JOURNAL OF Applied Polymer SCIENCE

Comparative Study of the Influence of Active Groups of Chitosan Derivatives on Antifungal Activity

Zhanyong Guo,[1] Jianming Ren,[1,2] Fang Dong,[1] Gang Wang,[1] Pengcheng Li[3]

[1]Yantai Institute of Coastal Zone Research, Chinese Academy of Sciences, Yantai 264003, China

[2]Graduate University of Chinese Academy of Sciences, Beijing 100039, China

[3]Institute of Oceanology, Chinese Academy of Sciences, Qingdao 266071, China

Correspondence to: Z. Guo (E-mail: qdioqdio@yahoo.com.cn) or P. Li (E-mail: pcli@ ms.qdio.ac.cn)

ABSTRACT: In this study, series of chitosan derivatives containing active groups were synthesized and evaluated for their antifungal activity against three crop-threatening fungi, *Fusarium oxysporum. f. sp. Vasinfectum, Alternaria solani*, and *Valsa mali*. Schiff bases of carboxymethyl chitosan (As: 2-(2-hydroxy-5-nitrobenzylideneamino)-6-carboxymethyl chitosan; Bs: 2-(2-hydroxyl-5-chlorobenzaldimino)-6-carboxymethyl chitosan), *N*-substituted carboxymethyl chitosan (An: 2-(2-hydroxyl-5-nitrobenzylamino)-6-carboxymethyl chitosan; Bn: 2-(2-hydroxyl-5-chlorobenzylamino)-6-carboxymethyl chitosan) and 2-urea-carboxymethyl chitosan (Au: 2-(2-nitrophenylurea)-6-carboxymethyl chitosan; Bu: 2-(2-chlorophenylurea)-6-carboxymethyl chitosan) were synthesized, and their antifungal activity was comparatively studied by hypha measurement *in vitro*, respectively. Results obtained from this study revealed that the active groups combined with Schiff bases functional groups (C=N) could strengthen the antifungal activity most effectively among the compounds studied in this work. © 2012 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 000: 000–000, 2012

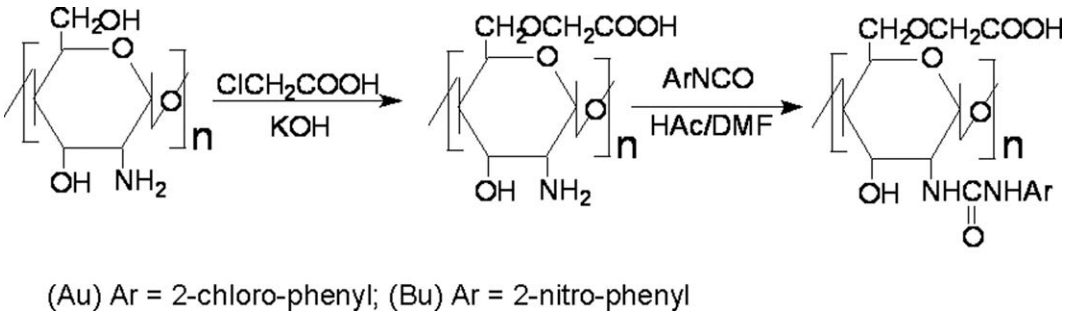
KEYWORDS: active groups; chitosan; antifungal activity

Received 2 March 2011; accepted 20 March 2012; published online
DOI: 10.1002/app.37747

INTRODUCTION

Chitosan, a kind of renewable, abundant natural polysaccharide, consists primarily of 2-amino-2-deoxy-glucopyranose units linked by β-(1–4) linkage.[1,2] It has already been well studied and sufficiently reported that chitosan is nontoxic, biodegradable, and biocompatible, which help it to earn much scientific research attention.[3–5] Among various bioactivities of chitosan, the antimicrobial activity of chitosan against different groups of fungi, has received considerable attention in recent years because of its biodegradability and many articles have been published on the potential applications of chitosan as antifungal agents in agriculture or in food industry.[6–8] However, to meet the requirements of this applications, chitosan usually needs to be modified. Chemical modification is a powerful tool and to promote the antifungal activity of chitosan, various chitosan derivatives were synthesized.[9–12] Earlier, we have reported that 2-(2-hydroxy-5-nitrobenzylideneamino)-6-carboxymethyl chitosan and 2-(5-chloro-2-hydroxybenzylideneamino)-6-carboxymethyl chitosan have superior antifungal activity to those of chitosan, carboxymethyl chitosan (CMC), and 2-(2-hydroxybenzylidenamino)-6-carboxymethyl chitosan.[9] It was hypothesized that the increased antifungal activity may be attributed to those active groups: nitryl and chloro groups, which are used in many fungicides such as pentachloronitrobenzene and chlorothalonil.[9] However, the relationships between these active groups and antifungal activity of chitosan derivatives are still unclear. This work principally aims to further determine the influence of active groups of chitosan derivatives on antifungal activity. For this, based on these active groups, we synthesized series of chitosan derivatives: Schiff bases of CMC, *N*-substituted CMC, and 2-urea-CMC, and their antifungal properties against three strains of phytopathogens were also evaluated by hypha measurement *in vitro*, respectively.

