Structural Characterization and Antimicrobial Activity of Chitosan (CS-40)/Nisin Complexes

Jun Cai,[1] Jianhong Yang,[2] Changgao Wang,[1] Ying Hu,[1] Jianguo Lin,[1] Lihong Fan[3]

[1]*Key Laboratory of Fermentation Engineering (Minister of Education), Hubei Provincial Key Laboratory of Industrial Microbiology, College of Bioengineering, Hubei University of Technology, Wuhan 430068, China*
[2]*Department of Environmental Science, College of Resource and Environmental Science, Wuhan University, Wuhan 430079, Hubei, China*
[3]*College of Chemical Engineering, Wuhan University of Technology, Wuhan 430070, Hubei, China*

Received 19 May 2008; accepted 10 December 2009
DOI 10.1002/app.31936
Published online 22 February 2010 in Wiley InterScience (www.interscience.wiley.com).

ABSTRACT: A complex of chitosan (CS-40) and nisin (CS-40/nisin) was prepared and characterized with Fourier transform infrared spectroscopy and thermal analysis (thermogravimetry, differential thermogravimetry, and differential scanning calorimetry). The results show that the complex formed mainly by electrostatic interaction between the protonated amino group in CS-40 backbone with the carboxylate ion of nisin. Minimum inhibitory concentrations (MICs) were evaluated against Gram-positive bacteria (*Staphylococcus aureus, Bacillus subtilis*, and *Bacillus stearothermophilus*), Gram-negative bacteria (*Escherichia coli, Salmonella enteritidis*, and *Proteus vulgaris*), and fungi (*Fusarium oxysporum*). The results show that the CS-40/nisin solution did inhibit or even more strongly inhibited the growth of all the tested microorganisms, whereas CS-40 did not inhibit the growth of *F. oxysporum* and nisin did not inhibit the growth of Gram-negative bacteria (*E. coli, S. enteritidis*, and *P. vulgaris*). The relative inhibition times of CS-40/nisin solutions with different concentrations and ratios of CS-40 and nisin were also investigated against the seven microorganisms. The results showed that CS-40/nisin solutions with CS-40/nisin concentration ratios of 0.05/0.005, 0.05/0.0025, 0.05/0.00125, and 0.025/0.0001% had higher antimicrobial activity against all tested bacteria and fungi. The relationship between complex formation and antimicrobial activity is discussed. © 2010 Wiley Periodicals, Inc. J Appl Polym Sci 116: 3702–3707, 2010

Key words: bioengineering; biological applications of polymers; biopolymers

INTRODUCTION

Chitosan, the *N*-deacetylated derivative of chitin, is soluble in acid solutions and has a wide range of uses, such as a natural insecticide,[1] a biopolymer for binding metals,[2] and a base for cosmetics.[3] It has attracted considerable interest because of its biological activity, including antimicrobial,[4–7] antitumor,[8,9] and immune-enhancing effects.[10] The antibacterial and antifungal activities of chitosan[11–13] and its derivatives, including *N*-sulfonated and *N*-sulfobenzoyl chitosan,[14] carboxymethylchitosan,[15] a water-soluble chitosan derivative with a fiber-reactive group,[16] quaternary ammonium salt of chitosan,[17] and a nanocomposite of chitosan and silver oxide, and its antibacterial properties[18] have been reported.

Nisin is a small peptide (3353 Da) produced by a common milk bacterium, *Lactococcus lactis*.[19] Its molecular structure includes unusual amino acids and thioether rings,[20] which are presumed to be important in its activity, although the actual mechanism of bactericidal action has yet to be clearly determined. Nisin has demonstrated activity against Gram-positive bacteria, especially spore formers. Although nisin is generally not active against Gram-negative bacteria and fungi, it can be an effective inhibitor of certain Gram-negative bacteria when used in combination with other compounds, such as chelating agents.[21,22] The application of nisin alone or in combination with other antimicrobials to meat surfaces has been shown to reduce the numbers of some meat-spoiling and/or pathogenic bacteria.[23–27] Studies have shown that combinations of lysozyme and nisin are synergistic for antimicrobials activity.[28,29] The antimicrobial activity of chitosan films by the incorporation of garlic oil, potassium sorbate, and nisin[30] and the antimicrobial activity of chitosan-coated paper with nisin and different acids[31] have been reported.

