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Antiprotozoal Activity of Deacetylated Chitosan Oligosaccharide (dp 2–8) on *Trichomonas vaginalis*

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Abstract Deacetylated chitosan oligosaccharide (COS) had effective antiprotozoal activity against *Trichomonas vaginalis* (Minimal Inhibitory Concentration, MIC 0.25%), whereas 80% acetylated COS showed no antiprotozoal activity (MIC >1%). On the other hand, 80% acetylated COS showed growth stimulatory activity against the protozoa. When *T. vaginalis* was treated with 98% deacetylated COS at 0.25% concentration, the viability of the protozoa was rapidly decreased within 15 min, and the protozoa completely died within 40 min. Ultrastructural changes of trichomonads treated with COS included a loss of defined nuclear membrane and endoplasmic reticulum membranes, an increase in the number of free ribosome, vacuolation, and ultimately lysis of the cell membrane. These results indicate that deacetylated COS can be used as an antitrichomonal agent, although its lethal mechanism is not known.

Key words: Chitosan, chitosan oligosaccharide, *Trichomonas vaginalis*, antitrichomonal agent

Trichomonas vaginalis is the causative agent of trichomoniasis, a common cause of vaginitis; however, few resources have yet been devoted to its control. Until recently, metronidazole was the only efficacious antibiotic available for the treatment of trichomoniasis [1]. More recently, however, appreciation of the high rates of disease and of associations of trichomoniasis in women with adverse outcomes of pregnancy, and increased risk for human immunodeficiency virus infection necessitated a need for increased efforts of control [2]. It is estimated that approximately 2.5 to 5% of all cases of trichomoniasis display some level of resistance to treatment with metronidazole [3], and therefore, the development of antitrichomonal agents is urgently needed.

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Previous reports have indicated that chitosan has an antitumor effect [4, 5], an antibacterial effect [6, 7], an antifungal effect [8], and a prebiotic effect [9]. However, antiprotozoal activities of chitosan have not yet been reported until now. Chitosan is prepared by alkaline deacetylation of chitin, an insoluble polymer obtained from the shells of crustaceans such as crabs and shrimps [10]. Polymeric chitosan is soluble in weakly acidified water. It has a high molecular weight, high viscosity, and is insoluble at pHs above 6.3 (the pKa of chitosan). However, chitosan oligomer has a low viscosity, and is freely soluble at neutral pH. Furthermore, the antibacterial effect of chitosan seems to be closely related to its degree of deacetylation [6, 11].

The main objective of this study was to investigate the feasibility of the growth inhibitory effect of COS on *T. vaginalis* using a defined COS. The COS used in the present study was prepared by enzymatic hydrolysis, and chitosan oligomer was isolated by size exclusion chromatography and analyzed by MALDI-TOF Mass spectrometry. The antiprotozoal activity of COS was tested on *Trichomonas* in terms of MIC, growth, and viability, and the effect of COS on the growth and fine structure of exponentially growing *T. vaginalis* cells was also examined.

Materials and Methods

Materials and Organisms

The chitosan polymer used had 98% deacetylation, and the chitosanase derived from *Bacillus* sp. was kindly provided by Kunpoong Bio Co., Ltd., Seoul, Korea.

Microbial culture media were purchased from Difco (U.S.A.). Other chemicals were obtained from Sigma-Aldrich (U.S.A.). Standard strains of *T. vaginalis* YS-10 and *Acanthamoeba culbertsoni* ATCC 30171 were obtained from the Korean National Institute of Health. The standard strains of bacteria were *Escherichia coli* ATCC 25922,

Staphylococcus aureus ATCC 25923, *Candida albicans* ATCC 36801, and *Lactobacillus acidophilus* KCTC 3111.

