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Water-solubility of chitosan and its antimicrobial activity

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Abstract

Chitosan samples with different molecular weights were prepared by depolymerization with hemicellusase, and water-soluble half *N*-acetylated chitosan samples were obtained by *N*-acetylation with acetic anhydride. The action of chitosans with molecular weights M_w from 1.4×10^3 to 4.0×10^5 on the growth of *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans* was explored by microcalorimetry. The water-soluble half *N*-acetylated chitosans and chitooligomers had no significant antimicrobial activity. Moreover, water-soluble chitosans and chitooligomers promoted the growth of *C. albicans*. In contrast, water-insoluble chitosan in acidic medium exhibited inhibitory effect against these microorganisms. The water-insoluble chitosans with M_w around 5×10^4 were the optimum for antimicrobial action in these tested samples. The antimicrobial mechanism of dissolved water-insoluble chitosan was hypothesized as forming an impervious layer around the cell. The results suggest that optimum chitosan as food preservative should be water-insoluble chitosan from mild depolymerization of native chitosan.

Keywords: Chitosan; Water-solubility; Microorganism; Microcalorimetry

1. Introduction

Chitosan, a natural linear biopolyaminosaccharide is obtained by alkaline deacetylation of chitin, which is the principal component of protective cuticles of crustaceans such as crabs, shrimps, prawns, lobsters and cell walls of some fungi such as *aspergillus* and *mucor*. Chitosan is a weak base and is insoluble in water and organic solvent. However, it is soluble in dilute aqueous acidic solution (pH < 6.5), which can convert glucosamine units into soluble form $R–NH_3^+$ (Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004). It gets precipitated in alkaline solution or with polyanions and forms gel at lower pH.

Chitosan is inexpensive, biodegradable, and nontoxic for mammals. This makes it suitable for use as an additive in the food industry (Koide, 1998; Shahidi, Arachchi, & Jeon, 1999), as a hydrating agent in cosmetics, and more recently as a pharmaceutical agent in biomedicine (Dodane & Vilivalam, 1998; Illum, 2003; Khor & Lim, 2003). The antimicrobial activity of chitosan against different groups of microorganisms has received considerable attention in recent years (Rabea, Badawy, Stevens, Smagghe, & Steurbaut, 2003). Chitosan, however, shows its antibacterial activity only in an acidic medium, which is usually ascribed to the poor solubility of chitosan at high pH (Liu, Du, Wang, & Sun, 2004; No, Lee, Park, & Meyers, 2003). These reported antimicrobial activities might be the effect of dissolved chitosan in acidic media such as acetic acid (Devlieghere, Vermeulen, & Debevere, 2004), lactic acid (Papineau, Hoover, Knorr, & Farkas, 1991), glutamic acid (Roller, & Covill, 1999; Sudharshan, Hoover, & Knorr, 1992) and hydrochloric acid (Chung, Wang, Chen, & Li, 2003). The antimicrobial activity of chitosan was reported to be dependent on its molecular weight and degree of deacetylation DD (Jeon, Park, & Kim, 2001; Yoshihiko, Mayumi, Takahiro, Hiroyuki, Yoshihiro and Ichiro, 2003). The antibacterial effect of chitosan oligomers was also investigated, but the media is still acidic (Choi, Kim, Yoo, Oh, Choi and Kim, 2001; Gerasimenko, Avdienko, Bannikova, Zueva, & Varlamov, 2004; No, Park, Lee, & Meyers, 2002).

Chitosan oligomers, which can be achieved by degradation of chitosan polymer chain, are water-soluble. In addition, water-soluble chitosan can be obtained through a chemical modification in which the degree of substitution is controlled. For instance, it is known that water-soluble chitosan with about 50% DD can be obtained from chitin by hydrolysis with alkali (Kurita, Sannan, & Iwakura, 1977) or from chitosan by

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N-acetylation with acetic anhydride (Kubota, Tatsumoto, Sano, & Toya, 2000).

But up to the present, special information on the correlation of water-solubility and antimicrobial activity of chitosan is lacking. Very few attempts have been made to date to assess the antibacterial activity of chitosan with free $-NH_2$ (not NH_3^+) in detail, although it is necessary for understanding correctly the antimicrobial effect of chitosan.

The main objective of this study was to investigate whether the chitosan with free $-NH_2$ itself has antimicrobial effect, and to explore preliminarily the relationship between the antimicrobial activity and water-solubility of chitosan. Chitosans with different molecular weights and water-solubility were prepared. Microcalorimetry method was employed to determine the action of chitosans on growth of three typical human pathogenic microorganisms in vitro.

2. Experimental

2.1. Materials and equipment

Native chitosan CS0 and hemicellusase solution were obtained from Hubei Yufeng Biology Engineering Co., Ltd (China). Peptone and yeast extract were biological reagents from Oxoid, all other chemicals were of analytical grade and were used without further purification.

The UF membranes (OMEGA) were purchased from PallFiltron Corporation (USA). FT-IR spectra were recorded with KBr pellets on a Perkin–Elmer Spectrum One B spectrophotometer. X-ray diffraction patterns of samples were measured by a Shimadzu XRD-6000 diffractrometer and used a Cu Kα target at 40 kV and 30 mA at 23 °C.

Staphylococcus aureus (CCTCC AB910393) and *Escherichia coli* (CCTCC AB91112) were provided by Chinese Center of Type Culture Collection, Wuhan University, China. *Candida albicans* was provided by the Hospital of Hubei Province, China. The three strains were grown to the stationary phase in nutrient broth at 37 °C for bacteria or at 30 °C for *C. albicans*, and these suspensions were used as the inocula for the microbiological assay.