Because chitosan and nisin have antimicrobial activity but their antimicrobial activity and spectrum

Correspondence to: J. Cai (hgcaijun@yahoo.com.cn).

Contract grant sponsor: National Science Foundation of Hubei province of China; contract grant numbers: 2009CDA059, 29977014.
Contract grant sponsor: International Cooperation Foundation of Hubei Province of China; contract grant number: 2009BFA004.
Contract grant sponsor: Key Laboratory of Fermentation engineering (Ministry of Education), Hubei University of Technology.

are low and restricted, the purpose of this study was to investigate the interaction of chitosan with nisin through structural characterization, to evaluate the antimicrobial activity, and to expand the spectrum of the complex of CS-40/nisin compared with chitosan and nisin alone.

EXPERIMENTAL

Materials and reagents

CS-40 from shrimp shells was purchased from Zhejiang Aoxing Biotechnology Co., Ltd. (Taizhou, China); its molecular weight was 412 kDa, and its deacetylation degree was 73.2%. Nisin was purchased from Sigma (USA). All other chemical reagents were of the highest purity commercially available. The dialysis membrane was purchased from Sigma, and the cutoff of the dialysis membrane was 10 kDa. The tested microbial strain included Gram-positive bacteria (*Staphylococcus aureus, Bacillus subtilis*, and *Bacillus stearothermophilus*), Gram-negative bacteria (*Escherichia coli, Salmonella enteritidis*, and *Proteus vulgaris*), and fungi (*Fusarium oxysporum*). *S. aureus, B. subtilis, B. stearothermophilus, E. coli, S. enteritidis*, and *P. vulgaris* were provided by the College of Biotechnology, Hubei University of Technology, China. *F. oxysporum* was provided by Typical Cultural Collection Center at Wuhan University, China.

Preparation of the CS-40/nisin complexes

CS-40 was dissolved in a 1% (w/v) acetate buffer (pH 4.8), and CS-40–acetate (CS-40–Ac) was obtained. The concentration of the CS-40–Ac solution was adjusted to 1% (w/v). Nisin was dissolved in a 0.1% (w/v) acetate buffer (pH 4.8), too, and the concentration of nisin was adjusted to 0.01% (w/v). The CS-40 solution was added to same volume of nisin solution. The resulting solution was carefully stirred for 3 h and then stored for at least 1 day at room temperature to allow equilibration. The solution, including chitosan, nisin, and their complex, was used directly for the assays of antimicrobial activity. The complex solution was lyophilized with a Speed Vac concentrator (Beijing Siping Scientific Instruments, LGJ-10) after dialysis (the cutoff of the dialysis membrane was 10 kDa) against distilled water for more than 3 days for structural characterization. Pure CS-40–Ac and nisin were also prepared under the same conditions. These samples were used for structural characterization.

Characterizations

The IR spectra of CS-40, nisin, and their complex were taken with KBr pellets on a Nicolet 360 Fourier transform infrared (FTIR) spectrophotometer.

Thermogravimetry (TG), differential thermogravimetry (DTG), and differential scanning calorimetry (DSC) curves of the samples were obtained with a Netzsch STA 449C TG/DAT/DSC (Germany) under a nitrogen atmosphere at 0.15 MPa and under an argon atmosphere at 0.10 MPa from 30 to 500°C at a heating rate of 10°C/min.

Cultural conditions of the microorganisms

Bacteria were incubated on nutrient agar (0.3% beef extract, 1% peptone, 0.5% NaCl, 2% agar, pH 7.4–7.6) at 37°C for 24 h. *F. oxysporum* was incubated in potato dextrose agar nutrient medium at 28°C for 48 h.

Assays for antimicrobial activity

To prepare the microorganism suspension, we put one or several colonies of microorganisms from agar plates with a sterile inoculator into sterile saline (0.9%) solution and then diluted the solution to 10^5–10^6 CFU/mL. Sample solutions were autoclaved at 121°C for 20 min. One milliliter of sample solution and 9 mL of autoclaved nutrient agar were poured into autoclaved Petri dishes and cooled; one ring of microorganism suspension was symmetrically spread onto cooled nutrient agar and then incubated at 37°C for bacteria and at 28°C for *F. oxysporum*. We observed and recorded whether colonies were visible with the naked eye after regular incubation times. All treatments were done in triplicate.