Fusarium oxysporum (ATCC 7808), *Alternaria solani* (ATCC 6396), and *Valsa mali* (ATCC 27054) are plant pathogens. They can cause some widely distributed diseases on vegetables, fruits, and even field crops throughout the word. Control of these plants-threatening fungi could benefit the production of related vegetables, fruits, and crops. So, antifungal activity of the synthesized chitosan derivatives against these three kinds of fungi was estimated.



(Au) Ar = 2-chloro-phenyl; (Bu) Ar = 2-nitro-phenyl

Scheme 1. Synthetic pathway of 2-urea-CMC.

MATERIALS AND METHODS

Materials

Chitosan was purchased from Qingdao Baicheng Biochemical Corp. (Qingdao, China). The degree of deacetylation was 97%, and the viscosity-average molecular weight was 2.0×10^5. The IR spectra were measured by a Nicolet Magne-Avatar 360 instrument (Winconsin, USA) using KBr disks. The elemental analyses (C, H, and N) were performed on a Carlo-Erba 1106 elemental analyzer (Milan, Italy). The other reagents were all analytic grade and used without further purification.

Synthesis of Chitosan Derivatives

Schiff bases of CMC (As: 2-(2-hydroxy-5-nitrobenzylideneamino)-6-carboxymethyl chitosan; Bs: 2-(2-hydroxyl-5-chlorobenzaldimino)-6-carboxymethyl chitosan) and *N*-substituted CMC (An: 2-(2-hydroxyl-5-nitrobenzylamino)-6-carboxymethyl chitosan; Bn: 2-(2-hydroxyl-5-chlorobenzylamino)-6-carboxymethyl chitosan) were prepared according to the method we described previously.[11–14] The synthesis of isocyanates was performed according to the procedures of Maya et al.[13] 2-Urea-CMC was synthesized based on the method of Silva et al.[15] To prepare 2-urea-CMC (Au: 2-(2-nitrophenylurea)-6-carboxymethyl chitosan; Bu: 2-(2-chlorophenylurea)-6-carboxymethyl chitosan), 1 g of CMC was dispersed into 20 mL dimethylformamide (DMF)/glacial acetic acid solution (1/1 v/v). Each isocyanate was then added to this mixture overnight, respectively, and the reactions were performed at 100°C with constant stirring for 4 h. The obtained products were separated by filtration and washed successively with DMF and acetone. The final derivatives were dried at 60°C for 24 h (Scheme 1).

Microorganisms and Biology

Three kinds of pathogenic fungi associated with plant diseases were assembled for this study, which were *F. oxysporum* (ATCC 7808), *A. solani* (ATCC 6396), and *V. mali* (ATCC 27054). They were kindly gifted from Qingdao Academy of Agricultural Sciences. Potato dextrose agar (PDA) was used as the growth medium of the tested fungi, and the PDA was prepared as follows: 300 g of sliced potato was kept boiling in water for 30 min, and then, the resulting boiled juice was strained the broth through cheesecloth. Distilled water was added such that the total volume of the suspension is 1000 mL and 20 g dextrose, 20 g agar powder was then added, and the medium was sterilized by autoclaving at 15 pounds per square inch (100 kPa) for 15 min.

