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Synthesis and antifungal properties of sulfanilamide derivatives of chitosan

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Abstract—Sulfanilamide derivatives of chitosan (2-(4-acetamido-2-sulfanimide)-chitosan (HSACS, LSACS), 2-(4-acetamido-2-sulfanimide)-6-sulfo-chitosan (HSACSS, LSACSS) and 2-(4-acetamido-2-sulfanimide)-6-carboxymethyl-chitosan (HSACMCS, LSACMCS)) were prepared using different molecular weights of chitosan (CS), carboxymethyl chitosan (CMCS) and chitosan sulfates (CSS) reacted with 4-acetamidobenzene sulfonyl chloride in dimethylsulfoxide solution. The structures of the derivatives were characterized by FT-IR spectroscopy and elemental analysis, which showed that the substitution degree of sulfanilamide group of HSACS, HSACSS, HSACMCS, LSACS, LSACSS and LSACMCS were 0.623, 0.492, 0.515, 0.576, 0.463 and 0.477, respectively. The solubility of the derivatives (pH < 7.5) was higher than that of chitosan (pH < 6.5). The antifungal activities of the derivatives against *Aiternaria solani* and *Phomopsis asparagi* were evaluated based on the method of Jasso et al. in the experiment. The results indicated that all the prepared sulfanilamide derivatives had a significant inhibiting effect on the investigated fungi in the polymer concentration range from 50 to 500 μg mL^{-1}. The antifungal activities of the derivatives increased with increasing the molecular weight, concentration or the substitution degree. The sulfanilamide derivatives of CS, CMCS and CSS show stronger antifungal activities than CS, CMCS and CSS.

Keywords: 2-(4-Acetamido-2-sulfanimide)-chitosan; 2-(4-Acetamido-2-sulfanimide)-6-sulfo-chitosan; 2-(4-Acetamido-2-sulfanimide)-6-carboxymethyl-chitosan; Antifungal activity

1. Introduction

Chitosan, a copolymer of glucosamine and *N*-acetyglucosamine units linked by 1–4 glucosidic bonds, was obtained by N-deacetylation of chitin, which is the second most naturally occurring biopolymer after cellulose.[1] As a kind of natural renewable resource, chitosan has a number of special properties such as biocompatibility, biodegradability and non-toxicity activity.[2–5] Amongst various bioactive properties of chitosan, its antifungal activity has received considerable interest due to problems associated with fungicidal agents.[6–8] EI Ghaouth et al. have reported that chitosan could inhibit the growth of *Alternaria alternate*, *Botrytis cinerea*, *Colletrotichum gloeosporioides* and *Rhizopus stolonifer* and that the inhibitory index was affected by the concentration of chitosan.[9] The growth of fungi such as *Fusarium oxysporum*, *R. stolonifer*, *Penicillium digitatum* and *C. gloeosporioides* can be inhibited completely by chitosan at a concentration of 3%.[10,11]

Although some studies proved chitosan had antifungal activities, it presents its antibacterial activities only in acidic medium because of its poor solubility above pH 6.5. Furthermore, acid also has antibacterial activities, which cannot be ignored in the investigation experiment of the antifungal activities of chitosan. Thus,

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water-soluble chitosan derivatives, which are soluble in both acidic and basic physiologic circumstances might be good candidates to be polycationic biocides. In an attempt to improve antifungal activity and solubility of chitosan, our paper reports the preparation of sulfanilamide derivatives of chitosan (2-(4-acetamido-2-sulfanimide)-chitosan (SACS), 2-(4-acetamido-2-sulfanimide)-6-sulfo-chitosan (SACSS) and 2-(4-acetamido-2-sulfanimide)-6-carboxymethyl-chitosan (SACMCS)). When sulfanilamide group is grafted onto chitosan, the solubility of chitosan is increased to a wider pH range (<7.5). Besides, the antifungal activities of the derivatives against two crop-threatening pathogenic *P. asparagi* and *Aiternaria solani* were studied in this paper, and the results show that the antifungal activities of the derivatives were much higher than that of chitosan.