The minimum inhibitory concentration was tested as follows: sample solutions were diluted serially twice and then operated as discussed previously. The minimum inhibitory concentration was defined as the lowest concentration of the tested sample at which the microorganism colonies were not visible with the naked eye within 18 to 48 h. The relative inhibition time (RIT) was indicated by the difference between the time when the microorganism colonies were visible in agar plates with tested samples and in the controlled plates.

RESULTS AND DISCUSSION

Complex formation of CS-40 with nisin

FTIR study

In our experiment, CS-40/nisin was dialyzed against distilled water for more than 3 days; if nisin did not form a complex with chitosan, it would have been removed from the system. Then, there would have been no special absorption band of nisin in the IR spectra of CS-40/nisin (dried from a solution containing chitosan and nisin with different proportions).

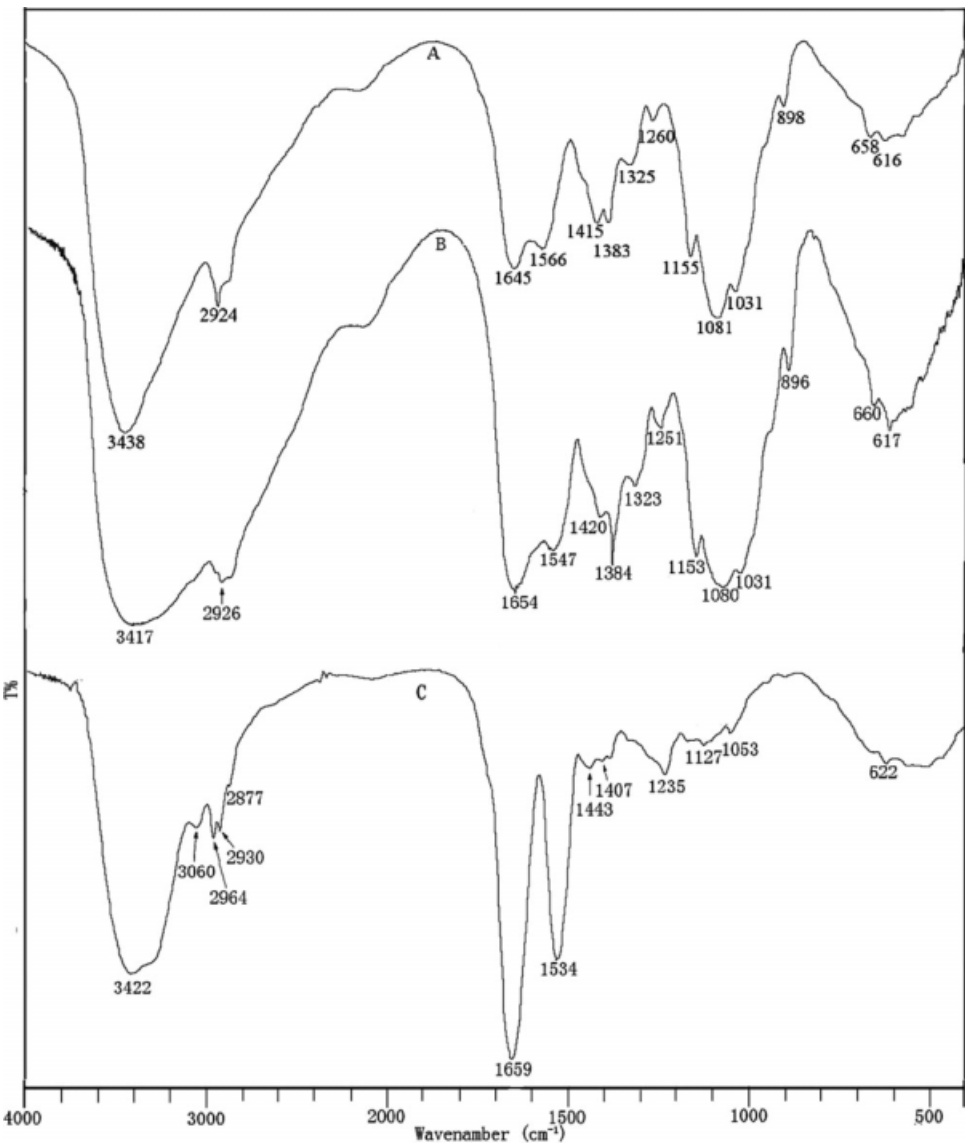

Figure 1 FTIR spectra of (A) CS-40–Ac, (B) CS-40/nisin, and (C) nisin.