Preparation of Chitosan Oligomer and N-Acetylated COS

Deacetylated COS (dp 2–8) was prepared by the method previously reported [9]. The chitosan solution (2 g/100 ml) was incubated with 2 units of chitosanase for 4 h at 50°C. To isolate the chitosan oligomer by size exclusion chromatography, the hydrolysate was applied to a Bio-gel P10 column (2×80 cm) and eluted with distilled water. The chitosan oligomer fraction was collected and dried in a freeze dryer. The white powder obtained was analyzed using a MALDI-TOF mass spectrometer (Voyager DE, Perkin-Elmer PerSeptive Biosystem). The composition of the oligomer was calculated from their peak intensities in the MALDI-TOF mass spectrum. The COS was found to be composed of 33.6% of disaccharide, 16.9% of trisaccharide, 15.8% of tetrasaccharide, 12.4% of pentasaccharide, 8.3% of hexasaccharide, 7.1% of heptasaccharide, and 5.9% of octasaccharide. N-Acetylated COS (acetylation 50–80%) was prepared by reaction of deacetylated COS with acetic unhydride (Fig. 1) [12]. Thus, COS (1 g) was dissolved in 100 ml of 10% acetic acid, and acetic anhydride was added. For the preparation of 50% and 80% acetylated COS, the molar ratio of acetic anhydride to glucosamine unit was adjusted to 1:10 and 1:30, respectively. After stirring at ambient temperature for 5 h, aqueous NaOH was added until the pH reached 8–9, in order to stop the reaction. The reaction mixtures were precipitated by metanolic KOH and repeatedly washed with methanol using a centrifuge. Finally, it was lyophilized. N-Acetyl glucosamine content of COSs was determined by Morgan-Elson reaction [13].

Culture

The microorganisms were incubated aerobically for 24 h at 37°C, and the stock cultures were stored in 10% glycerol at −70°C until required. Bacteria were cultured in brain-heart infusion medium, yeast was cultured in Sabouraud dextrose medium, and protozoa were cultivated axenically in trypticase-yeast extract-maltose (TYM) medium supplemented with 10% (v/v) heat-inactivated horse serum, penicillin (1,000 IU/ml), and streptomycin sulfate (100 μg/ml) in aerobiosis at 37°C. To examine the effects of various factors on cell growth, the parasite was cultivated overnight in the basal medium at 37°C, diluted to 10^6 cells/ml, and a 100-ml aliquot was then inoculated into 5 ml of basal medium containing COS. The protozoa were cultured in a 5% CO_2 incubator.

Growth Measurement

Total cell number was counted with a hematocytometer. Viable cell count was carried out by the trypan blue dye exclusion method. The diluted cell suspensions were mixed well with an equal volume of 0.1% trypan blue in PBS, and a 15 μl aliquot was transferred to the hematocytometer chamber, and the unstained cells were then counted under a light microscope as viable cells.

Antibacterial and Antiprotozoal Activity

The MICs of the various acetylated COS against bacteria were determined by using the two-fold agar dilution method. Overnight culture broth from a stock culture was diluted to McFarland No. 0.5 with phosphate-buffered saline (PBS; 0.8% NaCl, 0.02% KH_2PO_4, 0.115% Na_2HPO_4, w/v, pH 7.4), and inoculated into a basal medium agar plate supplemented with various concentrations of COSs (0–5%). Bacteria were cultured in aerobic incubators for 24–48 h. After incubation, the appearance of the colony on the agar plate was examined with the naked eye, and the minimum concentration to show no colony was determined as the MIC. MICs of COSs against protozoa were determined by the two-fold broth dilution method. Fresh cultured protozoa were counted, and the cells were then inoculated in 5 ml of TYM medium supplemented with various concentrations of COS (0–5%). After incubation for 48–72 h at 37°C, the turbidity of the broth was compared with not-inoculated control with the naked eye, and the minimum inhibitory concentration, which showed no turbidity of the broth, was determined.