TAM air (an eight-channel isothermal batch calorimeter for heat flow measurements, whose limit of detectibility is 2 μW) manufactured by Thermometric AB of Sweden was used to obtain the metabolic growth power-time curves of microorganisms, and PicoLog software (Pico Technology Ltd, UK) was used to treat the data (Wadsö, 2002).

2.2. Preparation of samples by enzymatic hydrolysis

CS0 (200 g) was completely dissolved in 3000 ml 5% (w/v) acetic acid with a water bath at 50 °C, and 25 ml of hemicellusase solution was added. At time intervals of 10, 20, 30 and 60 min, 300 ml mixture was respectively taken out, boiled for 10 min, and filtered to remove the enzyme. The filtrate was neutralized with NaOH to pH 10. The precipitate was washed thoroughly with distilled water. The samples CS1, CS2, CS3 and CS4 were collected after drying at 50 °C in vacuum for 48 h, respectively.

The left mixture continued to react for 4 h. Membrane of 10 kDa was used to remove enzyme, and the ultrafiltrate was further separated by membrane of 1 kDa. The filtrate was neutralized with NaOH to pH 10. The precipitate was washed thoroughly with distilled water to obtain sample CS5 after drying over phosphorus pentoxide in vacuum. The filtrate was concentrated, precipitated by adding ethanol, and washed thoroughly with ethanol to obtain water-soluble sample CS6 after drying over phosphorus pentoxide in vacuum.

2.3. Half-N-acetylation of chitosan

2.2 g chitosan was dissolved in 3% acetic acid solution (50 ml), and acetic anhydride (0.56 g) in 50 ml ethanol was added. After stirring at 40 °C for 12 h, the solution was neutralized with NaOH to pH 9, precipitated by adding ethanol, and washed thoroughly with ethanol to obtain half-acetylated sample (HCS1, HCS2 and HCS3) after drying in vacuum.

2.4. Potentioniometric determination of DD

The chitosan (0.3 g) was dissolved in a known excess of 0.1 M HCl acid (20 ml). From the titration of this solution with a 0.1 M NaOH solution, a curve with two inflexion points was obtained. The amount of the acid consumed between these two points was considered to correspond to the amount of the free amino groups in the solution (Tolaimate, Desbrieres, Rhazi, Alagui, Vincendon and Vottero, 2000). The titration was performed with a DELTA-320-S pH meter.

2.5. Molecular weight determination

Weight-average molecular weight (M_w), number-average molecular weight (M_n) and molecular weight dispersion (M_w/M_n) were measured by a gel permeation chromatography (GPC). GPC system incorporated a TSP P100 instrument (USA). The connected columns (TSK G5000-PW and TSK G3000-PW) were used. 0.2 M CH_3COOH/0.1 M CH_3COONa was used as the eluent. The flow rate was maintained at 1.0 ml/min. The eluent was monitored by RI 150 refractive index detector. The sample concentration was approximately 0.4% (w/v). The standards used to calibrate the column were TOSOH pullulan (Japan). All data provided by the GPC system were collected and analysed using the Jiangshen Workstation software package (Dalian, China).

2.6. Estimation of water-solubility

The pH dependence of water solubility of chitosan was evaluated from turbidity. The sample (100 mg) was dissolved in 1% (w/v) HAc (100 ml). Following stepwise addition of concentrated NaOH, the transmittance of the solution was recorded on a UV-1601 Shimadzu spectrophotometer (Japan) using a quartz cell with an optical path length of 1 cm at 600 nm.

2.7. Assays for action of chitosan on microorganisms by microcalorimetry

LB medium (NaCl 5 g, peptone 10 g, yeast extract 5 g/l, pH 7.0) was sterilized by autoclaving for 20 min at 120 °C.

The inocula were homogeneously distributed into 50 ml of LB medium at a concentration 10^6 CFU/ml for bacteria and 10^5 CFU/ml for *C. albicans* by gentle shaking. A liquor of 5 ml of the suspensions were added into 20 ml sterilized ampoules containing test samples and sealed tightly. Sterilized water was used as a control instead of sample. The ampoules were placed in the calorimeter and signals obtained during growth were detected. The experiments were run at 37.00 °C for bacteria or 30.00 °C for *C. albicans*. Each batch experiments were carried out at least twice, the standard error of the mean usually did not exceed 2%.

3. Results and discussion

3.1. Preparation of chitosan samples

A series of chitosan samples CS1, CS2, CS3, CS4, CS5 and CS6 were prepared by depolymerization of CS0 with catalysis of hemicellulase. HCS1, HCS2 and HCS3 were prepared by *N*-acetylation of degraded chitosan with acetic anhydride in acetic acid–water–ethanol complex solvent. The calculated values of M_w and M_w/M_n obtained by GPC are given in Table 1. In this paper, CS0, CS1, CS2, CS3 and CS4 were called as 'water-insoluble chitosan', which were not soluble in pure water (Qin, Du, Zong, Zeng, Liu and Zhou, 2003); HCS1 and HCS2 with about 50% DD were called as 'water-soluble chitosan', which were soluble in pure water (Kubota et al., 2000); CS6 and HCS3 with a low degree of polymerization (DP < 10) were called as chitooligomers, which were very easy to dissolve in pure water.