2. Experimental

2.1. Materials

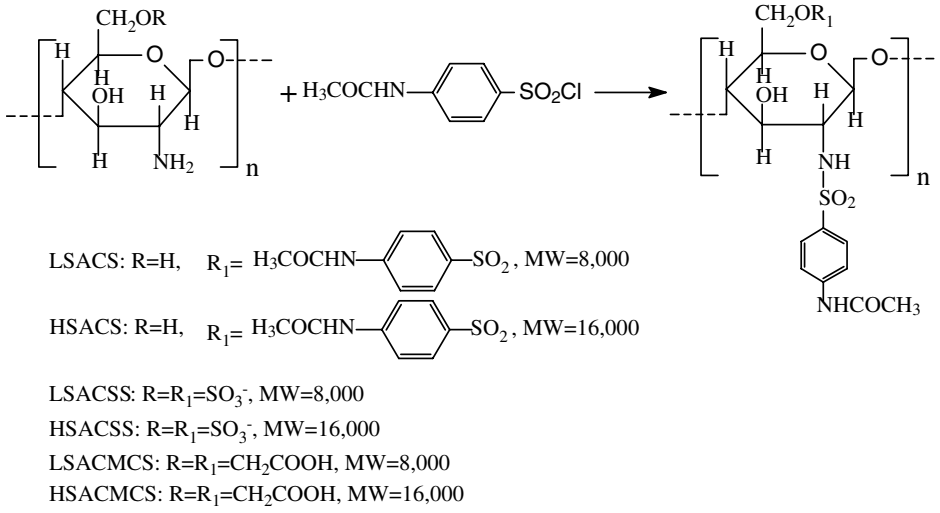
High molecular weight Chitosan (HCS) was supplied by Qingdao Baicheng Biochemical Corp. (China). Its deacetylation was 96%, average molecular weight 16 kDa. Low molecular weight (8 kDa) chitosan (LCS) was prepared in our laboratory by the method of acetic acid and hydrogen peroxide (H_2O_2) hydrolysis. Chitosan sulfates with high molecular weight (HCSS) and that with low molecular weight (LCSS) as well as carboxymethyl chitosan with high molecular weight (HCMCS) and low molecular weight (LCMCS) were prepared according to previous work.[12,13] Other reagents were of analytical grade and were used without further purification. Two crop-threatening pathogenic fungi *A. solani* and *P. asparagi* used for the antifungal assay were obtained from Qingdao Academy of Agricultural Sciences.

2.2. Analytical methods

Fourier transform infrared (FT-IR) spectra of the compounds were measured in the 4000–400 cm^{-1} regions using a Nicolet Magna-Avatar 360 FT-IR spectrometer with KBr disks. The elemental analysis (C, H, N) was performed on a Carlo-Erba 1106 elemental analyzer. The average viscometric molecular weight of chitosan and all of the derivatives was estimated from the intrinsic viscosity (mL g^{-1}) determined in the solvent 0.1 mol L^{-1} CH_3 COOH/0.2 mol L^{-1} NaCl using the Mark-Houwink parameter $\alpha = 0.96$, $K_\eta = 1.424$ at 25 °C.

2.3. The preparation of SACS, SACMCS or SACSS

The derivatives (SACS, SACSS or SACMCS) were synthesized according to Scheme 1. Two grams CS, CSS or CMCS was dissolved in dimethylsulfoxide, respectively. Dimethylsulfoxide solution contained 4-acetamidobenzene sulfonyl chloride was added to the system at a stated water bath temperature. After stirring for a few hours, the mixture was cooled to room temperature and poured into a beaker containing 400 mL acetone. Then a white precipitate was produced. After placing at 4 °C for 10 h, the mixture of products was filtered through a Bucher funnel under reduced pressure. The precipitate was rinsed with acetone, and redissolved in distilled water. The solution was dialyzed against distilled water for 48 h using a 3600 Da MW cut-off dialysis membrane. It was then concentrated and lyophilized to give SACS, SACSS or SACMCS. Table 1 shows the synthesis conditions of the compounds (Scheme 1 shows the synthesis pathway of the derivatives).



