisms, and (ii) by the hydrolysis of the peptidoglycans in the microorganism wall, leading to the leakage of intracellular electrolytes such as potassium ions, and other low molecular weight proteinaceous constituents (e.g. protein, nucleic acid, glucose, and lactate dehydrogenase) [27]. Since such mechanism is based on electrostatic interaction, it suggests that the greater the number of cationized amines, the higher will be the antimicrobial activity. Carboxymethylation of chitosan allowed the synthesis of CMCS with higher hydrophilicity, with better solubility in aqueous media and with greater positive charge density; where in CMCS the –COOH groups may react with the NH₃⁺ groups intra or intermolecular and changed these NH₂ groups into NH₃⁺ groups leading to increased polycationic character (non-pH dependent positive charges on CMCS). Further, the introduction of acyl thiourea moieties onto CMCS increases its solubility both in organic and aqueous media [28] and also increases its cationic centers; thus their C=O, NH, and C=S groups can be protonated and consequently the net positive charge was strengthened, leading to a better antibacterial activity. Another proposed mechanism is the binding of chitosan with microbial DNA, which leads to the inhibition of the mRNA and protein synthesis via penetration of chitosan into the nuclei of the microorganisms [29]. Acyl thiourea moieties grafted onto hydrophilic CMCS apart the CMCS chains away from each other, decrease their intermolecular hydrogen bonds, and increase their solubility; the reason for the easy of penetration of the acyl thiourea derivatives into the cells of microorganisms and prevent the growth of the cell by preventing the transformation of DNA to RNA to obtain a higher antibacterial activity. The third mechanism is the chelation of metals, suppression of spore elements and binding to essential nutrients to microbial growth [30]. It is well established that both the carboxylic and acyl thiourea groups have excellent metal-binding capacity. This explains the observed higher antibacterial activity of acyl thiourea derivatives relative to the parent CMCS.

Moreover, the acyl thiourea derivatives of CMCS were more active against the Gram-positive bacteria than against the Gram-negative bacteria (Table 2). As the strongest CATUCMCS derivative caused inhibition zone diameter of B. subtilis and S. aureus of 21.08 and 20.04 mm, respectively, corresponded to 15.6 mm of E. coli. This may be attributed to their different cell wall. The cell wall of Gram-positive bacteria is fully composed of peptide polyglycogen. The peptidoglycan layer is composed of net works with plenty of pores, which allow foreign molecules to come into the cell without difficulty and allow more rapid absorption of ions into the cell. But the cell wall of the Gram-negative bacteria is made up of a thin membrane of peptide polyglycogen and an outer membrane constituted of lipopolysaccharide, lipoprotein and phospholipids. Because of the complicated bilayer cell structure, the outer membrane is a potential barrier against foreign molecules with high molecular weight. Therefore, the derivatives have different effects on the two kinds of bacteria. An additional evidence for the greater activity of the acyl thiourea derivatives of CMCS against Gram-positive bacteria than that against Gram-negative bacteria comes from their minimum inhibitory concentration (MIC) values. MIC is defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation. The MIC of the CATUCMCS, ATUCMCS and BZTUCMCS are shown in Table 3 and Fig. 3. Since the MIC values of these

### Table 2

Inhibition indices of carboxymethyl chitosan derivatives against B. subtilis, S. aureus and E. coli.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Inhibition zone tested microorganisms (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B. subtilis (RCMB 000107)</td>
</tr>
<tr>
<td>CMCS</td>
<td>11.3</td>
</tr>
<tr>
<td>ATUCMCS</td>
<td>23.08</td>
</tr>
<tr>
<td>CATUCMCS</td>
<td>19.1</td>
</tr>
<tr>
<td>BZTUCMCS</td>
<td>14.2</td>
</tr>
</tbody>
</table>

### Table 3

MIC values of carboxymethyl chitosan derivatives against B. subtilis, S. aureus and E. coli.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Minimum inhibitory concentration (µg/ml) (MIC)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B. subtilis (RCMB 000107)</td>
</tr>
<tr>
<td>CATUCMCS</td>
<td>3.9</td>
</tr>
<tr>
<td>ATUCMCS</td>
<td>15.6</td>
</tr>
<tr>
<td>BZTUCMCS</td>
<td>62.5</td>
</tr>
</tbody>
</table>