The IR spectra of degraded chitosan product CS3 and the half *N*-acetylated product HCS1 were shown in Fig. 1. The absorption bands at 1650 and 1600 cm^{-1} in CS3 were, respectively, referenced as amide I band and N–H bending mode of $-NH_2$ (Dong, Xu, & Wang, 2001). HCS1 had the lower relative absorption intensity of $-NH_2$, and higher absorption intensity of amide III band at 1316 cm^{-1}, suggesting that HCS1 had lower DD. The absorption band at 1586 cm^{-1} in HCS1 was considered as the contribution of amide II band, also indicating that HCS1 had lower DD. These data coincided well with the data of potentiometric determination of DD.

Table 1
Parameters of chitosan samples

Sample	$M_w \times 10^{-3}$	M_w/M_n	DD (%)
CS0	400	4.12	85.5
CS1	130	3.69	85.6
CS2	78	3.44	86.1
CS3	48	3.32	86.2
CS4	17	2.91	86.4
CS5	2.8	1.61	87.5
CS6	1.4	1.52	88.0
HCS1	53	3.56	52.1
HCS2	18	2.27	55.6
HCS3	1.4	1.49	54.5

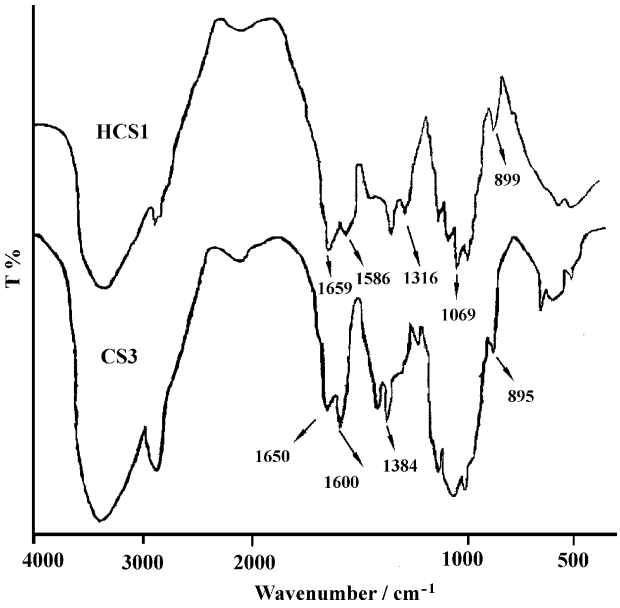

Fig. 1. IR spectra of CS3 and HCS1.

The X-ray diffraction patterns of CS3 and half *N*-acetylated chitosans were shown in Fig. 2. The WAXD pattern of CS3 exhibited its two characteristic peaks at $2\theta = 10.4°$, 19.8° and 21.8°, the 'tendon hydrate polymorph' (Belamie, Domard, & Giraud-Guille, 1997; Qin et al., 2003). HCS1 and HCS2 only displayed a sharply decreased wide peak at $2\theta = 19.8°$, which suggests that they were amorphous and had good water-solubility (Kurita et al., 1977).

3.2. Effect of pH on solubility of chitosan samples

The pH dependence of transmittance of chitosan solution was shown in Fig. 3. All tested samples had good solubility at

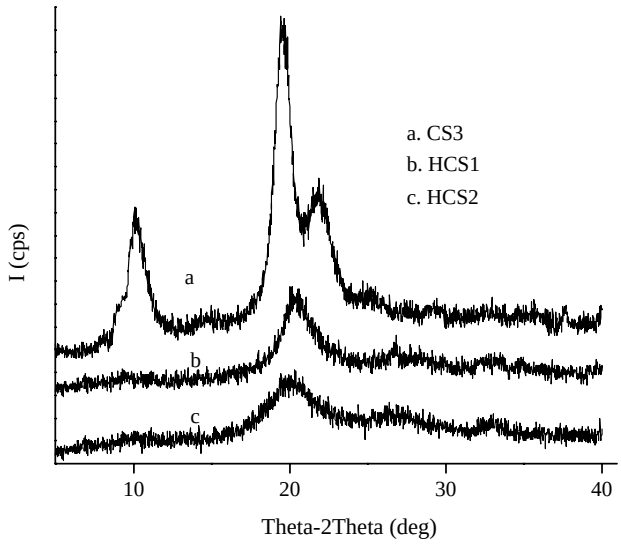

Fig. 2. X-ray diffraction patterns of CS3, HCS1 and HCS2.

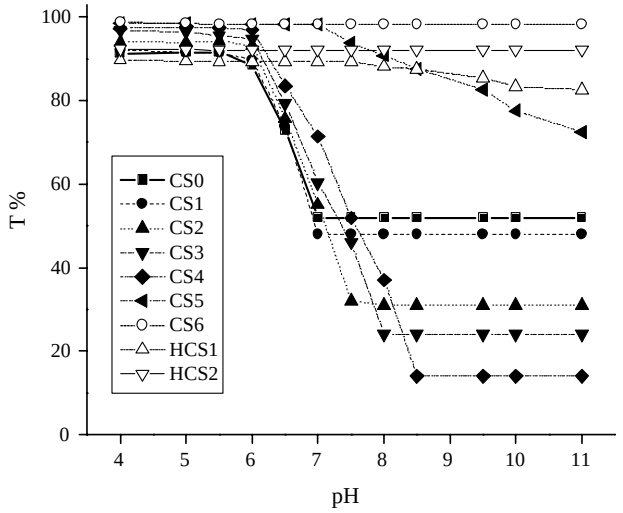

Fig. 3. pH dependence of solubility of chitosan.

pH < 6. The dissolved macromolecular CS0 was easily transferred to floc with addition of NaOH whereas the chitooligomer CS6 were water-soluble over a wide pH range. The water solubility obviously depended on the molecular weight of chitosan. As M_w of chitosan with 85–88% DD decreased, its solubility increased at pH 7. When chitosan was firstly dissolved in aqueous acetic acid, the solubility at neutral pH appeared to be higher than that in pure water. The ionic strength might be a cause for this phenomenon. The half-*N*-acetylated chitosans HCS1 and HCS2 had higher water-solubility than the corresponding chitosans with the similar molecular weight.