Antifungal Assays

Antifungal assays were performed based on the method of Jasso de Rodríguez et al.[16] Different samples of chitosan, Schiff bases of CMC, *N*-substituted CMC, 2-urea-CMC, and chlorothalonil, used as quality control, were added to sterile petri dishes (9-cm diameter) containing sterilized PDA to give a final concentration of 5, 50, and 500 μg mL^{-1}. The plates were inoculated with 5-mm-diameter plugs taken from the margins of 3 days old colonies of tested fungi on PDA. All the plates were incubated at 27°C till the mycelium reached the edges of the negative control plate (without adding samples), the antifungal index was calculated as follows:

$$\text{Antifungal index } (\%) = (1 - D_a/D_b) \times 100$$

D_a represents the diameter of the growth zone in the test plates and D_b is the diameter of growth zone in the negative control plate.

Each experiment was performed three times, and the data were shown with mean ± S.D. The Scheme method was used to evaluate the differences in antifungal index in the antifungal tests. Results with $P < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Structure of 2-urea-CMC

The elemental analyses results and the substitution degree of chitosan, CMC, and 2-urea-CMC are listed in Table I, and the IR spectra of chitosan, CMC, and 2-urea-CMC are shown in Figure 1. The IR spectra data of chitosan shows peaks assigned to the saccharide structure at 897 and 1154 cm^{-1} and characteristic amino peak at 1600 cm^{-1}. CMC is confirmed by the absorption bands at 1600 [$\nu_{sym}(CO_2)$] and 1416 cm^{-1} [$\nu_{as}(CO_2)$].[18] Compared with the IR spectra of chitosan and CMC, the derivatives of 2-urea-CMC have stronger peaks at about 1654 cm^{-1}, which are assigned to the characteristic

Table I. The Elemental Analyses Results and the Substitution Degree of Chitosan, CMC, and 2-urea-CMC

Compounds	Elemental analyses (%)			Substitution degree[a]
	C	N	H	
Chitosan	44.72	8.70	6.83	
CMC	43.86	6.46	5.96	0.96
Au	48.10	7.42	4.95	0.67
Bu	46.45	10.20	4.72	0.72

[a]Substitution degree, determined according to the method of Chen et al.,[17] refers to the C-6-O substitution degree of CMS, and C-2-NHCONH- of Au and Bu.

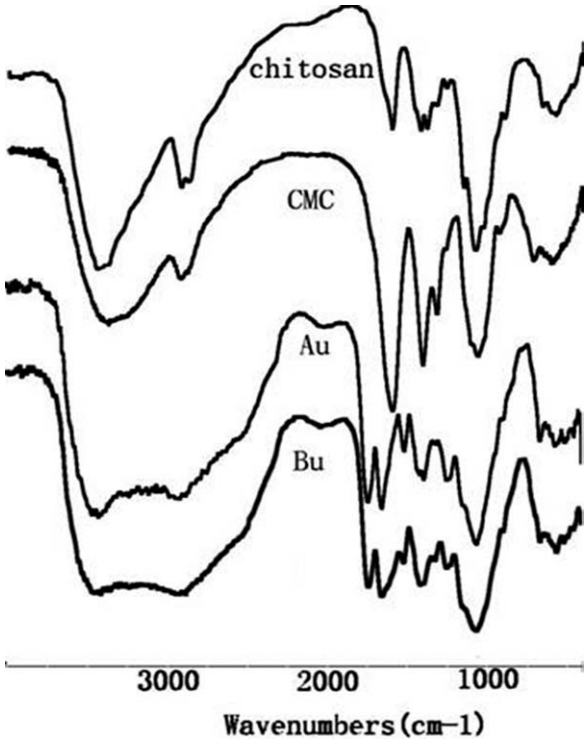


Figure 1. The IR spectra of chitosan, CMC, and 2-urea-CMC.

absorbance of the urea groups (—NHCONH—), and 1736 cm^{-1} assigned to the carboxyl group.[15] Moreover, there are strong peaks at about 1510, 1440, and 720 cm^{-1} corresponding to the benzene groups.[19] These results indicated that the compounds shown in Scheme 1 were successfully obtained.