Time-Kill Activity of COS Towards Protozoa

Time-killing activity of 98% deacetylated COS towards protozoa was determined by an *in vitro* killing assay. To obtain mid-logarithmic phase cells, 5 ml of TYM broth was inoculated with 100 μl of a fresh protozoa culture and incubated at 37°C for 24 h. The culture was centrifuged (2,000 ×*g*, 20 min, 4°C), and the pellet was washed once with PBS and then adjusted cells were inoculated into TYM medium containing COS. After the cells were

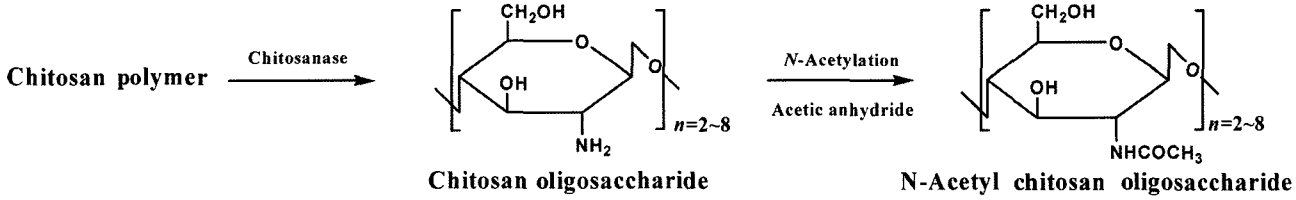

Fig. 1. Preparation of COS and N-acetylated COS.

treated with COS, the surviving cells were counted by the trypan blue dye exclusion assay.

Electron Microscopy

Samples for transmission electron microscopy were fixed 15–60 min after the addition of the COS to the culture medium. For transmission electron microscopy, protozoa were fixed overnight with 2.5% glutaradehyde in 0.1 M cacodylate buffer (pH 7.4), washed with three changes of buffer, and postfixed in 1% osmium tetraoxide in the same buffer for 1 h. After fixation, protozoa cells were washed three times in the same buffer, and dehydrated with an ethanol series (60%, 70%, 80%, 90%, and absolute ethanol for each 10 min). The dehydrated protozoa cells were treated with propylene oxide for 1 h, embedded in epon 812 resin, and hardened at 60°C for 2 days. The epon block was sectioned with an ultramicrotome, stained with uranyl acetate and lead nitrate for 30 min, and examined under an electron microscope.

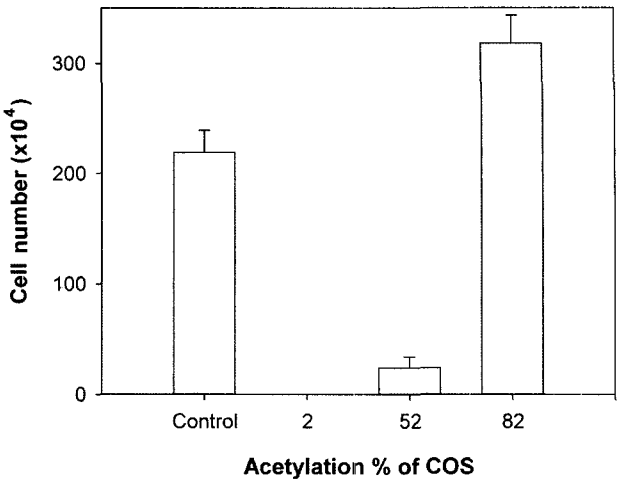

Fig. 2. Effect of acetylation of COS on growth of protozoa. *Trichomonas vaginalis* was cultured in TYM broth supplemented with 0.25% of various acetylated COS, and growth was measured after 72 h. Results are given as means±SD of triplicate samples.

RESULTS

MIC of COSs on *Trichomonas*

The antimicrobial effects of acetylated and deacetylated COSs against bacteria and protozoa were examined (Table 1). Fully deacetylated COS was found to have potent inhibitory effects against *T. vaginalis* (MIC 0.25%) but not against *Acanthamoeba* and bacteria (MIC >1%). The MICs of 52% and 82% acetylated COSs against *T. vaginalis* were 0.5% and >1%, respectively. The acetylated or deacetylated COSs had no inhibitory effect on bacteria.