3.3. Effect of chitosan on microorganisms

3.3.1. Verification of method

Many techniques are in use for testing microbial activity. Plate counts and MPN-techniques remain the most widely used ones because of their low cost and the unequivocal results. Microcalorimetry is a useful tool for quantitative evaluation of the growth activity of microbial cells based on detection of their metabolic heat (Wadsö, 2002). One of the most prominent features of the microbial growth process is the production of heat. Microbial activity may be quantified by the detection of heat output accompanying all biochemical redox reaction. When the heat is monitored by microcalorimeter, much useful information, both qualitative and quantitative, may be obtained. Calorimetry has also been particularly useful in monitoring cellular metabolism (Lin, Liu, Liu, Qu, & Yu, 2004), and heat measurements have long been used to study metabolism in cells and whole organism. The good reproducibility of calorimetric measurements on cellular systems has been demonstrated in a number of studies on heat production. Modern instruments allow heat quantities as small as microwatt, e.g. evolved by bacteria, to be recorded. The method further provides information on the bacteriostatic and bactericidal effects of various chemicals (Yan, Liu, & Wang, 1999). Moreover, it allows the rapid measurement of microbial activity on line, and is intact on the sample (Rège & Sand, 1998). Because of the possibility of testing batch-wise or in continuous culture, almost all conditions, which influence the growth of the cells, can rapidly be simulated. Thus, microcalorimetry can be used for measurements of metabolism of microorganism.

The power-time curves for growth of microorganisms are continuously recorded by computer. The calorimetric power, P, which reflects the multiplication of the cells, can be used as a parameter to characterize the growth of the cells. Since, $P = dQ/dt$, the area under the curve records the heat output Q released during the experimental period t (Wadsö, 2002).

3.3.2. Effect of water-solubility of chitosan

The optimal pH for growth of *S. aureus* and *E. coli* is 7.2–7.6, and of *C. albicans* is 5.5 (Shen, 2000). Acid has also an inhibitory effect on the growth of *S. aureus* and *E. coli* (Chung et al., 2003). The power-time curves for growth of *C. albicans*, *S. aureus* and *E. coli* were shown in Figs. 4–6, respectively. 10 mM HAc alone stimulated growth of *C. albicans* by comparison with the controls containing no additions. However, addition of HCS2 at concentration of 1.4 g/L greatly promoted growth of *C. albicans* (Fig. 4), although chitosan with free $-NH_2$ is an alkaline substance. Similarly, HCS1 at concentration of 2.8 g/L exhibited no inhibitory effect on *S. aureus* (Fig. 5) and *E. coli* (Fig. 6). The results suggest that the water-soluble chitosans themselves had no antimicrobial activity on yeast *C. albicans* and bacteria *S. aureus* and *E. coli*.

Acid might be very important for the antimicrobial effect of water-insoluble chitosan. In absence of acid, 5 g/L CS0, CS3 or CS4 alone had no inhibitory effect on development of these microorganisms in neutral LB media (data not shown) due to its indissolubility. However, in the presence of 10 mM HAc, 0.8 g/L CS4 completely inhibited growth of *C. albicans* whereas 1.4 g/L HCS2 still promoted growth of *C. albicans*, although both CS4 and HCS2 were dissolved in the acidic

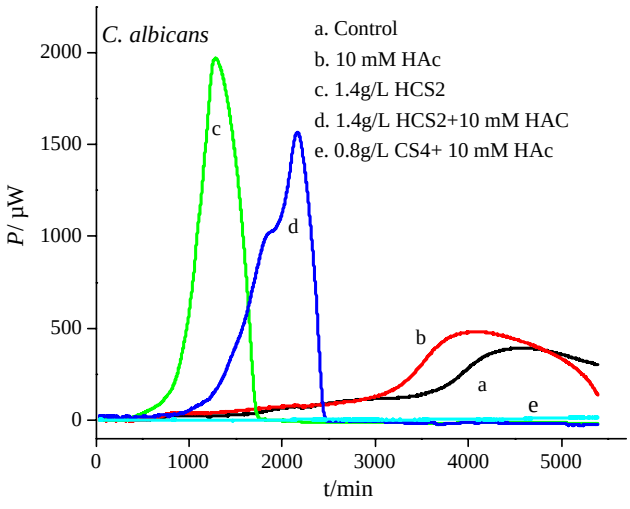

Fig. 4. Power-time curves for growth of *Candida albicans*.

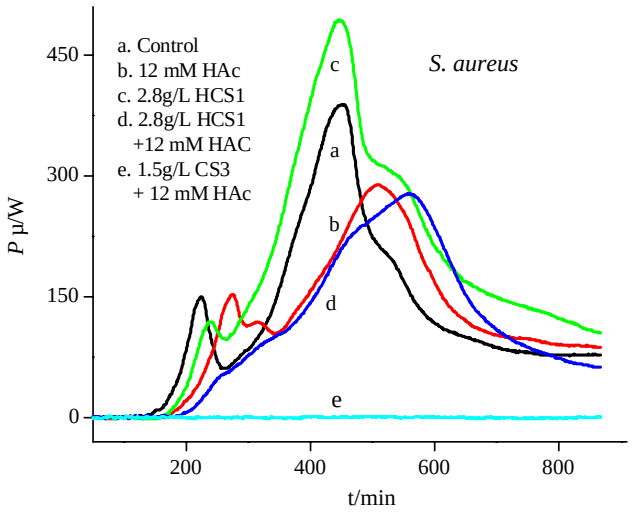

Fig. 5. Power-time curves for growth of *Staphylococcus aureus*.