Scheme 1. Synthesis pathway of sulfanilamide derivatives of chitosan, carboxymethyl chitosan and chitosan sulfates.

Table 1. The reaction conditions, yield, colour, elemental analyses results and the substitution degree of CS, CMCS, CSS, SACS, SACSS and SACMCS

Compounds	Molar ratio	Temperature (°C)	Reaction time (h)	Yield (%)	Elemental analyses			Substitution degree[a]	Colour	Dissoluble pH range
					C	N	H			
CS	—	—	—	—	44.28	8.52	7.36	—	Ivory	<6.5
CMCS	—	—	—	—	43.76	6.20	5.86	—	White	<7.0
CSS	—	—	—	—	41.62	8.14	6.36	—	Yellow	<7.5
HSACS	1:2	65	6	84.65	47.29	7.76	4.56	0.623	Orange	<6.8
LSACS	2:3	60	5	82.08	47.21	7.79	4.63	0.576	Brown	<7.0
HSACSS	1:2	65	6	79.89	44.17	7.74	5.35	0.492	Brown	<7.5
LSACSS	2:3	60	4	75.54	44.07	7.75	5.39	0.463	Brown	<7.5
HSACMCS	1:2	65	6	81.09	45.73	7.19	5.33	0.515	Orange	<7.0
LSACMCS	2:3	60	4	78.98	45.65	7.19	5.38	0.477	Brown	<7.0

[a] Substitution degree refers to the substitution degree of sulfanilamide group.

2.4. Antifungal assays

Antifungal assays were performed based on the method of Jasso et al.[14] Briefly, the compounds were dissolved in distilled water at a concentration of 2% (w/v). Then, each derivatives (HSACS, LSACS, HSACMCS, LSACMCS, HSACSS and LSACSS) solution was added to sterilized potato dextrose agar to give a final concentration of 50, 100 and 500 μg mL^{-1}. After the mixture was cooled, the mycelium of fungi were transferred to the test plate and incubated at 29 °C for 3 days. When the mycelium of fungi reached the edges of the control plate (without the added samples), the antifungal index was calculated as follows:

$$\text{Antifungal index } (\%) = (1 - D_t/D_c) \times 100,$$

where D_t is the diameter of the growth zone in the test plate and D_c is the diameter of growth zone in the control plate. Each experiment was performed three times, and the data were averaged. The Scheffe method was used to evaluate the differences in antifungal index in the tests. Results with $P < 0.05$ were considered statistically significant.[15]

3. Results

3.1. Structure and physicochemical characteristics of the compounds

Figure 1 shows the comparison of the FT-IR spectra for SACS, SACSS and SACMCS and original CS, CSS and CMCS. There were new strong peaks at 1358 cm^{-1} and 1163 cm^{-1} at the SACS spectrum compared to CS, which assigned to the $\nu(SO_2)_{asym}$ and $\nu(SO_2)_{sym}$ characteristic absorbance. In addition, there were strong peaks at about 1531 and 824 cm^{-1}assigned to the characteristic absorbance of phenyl-group. Furthermore, obvious translocation at 3447 cm^{-1} due to the O–H and N–H group stretching vibration were also observed, which is the result that O–H and N–H had reacted with 4-acetamidobenzene sulfonyl chloride. In SACS spectra, a new sorption band at 1627 cm^{-1} (C═O in $CONH_2$) appeared instead of the band at 1600 cm^{-1} ($-NH_2$) in pure chitosan spectra. All of the above results show SACS were obtained. As for the spectrum of SACSS and CSS were concerned, the new peaks appeared at 1669 (C═O), 1386 ($\nu(SO_2)_{asym}$), 1165 ($\nu(SO_2)_{sym}$), 1533 (phenyl) and 826 (phenyl), which show the sulfanilamide derivatives of SACSS were obtained. For the same reason, new peaks at 1633 (C═O), 1392 ($\nu(SO_2)_{asym}$), 1199 ($\nu(SO_2)_{sym}$), 1533 (phenyl) and 838 (phenyl) indicated that SACMCS were formed. The above-mentioned results demonstrated that the sulfanilamide derivatives of chitosan, carboxymethyl chitosan and chitosan sulfates were synthesized successfully.