Antifungal Activity

The antifungal assays indicated that although chitosan and its derivatives could not delay the growth of the pathogenic fungi as effectively as the quality control agent chlorothalonil, which could totally inhibit the growth at 5 $\mu g\ mL^{-1}$, appropriate chemical modifications of chitosan could strengthen its antifungal activity effectively. And all the samples exhibited concentration-dependent inhibitory effect on the hyphal growth of phytopathogen fungi. Figures 2–4 demonstrate the antifungal activity

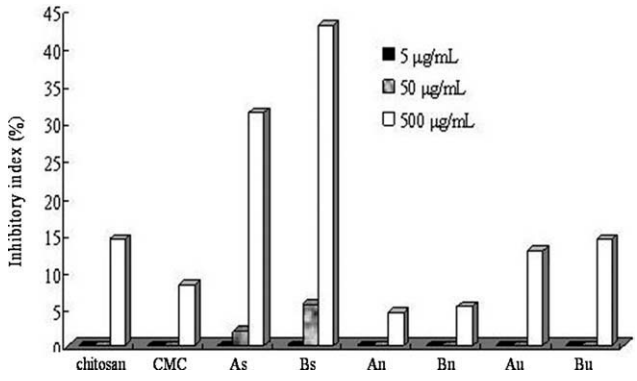


Figure 2. Antifungal activity of chitosan derivatives against *F. oxysporum f. sp. vasinfectum.*

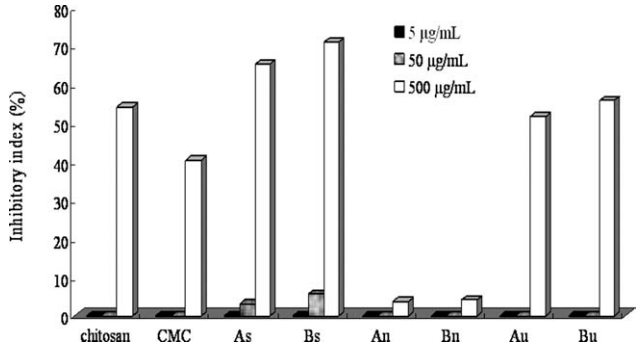


Figure 3. Antifungal activity of chitosan derivatives against *A. solani.*

of chitosan and its derivatives against three different plant pathogenic fungi.

The antifungal activity of chitosan, CMC, Schiff bases of CMC (As, Bs), *N*-substituted CMC (An Bn), and 2-urea-CMC (Au, Bu) against *F. oxysporum* was shown in Figure 2. From Figure 2, we could know that: (1) with a percentage of inhibition of 8.2, CMC has reduced antifungal activity than chitosan at the concentration of 500 $\mu g\ mL^{-1}$, which is consistent with the previous report[9]; (2) compared with chitosan and CMC, Schiff bases of CMC have improved antifungal activity and the inhibitory index is 31.2 % and 43.0% at the concentration of 500 $\mu g\ mL^{-1}$, respectively; (3) 2-urea-CMC has no obvious changes in antifungal ability compared with chitosan and CMC, and the inhibitory index is 12.7% and 14.3% at the concentration of 500 $\mu g\ mL^{-1}$; (4) *N*-substituted CMC has decreased antifungal activity than those of chitosan and CMC. An earlier research proposed that the increased antifungal activity of Schiff bases of CMC was principally due to the active chlorine atom or nitryl group grafted on the chitosan chain.[9] However, both *N*-substituted CMC and 2-urea-CMC possessing these active groups failed to enhance the antifungal activity and *N*-substituted CMC even decreased the antifungal activity. The result is conflict with the former mentioned hypothesis. The chlorine atom or nitro group is found in the molecule of *N*-substitute CMC or 2-urea-CMC in the absence of imino group (C=N). So, it is reasonable to infer that apart from the chlorine atom and nitryl group, the Schiff bases functional group (C=N) also contribute to enhance the antifungal activity of the products. The antifungal activity of chitosan, CMC, Schiff bases of CMC, *N*-substituted CMC, and 2-urea-

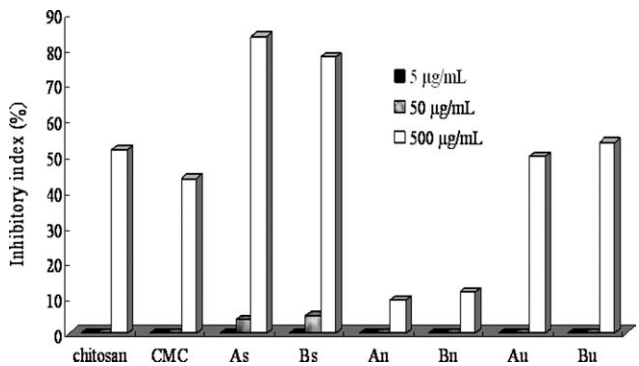


Figure 4. Antifungal activity of chitosan derivatives against *V. mali.*

CMC against *A. solani* and *V. mali* are shown in Figures 3 and 4, respectively. Similarly, Schiff bases of CMC have improved antifungal activity than those of chitosan, CMC, *N*-substituted CMC, and 2-urea-CMC. It should be for the same reason that the increased antifungal activity is caused by both the active antifungal groups and the grafted forms.