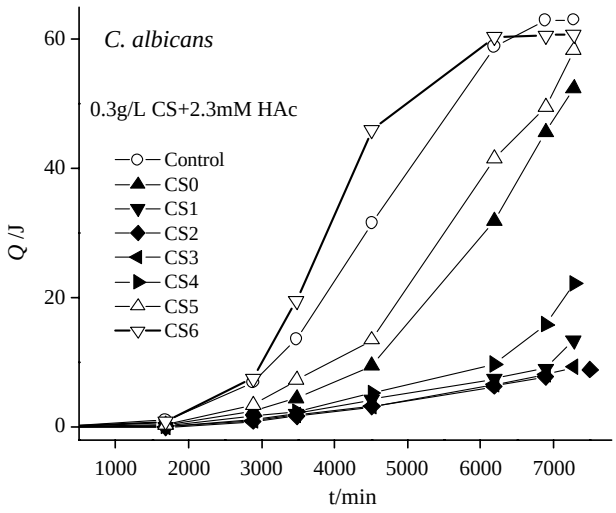

Fig. 7. Influence of chitosan with different M_w on the growth of *C. albicans*.

media. Moreover, CS5 with lower M_w also exhibited the inhibitory effect on growth of *C. albicans* in the acidic medium (Fig. 7), which was contributed to poorer water-solubility of CS5 than that of HCS2. Similarly, in the presence of 10 mM HAc, CS3 at a concentration of 1.5 g/L completely prevented growth of both target bacteria (Figs. 5 and 6), whereas HCS1 at a concentration of 2.8 g/L only slightly delayed the lag phases. These results revealed that the water-soluble chitosan HCS1 and HCS2 in the acetic media had no significantly different antimicrobial effect from the corresponding HAc, which were very different from the water-insoluble chitosan CS3 and CS4 in the acetic media.

3.3.3. Effect of M_w of chitosan

The effect of M_w on antimicrobial activity of chitosan was shown in Figs. 7–9. The DD of chitosans CS0 to CS6 was similar (85–88%). Thus, the effect of DD on antimicrobial activity of these chitosans was excluded. The molecular weight dependence of the antimicrobial activity of chitosan was more pronounced at a low concentration. CS2 and CS3 with middle M_w had higher inhibitory effect than others. Chitooligomer CS6 promoted the growth of *C. albicans*, but slightly inhibited growth of the bacteria. It is worth noting that CS0 with the highest M_w exhibited much weak inhibitory effect in comparison with CS2.

3.3.4. Effect of chitooligomers

The effect of chitooligomers on growth of the microorganisms was shown in Fig. 10. The chitooligomers, especially half *N*-acetylated sample HCS3 promoted growth of *C. albicans*. On the other hand, CS6 slightly inhibited growth of *S. aureus* and *E. coli*, and HCS3 possessed very weak effect. The results suggested that chitooligomers at neutral media have no significant antimicrobial effect.

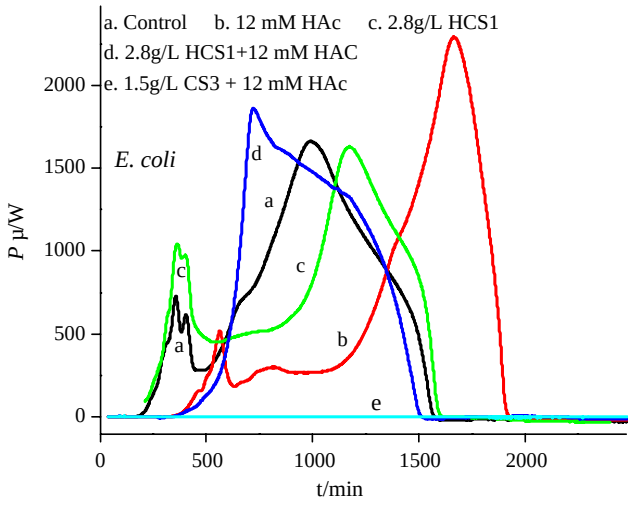

Fig. 6. Power-time curves for growth of *Escherichia coli*.

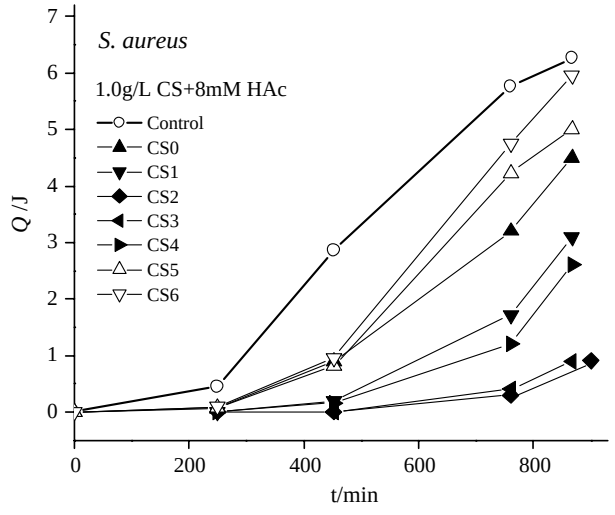

Fig. 8. Influence of chitosan with different M_w on the growth of *S. aureus*.