Figure 1 shows the FTIR spectra of CS-40–Ac, CS-40/nisin, and nisin. In these spectra, the absorbance bands at 3438 cm^{-1} of CS-40–Ac and 3422 cm^{-1} of nisin were due to O—H stretching vibrations. In the spectra of CS-40–Ac/nisin, the band shifted to the place at 3417 cm^{-1}. The bands became wider, too. This indicated that a hydrogen bond between chitosan and nisin was formed. The absorb bands at 1645 cm^{-1} of CS-40–Ac and 1659 cm^{-1} of nisin were assigned to C=O stretching vibrations of the amide I band.[32–34] For CS-40–Ac, the absorbance bands at 1566 and 1415 cm^{-1} were attributed to asymmetrical and symmetrical COO^- stretching vibrations, respectively. In the spectra of CS-40–Ac/nisin, the absorbance bands of asymmetrical and symmetrical COO^- stretching vibrations shifted to 1547 and 1384 cm^{-1}.[32–34] This indicated that electrostatic interaction between CS-40 and nisin occurred.

In addition, in the spectra of CS-40–Ac, the absorbance band at about 898 cm^{-1} belonged to the vibration of the sugar ring, and this indicated that the C_1 configuration of the sugar residue was β type. The band still remained in the spectra of CS-40–Ac/nisin.[32–34] This indicated that the CS-40's β configuration was not changed in the process of CS-40/nisin complex formation.

Thermal analysis

The TG and DTG curves of CS-40–Ac, CS-40–Ac/nisin, and nisin are shown in Figure 2. The TG and DTG curves of CS-40 showed two stages. The first degradation stage of CS-40 was due to the loss of water. The second stage was due to the degradation of chitosan, and the greatest weight loss point was at 298°C.[35] In the TG and DTG curves of nisin, two

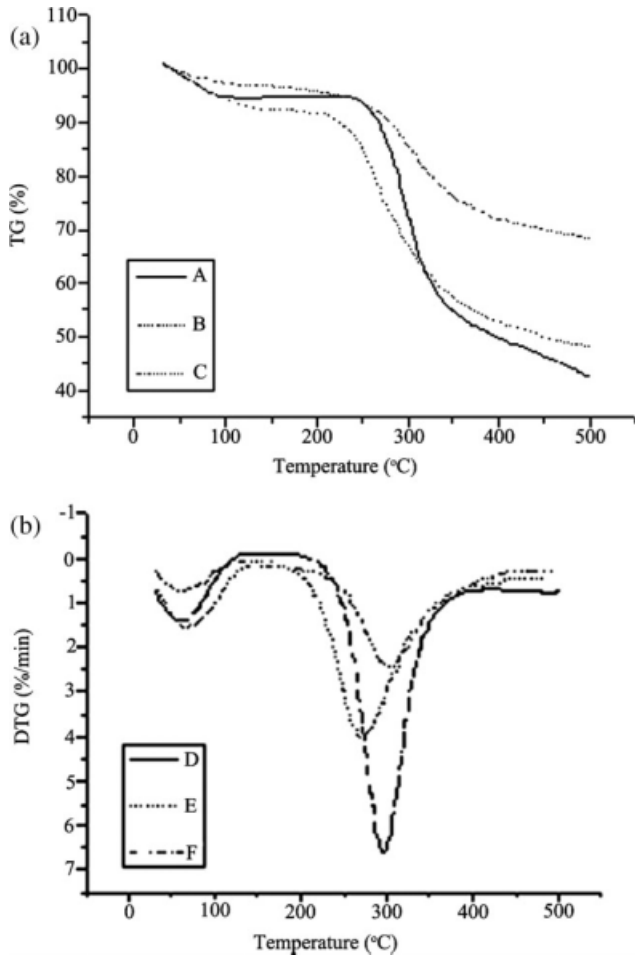

Figure 2 (a) TG curves of (A) CS-40–Ac, (B) CS-40/nisin, and (C) nisin and (b) DTG curves of (D) CS-40–Ac, (E) CS-40/nisin, and (F) nisin.