The results of elemental analysis and the substitution degree of the compounds are listed in Table 1. From Table 1, the solubility of the derivatives (<7.5) was higher than that of chitosan (<6.5), and the lowest yield was 75.54%. The elemental analysis indicated that the N,O-substitution degree of CMCS was about 0.56, and the C6–O-substitution of CSS was about 0.152. Furthermore, the substitution degree of sulfanilamide group of HSACS, HSACSS and HSACMCS was higher than LSACS, LSACSS and LSACMCS, respectively.

3.2. Antifungal activity

3.2.1. Antifungal activities of the derivatives against *P. asparagi*. *P. asparagi* can cause severe stem blight of asparagus, and the disease has been discovered on the leaves and in any part of the stem of asparagus. When asparagus is affected by this pathogen, lesions were formed on the stems. At first the lesions appear light brown and later turn dark reddish brown. Asparagus will die in areas where the lesions have been formed around. Thus, *P. asparagi* is a kind of destructive fungi to the production of asparagus. The antifungal activities of SACS, SACMCS and SACSS against *P. asparagi* are shown in Figure 2. The inhibitory index of HSACS,

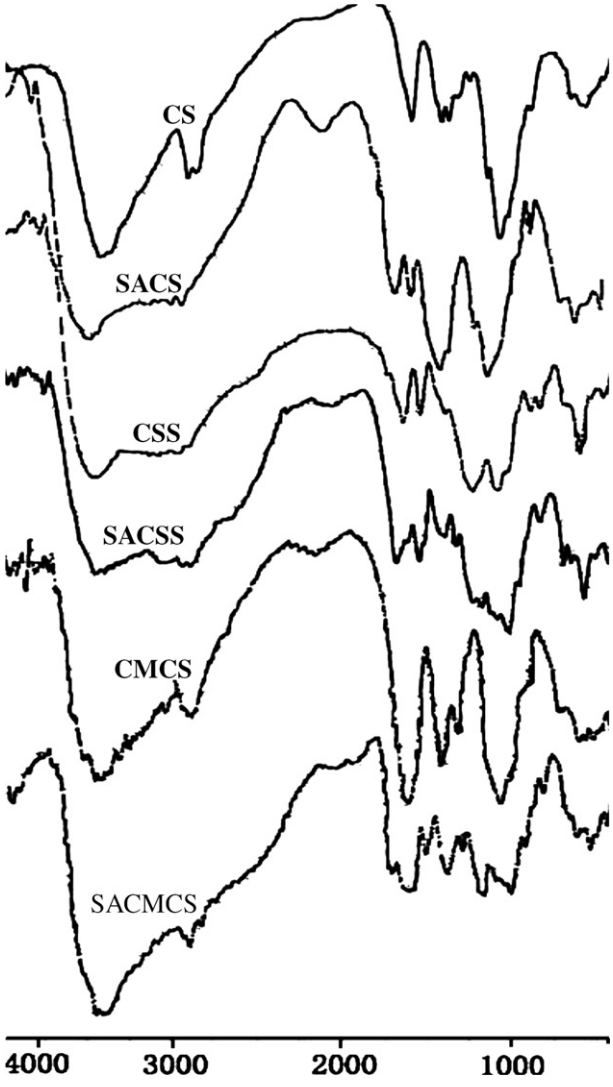


Figure 1. FT-IR spectrum data of CS, SACS, CSS, SACSS, CMCS, SACMCS.