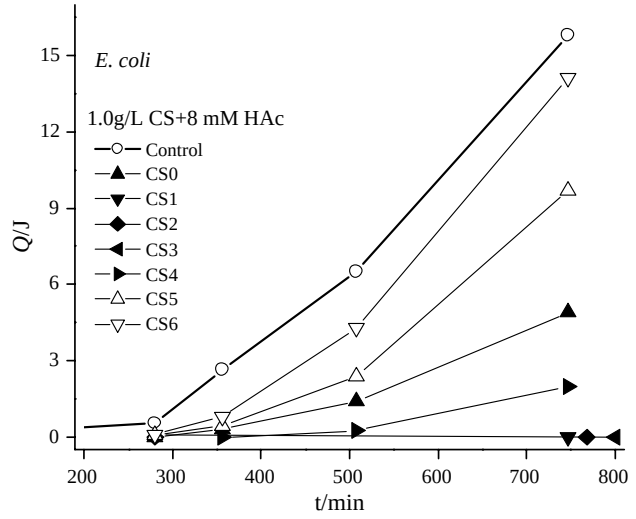

Fig. 9. Influence of chitosan with different M_w on the growth of *E. coli*.

3.4. Discussion

It has been postulated that the antimicrobial action of chitosan occurs as a result of several mechanisms. Chitosan exhibited antibacterial activity only in an acidic medium, which was usually attributed to the poor solubility of chitosan above pH 6.5 and more positively charged polycation with stronger affinity for cells (Rabea et al., 2003). We found that water-soluble chitosan promoted the growth of *C. albicans* even in acidic media whereas dissolved water-insoluble chitosan exhibited inhibitory effect. Actually, the theoretical likelihood of strong interaction between microbial proteins and chitosan at very acidic pH values is low. This was proven by Strand's experiments. The adsorption of chitosans to *E. coli* cells increased strongly with increasing pH. Despite their low charge density, chitosans with lower DD were adsorbed in higher amounts and reversed the cell surface charge most effectively. Chitosan with low molecular weight was adsorbed most. Water-soluble chitosan with DD around 50% had the highest affinity for *E. coli* cells and was adsorbed in highest amounts (Strand, Vårum, & Østgaard, 2003). However, many research results proved that chitosan oligomers had weak or no antimicrobial activity although chitosan oligomers were well water-soluble (Jeon et al., 2001; No et al., 2002). Moreover, Park, Je, Byun, Moon, and Kim (2004) reported that 75% deacetylated chitosan and its degraded products showed the highest antimicrobial activity as compared with 90 and 50% deacetylated chitosan and their degraded products in medium of pH 5.5, suggesting that the antimicrobial activity of chitosan is not proportional to its DD value. In addition, the quaternary ammonium salt of chitosan had stronger antimicrobial activity against these three microorganisms in weak basic conditions than in weak acidic conditions (Qin, Xiao, Li, Fang, Liu and Chen, 2004), which coincided well with the regulation of general quaternary ammonium disinfectants (Gu & Zhang, 1990). Thus, the mode of polycation for antimicrobial activity of chitosan in acidic media should be further discussed.

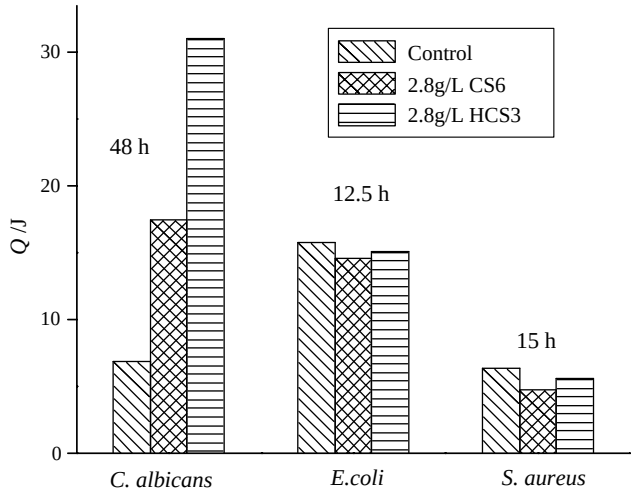

Fig. 10. Effect of chitooligomers on growth of the microorganisms.

Some reported experiments confirmed that the dissolved water-insoluble chitosan increased the permeability of cell membrane, and ultimately disrupted bacterial cell membranes with the release of cellular contents (Helander, Nurmiaho--Lassila, Ahvenainen, Rhoades, & Roller, 2001; Liu et al, 2004). Our research result revealed that the dissolved water-insoluble chitosan exhibited the antimicrobial effect, whereas water-soluble chitosan itself had no significant antimicrobial effect against both bacteria and yeast. Thus, it is conceivable that chitosan molecules have the ability to interact with bacterial surface compounds, and is absorbed on surface of the cells. However, physiological pH in the cell is around neutral, so water-insoluble chitosan molecules can precipitate, and stack on the microbial cell surface, thereby forming an impervious layer around the cell and blocking the channels, which are crucial for living cells. 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 (Ralston, Tracey, & Wrench, 1964). Rhoades & Roller (2000) reported that mild hydrolysis of chitosan enhanced its inhibitory activity against some species of spoilage yeasts grown in complex media, whereas highly degraded chitosan oligomers showed no antimicrobial activity, which was also found by other authors (Knill, Kennedy, Mistry, Miraftab, Smart and Groocock, 2004; No et al., 2002). When the chitosan molecules are too large, the chitosan layer may be not very compact. Contrarily, chitosan with lower molecular weight is more difficult to precipitate and form the layer. The water-soluble chitosan could not form such a layer so that water-soluble chitosan had no antimicrobial activity. Interestingly, all water-soluble chitosan and chitooligomer as alkaline substances promoted growth of *C. albicans* although the optimal pH for growth of *C. albicans* is 5.5. A possible reason is that *C. albicans*' cell wall contains chitin (Maoz, & Neeman, 2000), which has the structural affinity with GlcN and GlcNAc residues. This also proved that the water-soluble chitosan has low cell-toxicity. Many papers have reported that water-soluble chitosan had better physiological