weight loss stages were shown, too. The first degradation stage was due to the loss of water. The second stage was due to the degradation of nisin, and the greatest weight loss point was at 302°C. Compared with CS-40–Ac and nisin, the greatest weight loss temperature of CS-40/nisin was 268°C, which was shifted to a lower temperature. The reason was the stronger interaction of the chitosan amido and nisin carboxyl group.

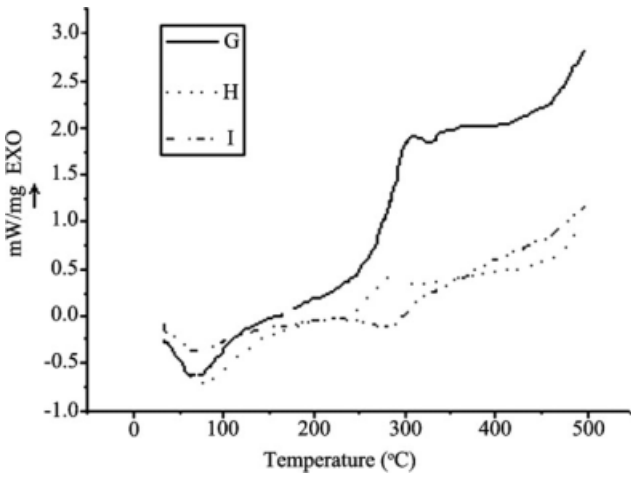

Figure 3 DSC curves of (G) CS-40, (H) CS-40/nisin, and (I) nisin.

The DSC curves of CS-40–Ac, CS-40–Ac/nisin, and nisin are shown in Figure 3. CS-40–Ac had an exothermic peak at 70°C that was associated with the evaporation of water and an exothermic peak at 306°C that corresponded to the decomposition temperature of chitosan.[36,37] As shown in Figure 3, the DSC curve of nisin had two endothermic peaks at 68 and 274°C, they were attributed to the evaporation of water and the decomposition of nisin, respectively. In the curve of complex CS-40/nisin, the endothermic peak of nisin at 274°C disappeared. Compared with CS-40 (306°C), the exothermic peak of CS-40/nisin (286°C) was shifted to a lower temperature. These changes were attributed to the electrostatic interaction of protonated amino groups in the chitosan backbone with the carboxylate ions of nisin. These results were in agreement with those from the FTIR spectra.

TABLE I
MICs of CS-40/Nisin Versus CS-40 and Nisin

Treatment	MIC (%)			
	Acetate buffer	CS-40–Ac	Nisin	CS-40/nisin
S. aureus[a]	0.1	0.05	0.0002	0.05/0.0001
B. subtilis[a]	0.2	0.025	0.0001	0.0125/0.00005
B. stearothermophilus[a]	0.05	0.05	0.00005	0.0125/0.000025
E. coli[b]	0.1	0.05	0.03	0.05/0.005
S. enteritidis[b]	0.2	0.1	0.02	0.05/0.0025
P. vulgaris[b]	0.05	0.025	0.02	0.025/0.0025
F. oxysporum[c]	0.2	0.1	0.01	0.025/0.00125

[a] Gram-positive bacterium.
[b] Gram-negative bacterium.
[c] Fungus.

TABLE II
RITs of CS-40/Nisin Samples with Different Concentrations and Ratios of CS-40 to Nisin

CS-40/nisin concentration ratio (%)	RIT (h)						
	S. aureus[a]	*B. subtilis*[a]	*B. stearothermophilus*[a]	*E. coli*[b]	*S. enteritidis*[b]	*P. vulgaris*[b]	*F. oxysporum*[c]
0.05/0.005	>144	>144	>144	>144	>144	>144	>144
0.05/0.0025	>144	>144	>144	108	>144	>144	>144
0.05/0.00125	>144	>144	>144	48	72	72	96
0.05/0.000625	>144	>144	>144	24	36	36	72
0.05/0.0003125	>144	>144	>144	0	0	0	24
0.05/0.00015625	>144	>144	>144	0	0	0	0
0.025/0.0001	>144	>144	>144	0	0	0	0
0.0125/0.0001	108	>144	>144	0	0	0	0
0.00625/0.0001	24	72	72	0	0	0	0

[a] Gram-positive bacterium.
[b] Gram-negative bacterium.
[c] Fungus.