Effect of Acetylation of COS on the Growth of *T. vaginalis*

In order to study the acetylation effect of COS on the growth of protozoa, the protozoa were cultured in a TYM medium supplemented with COSs for 48 h at 37°C (Fig. 2). Deacetylated COS completely inhibited the growth of the protozoa at 0.25% concentretion. Furthermore, 50% acetylated COS inhibited the growth of the protozoa by 90% of that achieved by 0.25% COS, whereas 82% acetylated COS showed a growth stimulatory effect on the protozoa.

Table 1. MIC of COS against microorganisms.

Organisms	MIC (%)		
	Acetylation of COS		
	2%	52%	82%
Escherichia coli ATCC 25922	>1	>1	>1
Lactobacillus acidophilus KCTC 3111	>1	>1	>1
Staphylococcus aureus ATCC 25923	>1	>1	>1
Candida albicans ATCC 36801	>1	>1	>1
Trichomonas vaginalis YS-10	0.25	0.5	>1
Acanthameba culbertsoni ATCC 30171	>1	>1	>1

MICs against bacteria and yeast were measured by the agar dilution method, and MICs against protozoa were tested by the broth dilution method according to Materials and Methods.

Effect of Concentration of COS on the Growth of *Trichomonas*

After cultivating the protozoa in the TYM broth supplemented with various concentrations of acetylated or deacetylated COSs (0–0.5%) for 72 h at 37°C, the cell growth was measured. As shown in Fig. 3, deacetylated COS was found to have a more potent growth inhibitory effect on the protozoa than that of 50% acetylated COS. Deacetylated COS completely inhibited the growth of the protozoa at the concentration of 0.25% COS, and inhibited 60% of the protozoa growth at 0.125% COS within 72 h of cultivation. When the cells were cultivated in 50% acetylated COS, the amount of cell growth was completely inhibited at 0.5% COS, whereas the cell growth was inhibited by 50–60% at 0.1–0.25% COS. When the protozoa were cultivated with 0.125–1% of 80% acetylated COS, the specific growth rate and the maximum growth were increased.

In Vitro Killing Assay for Antiprotozoal Activity

The time-kill studies were performed by exposing the protozoa to 1 or 2×MIC of 98% deacetylated COS for 1 h. Fig. 4 shows that 98% deacetylated COS at 1×MIC (0.25%) exhibited a large initial drop (90% lethality) of viability after 15 min, and the viable cells were slowly decreased for 1 h. The COS at 2×MIC (0.5%) completely eradicated the protozoa after only 20 min. On the other hand, metronidazole did not affect the viability of *T. vaginalis*, even at 2×MIC (2 μg/ml).