activity such as antitumor activity and immuno-enhancing effects in vivo (Jeon & Kim, 2002; Qin, Du, Xiao & Zhan, 2002; Suzuki, 1996).

4. Conclusions

The water-soluble half-acetylated chitosans and chitooligomers had no significant antimicrobial activity against these microorganisms. Moreover, water-soluble chitosan and chitooligomer promoted the growth of *C. albicans*. However, water-insoluble chitosan in acidic medium exhibited inhibitory effect against the three tested microorganisms. The water-insoluble chitosans with M_w around 5×10^4 in these tested chitosan samples exhibited the best antimicrobial action. The results suggest that water-soluble chitosan is not appropriate for antimicrobial agent, and water-insoluble chitosans with M_w around 5×10^4 have potential for use as a preservative in acidic foods.

Acknowledgements

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References

Belamie, E., Domard, A., & Giraud-Guille, M. M. (1997). Study of the solid state hydrolysis of chitosan in presence of HCl. *Journal of Polymer Science. Part A*, *35*, 3181–3191.

Choi, B. K., Kim, K. Y., Yoo, Y. J., Oh, S. J., Choi, J. H., & Kim, C. Y. (2001). In vitro antimicrobial activity of a chitooligosaccharide mixture against *Actinobacillus actinomycetemcomitans* and *Streptococcus mutans*. *International Journal of Antimicrobial Agents*, *18*, 553–557.

Chung, Y. C., Wang, H. L., Chen, Y. M., & Li, S. L. (2003). Effect of abiotic factors on the antibacterial activity of chitosan against waterborne pathogens. *Bioresource Technology*, *88*, 179–184.

Devlieghere, F., Vermeulen, A., & Debevere, J. (2004). Chitosan: Antimicrobial activity, interactions with food components and applicability as a coating on fruit and vegetables. *Food Microbiology*, *21*, 703–714.

Dodane, V., & Vilivalam, V. D. (1998). Pharmaceutical applications of chitosan. *Pharmaceutical Science and Technology Today*, *1*, 246–253.

Dong, Y. M., Xu, Z. Y., & Wang, J. W. (2001). FT-IR spectroscopy as a tool to determine the degree of substitution in acylated chitosan. *Science in China. Series B*, *31*(2), 153–160.

Gerasimenko, D. V., Avdienko, I. D., Bannikova, G. E., Zueva, O. Y., & Varlamov, V. P. (2004). Antibacterial effects of water-soluble low-molecular-weight chitosans on different microorganisms. *Applied Biochemistry and Microbiology*, *40*(3), 253–257.

Gu, D. H., & Zhang, H. X. (1990). *Science of medical disinfection*. Bejing: Bejing Science and Technology Press.

Helander, I. M., Nurmiaho-Lassila, E. L., Ahvenainen, R., Rhoades, J., & Roller, S. (2001). Chitosan disrupts the barrier properties of the outer membrane of gram-negative bacteria. *International Journal of Food Microbiology*, *71*, 235–244.

Illum, L. (2003). Nasal drug delivery—possibilities, problems and solutions. *Journal of Controlled Release*, *87*, 187–198.

Jeon, Y. J., & Kim, S. K. (2002). Antitumor activity of chitosan oligosaccharides produced in ultrafiltration membrane reactor system. *Journal of Microbiology and Biotechnology*, *12*, 503–507.

Jeon, Y. J., Park, P. J., & Kim, S. K. (2001). Antimicrobial effect of chitooligosaccharides produced by bioreactor. *Carbohydrate Polymers*, *44*, 71–76.

Khor, E., & Lim, L. Y. (2003). Implantable applications of chitin and chitosan. *Biomaterials*, *24*, 2339–2349.

Knill, C. J., Kennedy, J. F., Mistry, J., Miraftab, M., Smart, G., Groocock, M. R., et al. (2004). Alginate fibres modified with unhydrolysed and hydrolysed chitosans for wound dressings. *Carbohydrate Polymers*, *55*, 65–76.

Koide, S. S. (1998). Chitin–chitosan, properties, benefits and risks. *Nutrition Research*, *18*, 1091–1101.

Kubota, N., Tatsumoto, N., Sano, T., & Toya, K. (2000). A simple preparation of half *N*-acetylated chitosan highly soluble in water and aqueous organic solvents. *Carbohydrate Research*, *324*, 268–274.

Kumar, M. N. V. R., Muzzarelli, R. A. A., Muzzarelli, C., Sashiwa, H., & Domb, A. J. (2004). Chitosan chemistry and pharmaceutical perspectives. *Chemical Reviews*, *104*, 6017–6084.