Antimicrobial activity

As shown in Table I, under the tested conditions, CS-40 had inhibition activity on all of tested microorganisms except *B. stearothermophilus*. Compared with the Gram-positive bacteria, nisin did not have inhibition activity to Gram-negative bacterial and fungi. Boziaris et al.[21] and Ilkka et al.[22] report the same results.

CS-40/nisin inhibited the growth of all seven tested microorganisms. It showed a broader antimicrobial range. The MICs of CS-40 in the CS-40/nisin complex against *S. aureus, E. coli*, and *P. vulgaris* were same as that of CS-40–Ac, but that against *B. subtilis, B. stearothermophilus, S. enteritidis*, and *F. oxysporum* were lower by two, four, two, and four times, respectively. The MICs of nisin in the complex was reduced enormously for every tested microorganism. Hence, the antimicrobial activity of CS-40/nisin was strengthened compared with single CS-40–Ac and nisin. The antimicrobial rang of CS-40/nisin was enlarged. Particularly, the phenomena that single nisin did not have activity toward Gram-negative bacteria was changed by the CS-40/nisin complex. The activity toward Gram-negative bacteria was obvious after the complex was formed. The activity of these complexes toward Gram-positive bacteria increased.

Table II shows the RIT of CS-40/nisin with different concentrations and ratios of CS-40 to nisin. For CS-40/nisin, results could be seen when the concentration of CS-40 was kept at 0.05%. When the concentration of nisin was reduced from 0.005 to 0.00015625%, CS-40/nisin showed inhibition effects on the three tested Gram-positive bacteria. When the concentration of nisin was increased from 0.000625 to 0.005%, the complex showed inhibition effects on the three tested Gram-negative bacteria. When the nisin concentration was lower than 0.0025%, the complex showed shorter RITs for the three tested Gram-negative bacteria. The complex showed inhibition effects on the fungi *F. oxysporum* when the concentration of nisin was changed from 0.0003125 to 0.005%. When the nisin concentration was lower than 0.0025%, the complex showed a shorter RIT for *F. oxysporum*. When the concentration of nisin was kept at 0.0001% and the concentration of CS-40 was decreased from 0.025 to 0.003125%, the RIT of CS-40/nisin decreased obviously, and some lost activity.

In a word, the concentrations of CS-40 and nisin and the ratio of CS-40 to nisin markedly affected the inhibition effect. The results show that CS-40/nisin with CS-40/nisin concentration ratios of 0.05/0.005, 0.05/0.0025, and 0.05/0.00125% had higher antimicrobial activities against all of the tested bacteria and fungi.

CONCLUSIONS

The results of IR spectra and thermal analysis show that the complex of CS-40/nisin formed mainly due to the electrostatic interaction between protonated amino groups in the CS-40 backbone with the carboxylate ions of nisin. The complex showed stronger antimicrobial activity and a broader inhibition range than CS-40–Ac and nisin alone.

References

1. Muzzarelli, R. A. A. In Chitin in Nature and Technology; Muzzarelli, R. A. A.; Jeuniaux, C.; Gooday, G. W., Eds.; Plenum: New York, 1986; p 551.
2. Wan, M.; Petrisor, I. G.; Lai, H.-T.; Kim, D.; Yen, T. F. Carbohydr Polym 2004, 55, 249.
3. Davies, D. H.; Elson, C. M.; Hayes, E. R. In Chitin and Chitosan; Skjak-Braek, G.; Anthonsen, T.; Sandford, P., Eds.; Elsevier Applied Science: London, 1989; p 37.
4. Choi, B. K.; Kim, K. Y.; Yoo, Y. J.; Oh, S. J.; Choi, J. H.; Kim, C. Y. Int J Antimicrob Agents 2001, 18, 553.
5. Jeon, Y. J.; Park, P. J.; Kim, S. K. Carbohydr Polym 2001, 44, 71.