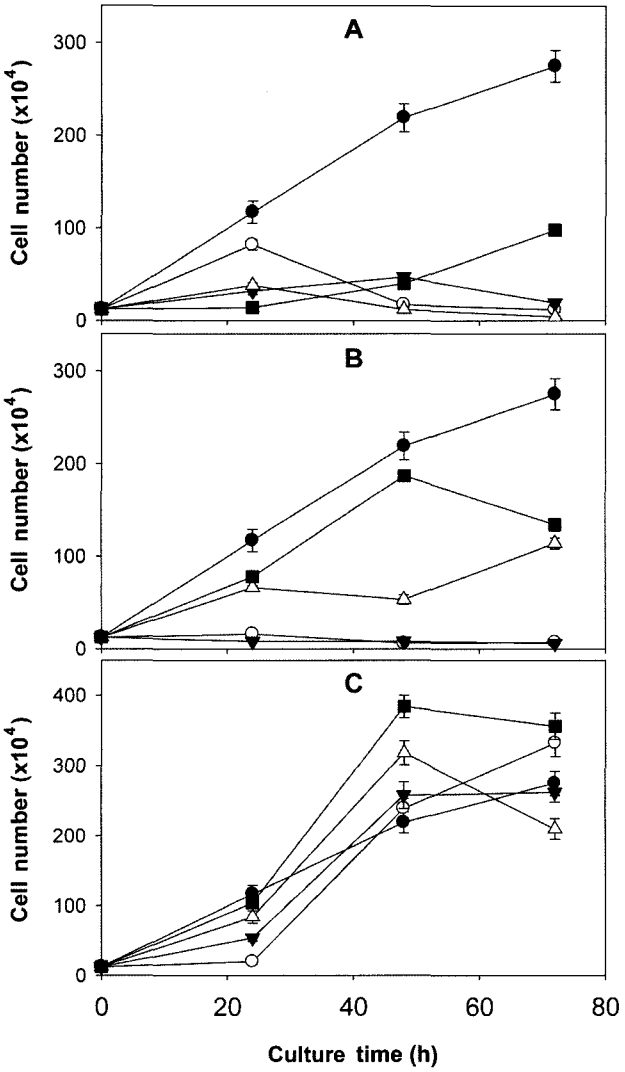

Fig. 3. Effect of concentration of COS on protozoa growth at different cultivation times.
Trichomonas vaginalis was cultured in TYM broth supplemented with 2% acetylated COS (**A**), 50% acetylated COS (**B**), or 80% acetylated COS (**C**), and growth was measured every 24 h. Control (●), 1% (○), 0.5% (▼), 0.25% (△), 0.125% (■). Results are given as means±SD of triplicate samples.

Effect of COS on Ultrastructure *In Vitro*

The first ultrastructural changes seen in electron micrographs occurred after 15 min, showing alteration of the matrix of the hydrogenosomes and decreased movement of flagella and undulating membrane (Figs. 5A and 5B). The fine structure of the cytoplasm was changed within 60 min; degenerative changes of the endoplasmic reticulum and Golgi apparatus occurred, and the vacuoles were large and their size and numbers increased (Fig. 5C). The electron-density of the cytoplasmic matrix was higher in the cells treated with COS than in control culture cells. In the cells exposed to metronidazole for 60 min, no morphological changes were seen (Fig. 5D).

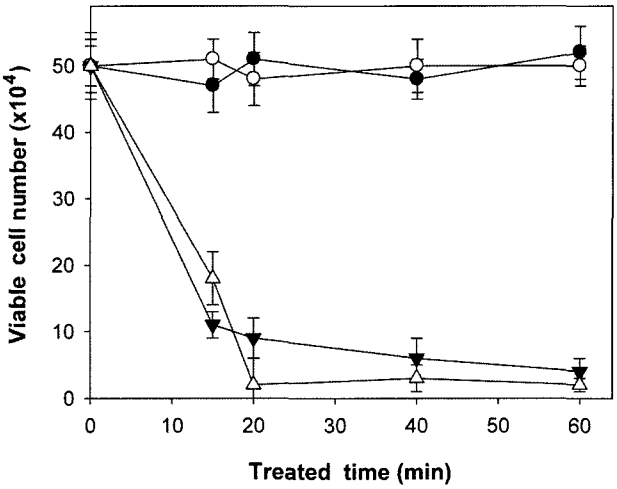

Fig. 4. Time-killing curve of deacetylated COS against protozoa.
Trichomonas vaginalis was treated in TYM broth supplemented with 2% acetylated COS for 60 min. Viable cells were counted by the tryphan blue dye exclusion method according to Materials and Methods. Control (●), metronidazole 2×MIC (○), COS 1×MIC (▼), COS 2×MIC (△). Results are given as means±SD of triplicate samples.