Kurita, K., Sannan, T., & Iwakura, Y. (1977). Evidence for formation of block and random copolymers of *N*-acetyl-D-glucosamine and D-glucosamine by heterogeneous and homogeneous hydrolyses. *Makromolekulare Chemie*, *178*, 3197–3202.

Lin, X. Y., Liu, Y., Liu, P., Qu, S. S., & Yu, Z. N. (2004). Microcalorimetric investigation on the growth model and the protein yield of *Bacillus thuringiensis*. *Journal of Biochemical and Biophysical Methods*, *59*, 267–274.

Liu, H., Du, Y. M., Wang, X. H., & Sun, L. P. (2004). Chitosan kills bacteria through cell membrane damage. *International Journal of Food Microbiology*, *95*, 147–155.

Maoz, M., & Neeman, I. (2000). Effect of inula viscose extract on chitin synthesis in dermatophytes and *Candida albicans*. *Journal of Ethnopharmacology*, *71*, 479–482.

No, H. K., Lee, S. H., Park, N. Y., & Meyers, S. P. (2003). Comparison of physicochemical, binding, and antibacterial properties of chitosans prepared without and with deproteinization process. *Journal of Agricultural Food Chemistry*, *51*, 7659–7663.

No, H. K., Park, N. Y., Lee, S. H., & Meyers, S. P. (2002). Antibacterial activity of chitosans and chitosan oligomers with different molecular weights. *International Journal of Food Microbiology*, *74*, 65–72.

Papineau, A. M., Hoover, D. G., Knorr, D., & Farkas, D. F. (1991). Antimicrobial effect of water-soluble chitosans with high hydrostatic pressure. *Food Biotechnology*, *5*, 45–57.

Park, P. J., Je, J. Y., Byun, H. G., Moon, S. H., & Kim, S. E. (2004). Antimicrobial activity of hetero-chitosans and their oligosaccharides with different molecular weights. *Journal of Microbiology and Biotechnology*, *14*, 317–323.

Qin, C. Q., Du, Y. M., Xiao, L., Zhan, L., & Gao, X. H. (2002). Enzymic preparation of water-soluble chitosan and their antitumor activity. *International Journal of Biological Macromolecules*, *31*, 111–117.

Qin, C. Q., Du, Y. M., Zong, L. T., Zeng, F. G., Liu, Y., & Zhou, B. (2003). Effect of hemicellulase on the molecular weight and structure of chitosan. *Polymer Degradation and Stability*, *80*, 435–441.

Qin, C. Q., Xiao, Q., Li, H. R., Fang, M., Liu, Y., Chen, X. Y., et al. (2004). Calorimetric studies of the action of chitosan-*N*−2-hydroxypropyl trimethyl ammonium chloride on the growth of microorganisms. *International Journal of Biological Macromolecules*, *34*, 121–126.

Rabea, E. I., Badawy, M. E. T., Stevens, C. V., Smagghe, G., & Steurbaut, W. (2003). Chitosan as antimicrobial agent: Applications and mode of action. *Biomacromolecules*, *4*, 1457–1465.

Ralston, G. B., Tracey, M. V., & Wrench, P. M. (1964). The inhibition of fermentation in baker's yeast by chitosan. *Biochimica et Biophysica Acta*, *93*, 652–655.

Rège, H., & Sand, W. (1998). Evaluation of biocide efficacy by microcalorimetric determination of microbial activity in biofilms. *Journal of Microbiological Methods*, *33*, 227–235.

Rhoades, J., & Roller, S. (2000). Antimicrobial actions of degraded and native chitosan against spoilage organisms in laboratory media and foods. *Applied and Environmental Microbiology*, *66*, 80–86.

Roller, S., & Covill, N. (1999). The antifungal properties of chitosan in laboratory media and apple juice. *International Journal of Food Microbiology*, *47*, 67–77.

Shahidi, F., Arachchi, J. K. V., & Jeon, Y. J. (1999). Food applications of chitin and chitosan. *Trends in Food Science and Technology*, *10*, 37–51.

Shen, P. (2000). *Microbiology*. Beijing, China: Higher Education Press.

Strand, S. P., Vårum, K. M., & Østgaard, K. (2003). Interactions between chitosans and bacterial suspensions: Adsorption and flocculation. *Colloids and Surfaces B: Biointerfaces*, *27*, 71–81.

Sudharshan, N. R., Hoover, D. G., & Knorr, D. (1992). Antibacterial action of chitosan. *Food Biotechnology*, *6*, 257–272.

Suzuki, S. (1996). Studies on biological effects of water soluble lower homologous oligosaccharides of chitin and chitosan. *Fragrance Journal*, *15*, 61–68.

Tolaimate, A., Desbrieres, J., Rhazi, M., Alagui, A., Vincendon, M., & Vottero, P. (2000). On the influence of deacetylation process on the physicochemical characteristics of chitosan from squid chitin. *Polymer*, *41*, 2463–2469.

Wadsö, I. (2002). Isothermal microcalorimetry in applied biology. *Thermochimica Acta*, *394*, 305–311.

Yan, C. N., Liu, Y., & Wang, T. Z. (1999). Thermochemical studies of the toxic actions of heavy metal ions on *Rhizopus nigricans*. *Chemosphere*, *38*, 891–898.

Yoshihiko, O., Mayumi, S., Takahiro, A., Hiroyuki, S., Yoshihiro, S., Ichiro, N., et al. (2003). Antimicrobial activity of chitosan with different degrees of acetylation and molecular with different degrees of acetylation and molecular weights. *Biocontrol Science*, *8*, 25–30.