6. No, H. K.; Park, N. Y.; Lee, S. H.; Meyers, S. P. Int J Food Microbiol 2002, 74, 65.
7. Liu, X. F.; Song, L.; Li, L.; Li, S. Y.; Yao, K. D. J Appl Polym Sci 2007, 103, 3521.
8. Mori, T.; Murakami, M.; Okumura, M.; Kadosawa, T.; Uede, T.; Fujinaga, T. J Vet Med Sci 2005, 67, 51.
9. Prashanth, K. V. H.; Tharanathan, R. N. Biochim Biophys Acta 2005, 1722, 22.
10. Suzuki, Y.; Miyatake, K.; Okamoto, Y.; Muraki, E.; Minami, S. Carbohydr Polym 2003, 54, 465.
11. Shahidi, F.; Arachchi, J. K. V.; Jeon, Y. J. Trends Food Sci Technol 1999, 10, 37.
12. Devlieghere, F.; Vermeulen, A.; Debevere, J. Food Microbiol 2004, 21, 703.
13. Zheng, L. Y.; Zhu, J. F. Carbohydr Polym 2003, 54, 527.
14. Chen, C.; Liau, W.; Tsai, G. J Food Prot 1998, 61, 1124.
15. Chen, L. Y.; Du, Y. M.; Liu, Y. J Wuhan Univ Nat Sci Ed 2002, 46, 191.
16. Lim, S. H.; Hudson, S. M. Carbohydr Res 2004, 339, 313.
17. Jia, Z. S.; Shen, D. F.; Xu, W. L. Carbohydr Res 2001, 333, 1.
18. Hu, Z. G.; Chan, W. L.; Szeto, Y. S. J Appl Polym Sci 2008, 108, 52.
19. Martin, I.; Ruysschaert, J. M.; Sanders, D.; Giffard, C. J. Eur J Biochem 1996, 239, 156.
20. Shiba, T.; Wakamiya, T.; Fukase, K.; Ueki, Y.; Teshima, T.; Nishikawa, M. In Nisin and Novel Lantibiotics; Jung, G.; Sahl, H. G., Eds.; ESCOM Science: Leiden, the Netherlands, 1991; p 113.
21. Boziaris, T. S.; Humpheson, L.; Adams, M. R. Int J Food Microbiol 1998, 43, 7.
22. Helander, I. M.; Mattila-Sandholm, T. Int J Food Microbiol 2000, 60, 153.
23. Chung, K.-T.; Dickson, J. S.; Crouse, J. D. Appl Environ Microbiol 1989, 55, 1329.
24. Cutter, C. N.; Siragusa, G. R. Food Microbiol 1996, 13, 23.
25. Gill, A. O.; Holley, R. A. Food Res Int 2000, 33, 83.
26. Holzapfel, W. H.; Geisen, R.; Schillinger, U. Int J Food Microbiol 1995, 24, 343.
27. Zhang, S.; Mustapha, A. J Food Prot 1999, 62, 1123.
28. Chung, W.; Hancock, R. E. Int J Food Microbiol 2000, 60, 25.
29. Nattress, F. M.; Yost, C. K.; Baker, L. P. Int J Food Microbiol 2001, 70, 111.
30. Pranoto, Y.; Rakshit, S. K.; Salokhe, V. M. LWT 2005, 38, 859.
31. Vartiainen, J.; Motion, R.; Kulonen, H.; Marjaana, R.; Skytta, E.; Ahvenainen, R. J Appl Polym Sci 2004, 94, 986.
32. Lillo, E.; Matsuhiro, B. Carbohydr Polym 1997, 34, 397.
33. Qu, X.; Wirsén, A.; Albertsson, A. C. J Appl Polym Sci 1999, 74, 3186.
34. Van de Velde, K.; Kiekens, P. Carbohydr Polym 2004, 58, 409.
35. Tirkistani, F. A. A. Polym Degrad Stab 1998, 60, 67.
36. Qin, C. Q.; Du, Y. M.; Xiao, L. Polym Degrad Stab 2002, 76, 211.
37. Ritthidej, G. C.; Thawatchai, P.; Koiaumi, T. Int J Pharm 2002, 232, 11.