CONCLUSIONS

The environmental issues caused by fungicides, especially chemical antifungal agents, cannot be ignored. New environmental-friendly effective substitutes of chemical fungicides need to be explored. In this article, series of chitosan derivatives, CMC, Schiff bases of CMC (As, Bs), *N*-substituted CMC (An Bn), and 2-urea-CMC (Au, Bu), were synthesized, and their antifungal activity against three kinds of phytopathogens was estimated by hypha measurement *in vitro*, respectively. The data showed that of all the derivatives, Schiff bases of CMC (As, Bs) had the best antifungal activity. The antifungal assays *in vitro* models suggested that Schiff bases functional group (C=N) together with the chlorine atom or nitro group could help to strengthen the antifungal activity effectively. Among various chemical modifications of chitosan studied in this article, the most recommended derivative should be Schiff bases of CMC (As, Bs). However, for antifungal applications, comprehensive studies need to be performed to ascertain the safety of chitosan derivatives in experimental animal models and field experiment.

ACKNOWLEDGMENTS

This work was supported by the Knowledge Innovation Program of the Chinese Academy of Sciences, Grant No. kzcx2-yw-225.

REFERENCES

1. Muzzarelli, R. A. A. *Carbohydr. Polym.* **1983,** *3*, 53.
2. Kurita, K. *Progr. Polym. Sci.* **2001,** *26*, 1921.
3. Ravi Kumar, M. N. V. *React. Funct. Polym.* **2000,** *46*, 1.
4. Allan, C. R.; Hadwiger, L. A. *Exp. Mycol.* **1979,** *3*, 285.
5. Hadwiger, L. A.; Loschke, D. C. *Exp. Mycol.* **1981,** *71*, 756.
6. Roller, S.; Covill, N. *Int. J. Food Microbiol.* **1999,** *47*, 67.
7. Ben-Shalom, N.; Ardi, R.; Pinto, R.; Aki, C.; Fallik, E. *Crop Protect.* **2003,** *22*, 285.
8. Benhamou, N. *Trends Plant Sci.* **1996,** *1*, 233.
9. Guo, Z. Y.; Chen, R.; Xing, R. E.; Liu, S.; Yu, H. H.; Wang, P. B.; Li, C. P. *Carbohydr. Res.* **2006,** *341*, 351.
10. Peng, Y. F.; Han, B. Q.; Liu, W. S.; Xu, X. J. *Carbohydr. Res.* **2005,** *340*, 1864.
11. Guo, Z. Y.; Xing, R. E.; Liu, S.; Zhong, Z. M.; Ji, X.; Wang, L.; Li, P. C. *Carbohydr. Res.* **2007,** *342*, 1329.
12. Guo, Z. Y.; Xing, R. E.; Liu, S.; Zhong, Z. M.; Ji, X.; Wang, L.; Li, P. C. *Int. J. Food Microbiol.* **2007,** *118*, 214.
13. Maya, I.; López, Ó.; Maza, S.; Fernández-Bolaños, J. G.; Fuentes, J. *Tetrahedron Lett.* **2003,** *44*, 8539.
14. Guo, Z. Y.; Xing, R. E.; Liu, S.; Zhong, Z. M.; Li, P. C. *Carbohydr. Polym.* **2008,** *73*, 173.
15. Silva, S. S.; Menezes, S. M. C.; Garcia, R. B. *Eur. Polym. J.* **2003,** *39*, 1515.
16. Jasso de Rodríguez, D.; Hernández-Castillo, D.; Rodríguez-García, R.; Angulo-Sánchez, J. L. *Ind. Crop. Prod.21*, 81**2005.**
17. Chen, L. Y.; Du, Y. M.; Liu, Y. *J. Wuhan Univ. (Nat. Sci. Ed.)***2000,** *16*, 191.
18. Sun, T.; Xie, W. M.; Xu, P. X. *Carbohydr. Polym.* **2004,** *58*, 379.
19. Matsumoto, M.; Shimizu, T.; Kondo, K. *Sep. Purif. Technol.* **2002,** *29*, 229.