LSACS, HSACSS, LSACSS, HSACMCS, LSACMCS, HCS, LCS, HCSS, LCSS, HCMCS and LCMCS at 500 μg mL^{-1} was 73.16, 43.33, 58.53, 42.44, 59.23, 44.44, 40.89, 30.84, 22.57, 21.23, 42.51 and 35.08, respectively. It indicated that the derivatives had effective activities against *P. asparagi,* although there was difference between them. Generally, SACS, SACSS and SACMCS showed stronger antifungal properties than CS, CSS and CMCS. Furthermore, the antifungal activities of SACS were obviously higher than SACSS and SACMCS. These results might be due to the fact that the sulfanilamide substitution degree of SACS was higher than SACSS and SACMCS, for 4-acetamidobenzene sulfonyl chloride reacted with CS at both –OH and $–NH_2$ group station but it reacted with CSS and CMCS only at $–NH_2$ group station. Compared with SACSS, SACMCS had much better antifungal activities against *P. asparagi*. These results were caused by the carboxymethyl group, which had antifungal activities and the higher substitution degree of sulfanilamide group in SACMCS. In addition, the inhibitory index of all of the compounds enhanced with the increase of the concentration of them. Moreover, the higher the molecular weight the stronger the antifungal activities.

3.2.2. Antifungal activities of CS, SACS, CSS, SACSS, CMCS and SACMCS against *A. solani*. *A. solani* is the causal agent of early blight disease of tomato. This pathogen colonizes various plant tissues including stems, leaves and fruit, and subsequently derives nutrients from host cells killed by the deleterious action of non-host specific, toxic secondary metabolites such as alternaric acid and zinniol.[16] Epidemics caused by this economically important pathogen can cause severe tomato crop defoliation in areas with high humidity and frequent nightly dew. Therefore, the study on antifungal agents is significative. In this paper, the fungicidal activity of the compounds towards *A. solani* was investigated and the results are depicted in Figure 3. As shown in Figure 3, all of the compounds show antifungal activities against *A. solani*, and SACS, SACSS and SACMCS had stronger inhibitory index than that of the original CS, CSS and CMCS. The rule of the compounds against *A. solani* was similar to that of them against *P. asparagi.* The antifungal index of HSACS, LSACS, HSACSS, LSACSS, HSACMCS and LSACMCS at 500 μg mL^{-1} was 66.43%, 56.14%, 48.57%, 44.29%, 61.11% and 52.14%, respectively.

4. Discussion

The Above-mentioned results indicated that the increased antifungal activities of SACS, SACSS and SACMCS against *P. asparagi* and *A. solani* might be attributed to the fact that sulfanilamide group that was grafted onto the chitosan chain and the $–SO_2NH$ group enhanced the antifungal activity of CS, CSS and CMCS. Furthermore, the results also demonstrated that the antifungal activity of them was affected by their molecular weight obviously. Higher molecular weight resulted in better antifungal ability. These results agreed with the previous work.[17] In addition, the results show that the antifungal activity of the compounds had a relationship to their concentration, and higher concentration resulted in higher antifungal activity. These results were consistent with the work of Liu et al.[8] who had demonstrated that with the increase of the concentration, the antibacterial activities of chitosan enhanced.