**Fig. 3.** The antibacterial activity of BZTUCMCS, ATUCMCS and CATUCMCS against B. subtilis, S. aureus and E. coli
derivatives against B. subtilis were 3.9, 15.6 and 62.5 μg/ml and against S. aureus were 7.8, 31.25 and 62.5 μg/ml, the MIC values against E. coli were 62.5, 125 and 500 μg/ml, respectively. It is worth mentioning that the activity of acyl thiourea CMCS derivatives against Gram-positive bacteria is greater than that of the corresponding acyl thiourea chitosan derivatives reported by Zhong et al. [12].

It is interesting to note that the benzoyl thiourea derivative showed the lowest antibacterial activity, relative to the other derivatives, as judged by the lowest inhibition zones (Table 2) and by the highest minimum inhibitory values (Table 3). On the other hand, the chloroacetyl thiourea derivative exhibited the highest antibacterial activity. The activity of the acetyl thiourea derivative lies in between these two cases. As for the derivatives, with comparable N-substitution degree, the charge densities are very closed to each other, thus, their different inhibitory effects may be attributed to the lipophilic character of the substituted group. The lipophilicity, which correlates well with the bioactivity of chemicals, is a very important molecular descriptor and different lipophilic behavior of compounds plays an important role in their biological mechanisms. The derivative with chloroacetyl group has a relatively higher lipophilicity than the derivative with acetyl group which in its turn has greater lipophilicity than the derivative with benzoyl group. Thus, the benzoyl thiourea derivative does not penetrate into the microorganisms as easily as the thiourea derivatives with chloroacetyl and acetyl groups do.

The antifungal activities of acyl thiourea CMCS derivatives against A. fumigatus, G. candidum and C. albicans are shown in Tables 4 and 5 and Fig. 4. The results show that all the derivatives had effective activities against the tested fungi, compared with the parent CMCS, with inhibitory indices ranging from 9.4 to 20.1 mm inhibition zone (Table 4) and with MIC values ranging from 7.8 up to 250 μg/ml according to the type of the derivative (Table 5). Generally chitosan has been reported as being very effective in inhibiting spor germination, germ tube elongation and radial growth [31]. The antifungal mechanism of chitosan involves cell wall morphogenesis with chitosan molecules interfering directly with fungal growth, similarly to the effects observed in bacteria cells [31]. The microscopic observation reported that chitosan molecules diffuse inside hyphae interfering on the enzymes activity responsible for the fungus growth [11]. The solubility of the acyl thiourea derivatives of CMCS is expected to enhance the diffusion of the active ingredient inside the pathogens, which may lead to a disturbance of the enzyme activities responsible for the growth criteria, instead of the adsorption of the insoluble compounds on the fungal hyphal surface. The results also showed that the highest antifungal activity was observed for CATUMCS. This may be due to the presence of chlorine atom. The chloro-group is used in many fungicides such as pentachloronitrobenzene and chlorothalonil [32]. However, these fungicides constitute a big problem in the environment due to their toxicities, when these groups are grafted onto chitosan and consequently onto CMCS, they might be released slowly and may induce lower pollution to the environment [33]. These results as shown are much higher than those obtained by the corresponding acyl thiourea derivatives of chitosan stated by Zhong et al. [12].

### 4. Conclusions

Three different acyl thiourea derivatives of CMCS having a comparable grating degree have been successfully synthesized via carboxymethylation of chitosan followed by reacting the produced CMCS with either acetyl, chloroacetyl or benzoyl thiocyanate. Compared with carboxymethyl chitosan, its acyl thiourea derivatives have a higher antimicrobial activity. The results showed that acyl thiourea derivatives of CMCS have stronger activity against Gram-positive bacteria than against Gram-negative bacteria. These CMCS derivatives also show a significant inhibitory effect on the fungi. The CATUMCS has a noticeably higher antifungal activity than those of ATUMCS and BZTUMCS. Thus, it can be concluded that acyl thiourea derivatives of CMCS have an interesting usage as antimicrobial substances.

### References