DISCUSSION

Trichomoniasis is a common cause of vaginitis, but only a few resources have been devoted to its control. Until recently, metronidazole was the only efficacious antibiotic available for the treatment of trichomoniasis [1]. However, the advent of metronidazole-resistant strains and increased risk for human immunodeficiency virus infection has indicated a need for increased control efforts such as development of antiprotozoal agents [2]. It was reported that the molecular weight and amine moiety of chitosan are important to its antibacterial activity [14, 15], and a novel biological activity of COS has also been reported [16]. Therefore, we examined the antiprotozoal activity of COS and found that deacetylated COS showed effective antiprotozoal activity against only *T. vaginalis* (Table 1). The antitrichomonal effect of COS was correlated to the degree of its deacetylation (Table 1, Fig. 2). On the other hand, acetylated COS showed a growth stimulatory effect on *Trichomonas* (Figs. 2 and 3). These findings indicate that regardless of whether COS is acetylated or not, COSs at biological concentrations do not inhibit bacteria. In addition, the results indicated that the amine moiety of COS is important for antitrichomonas activity, and that the acetyl moiety of COS has a growth stimulatory effect on *T. vaginalis*. When *Trichomonas* was treated with 98% deacetylated COS at 1×MIC for 60 min, the protozoa died rapidly within 15 min, whereas the protozoa was alive at 2×MIC of metronidazole (Figs. 4 and 5). Metronidazole-induced damage to *T. vaginalis* cells *in vitro* has been studied previously by electron microscopy [17, 18]. However, the effect of COS on the ultrastructure of *T. vaginalis* has

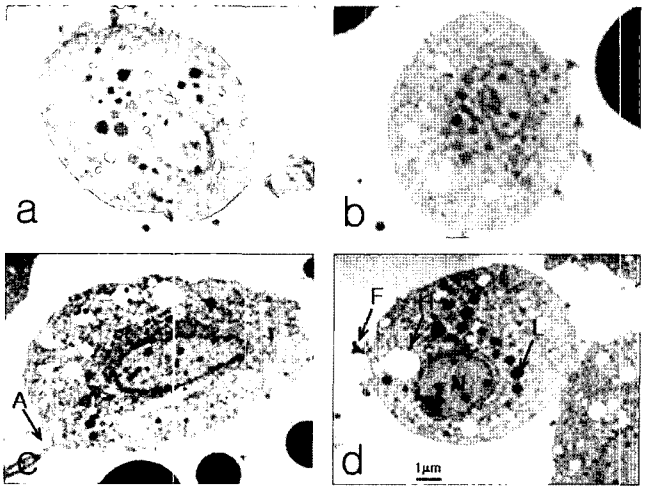

Fig. 5. Electron micrographs of *Trichomonas vaginalis*.
a. Normal cell in absence of COS; **b**. After exposure for 15 min; **c**. After 60 min of exposure to COS; and **d**. After exposure to metronidazole for 60 min. Abbreviations: A, axostyle; F, flagella; H, hydrogenosome; L, lysosome; N, nucleus.

not been examined *in vitro*. *T. vaginalis* is a zooflagellate and parasitic in the human urogenital tract. Some studies have been carried out on the fine structure of this organism, such as electron micrographs of various cytoplasmic inclusions, including axostyle, Golgi, endoplasmic reticulum, and granules [17–21]. In this study, the first sign of electron morphological damage to *T. vaginalis* cells was found in the cytoplasm 15 min after cells were exposed to the COS. The cytoplasmic effects of COS might have been due to the disruption of chromatic granules or hydrogenosomes. Müller [22] and Lindmark and Müller [23] demonstrated in *T. foetus* chromatic granules that contain a pyruvate phosphoroclastic system of the clostridial type.

Deacetylated COS at 0.25%, when added to growing *T. vaginalis* in culture, resulted in extensive disruption of the organisms. The lethal mechanism of COS on *Trichomonas* appears to be different from that of metronidazole, although the mophological change induced is similar. In conclusion, deacetylated COS has potential as an effective antitrichomonal agent, and therefore, further research on this interaction is warranted.