The exact mechanism of the antimicrobial action of chitosan is still unknown, but different mechanisms have been proposed. Helander reported that the dissolved water-insoluble chitosan increased the permeability of

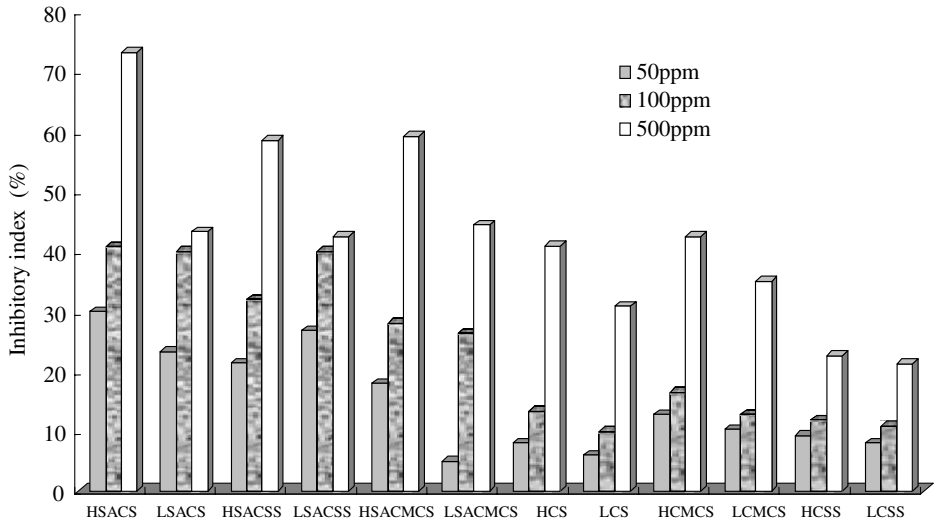


Figure 2. The antifungal activities of CS, SACS, CSS, SACSS, CMCS and SACMCS on *Phomopsis asparagi*.

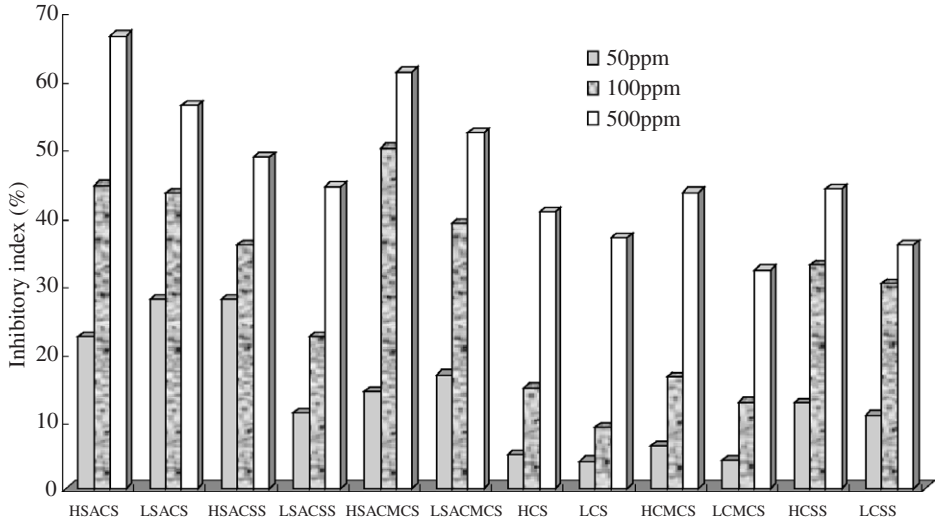


Figure 3. The antifungal activities of CS, SACS, CSS, SACSS, CMCS, SACMCS against *Alternaria solani*.

cell membrane, and ultimately disrupted bacterial cell membranes with the release of cellular contents.[18] Rhoades et al proposed that water-insoluble chitosan molecules could precipitate and stack on the microbial cell surface, thereby forming an impervious layer around the cell. Such a layer can be expected to prevent the transport of essential solutes and may also destabilize the cell wall beyond repair thereby causing severe leakage of cell constituents and ultimately cell death.[19] In this paper, the possible reasons for the antimicrobial activity of the sulfanilamide derivatives of chitosan was supposed as follows: (i) Chitosan could bind on the microbial cell surface to form a film around the cells, so the transport of nutrient into the cells was disturbed. (ii) The sulfanilamide group could cause microbial cell to death.

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