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References

1. Schwebke, J. R. and D. Burgess. 2004. Trichomoniasis. *Clin. Microbiol. Rev.* **17:** 794–803.
2. Cudmore, S. L., K. L. Delgaty, S. F. Hayward-McClelland, D. P. Petrin, and G. E. Garber. 2004. Treatment of infections caused by metronidazole-resistant *Trichomonas vaginalis*. *Clin. Microbiol. Rev.* **17:** 783–793.
3. Schmid, G., E. Narcisi, D. Mosure, and E. Secor. 2001. Prevalence of metronidazole resistant *Trichomonas vaginalis* in a gynecology clinic. *J. Reprod. Med.* **46:** 545–549.
4. Jeon, Y. J. and S. K. Kim. 2002. Antitumor activity of chitosan oligosaccharides produced in ultrafiltration membrane reactor system. *J. Microbiol. Biotechnol.* **12:** 503–507.
5. Seo, W. G., H. O. Pae, N. Y. Kim, G. S. Oh, I. S. Park, Y. H. Kim, Y. M. Kim, Y. H. Lee, C. D. Jun, and H. T. Chung. 2000. Synergistic cooperation between water soluble chitosan oligomers and interferon-γ for induction of nitric oxide synthesis and tumoricidal activity in murine peritoneal macrophages. *Cancer Lett.* **159:** 189–195.
6. Jeon, Y. J. and S. K. Kim. 2001. Effect of antimicrobial activity by chitosan oligosaccharide N-conjugated with asparagine. *J. Microbiol. Biotechnol.* **11:** 281–286.
7. Choi, B. K., K. Y. Kim, Y. J. Yoo, S. J. Oh, J. H. Choi, and C. Y. Kim. 2001. *In vitro* antimicrobial activity of a chitooligosaccharide mixture against *Actinobacillus actinomycetemcomitans* and *Streptococcus mutans*. *Int. J. Antimicrob. Agents* **18:** 553–557.
8. Roller, S. and N. Covill. 1999. The antifungal properties of chitosan in laboratory media and apple juice. *Int. J. Food Microbiol.* **47:** 67–77.
9. Lee, H. W., Y. S. Park, J. S. Jung, and W. S. Shin. 2002. Chitosan oligosaccharides, dp 2–8, have prebiotic effect on the *Bifidobacterium bifidium* and *Lactpbacillus* sp. *Anaerobe* **8:** 319–324.
10. Furda, I. 1983. Aminopolysaccharides - their potential as dietary fiber, pp. 105–122. *In* I. Furda (ed.), *Unconventional Sources of Dietary Fiber*. ACS Symposium series 214, American Chemical Society, Washington, D.C.
11. Shahidi, F., J. K. V. Arachchi, and Y. J. Jeon. 1999. Food applications of chitin and chitosans. *Trends Food Sci. Technol.* **10:** 37–51.
12. Kubota, N., N. Tatsumoto, T. Sano, and K. Toya. 2000. A simple preparation of half N-acetylated chitosan highly soluble in water and aqueous organic solvents. *Carbohydr. Res.* **324:** 268–274.
13. Chaplin, M. 1986. Monosaccharides, pp 4–5. *In* M. F. Chaplin and J. F. Kennedy (eds.), *Carbohydrate Analysis: A Practical Approach*. IRL press, Washington, D.C.
14. Park, P. J., J. Y. Je, H. G. Byun, S. H. Moon, and S. K. Kim. 2004. Antimicrobial activity of hetero-chitosans and their oligosaccharides with different molecular weights. *J. Microbiol. Biotechnol.* **14:** 317–323.
15. Park, P. J., H. K. Lee, and S. K. Kim. 2004. Preparation of hetero-chitooligosaccharides and their antimicrobial activity on *Vibrio parahaemolyticus*. *J. Microbiol. Biotechnol.* **14:** 41–47.
16. Shon, Y. H. and K. S. Nam. 2005. Induction of phase 2 enzymes and inhibition of cytochrome P450 isozymes by chitosanoligosaccharides. *J. Microbiol. Biotechnol.* **15:** 183–187.

17. Buchner, Y. and D. R. Edwards. 1975. The effect of metronidazole and nitrofurans on the morphology of *Trichomonas vaginalis*. *J. Antimicrob. Chemother.* **1:** 229–234.
18. Ings, R. M. and F. Constable. 1975. An investigation into the effect of metronidazole on the morphology of *Trichomonas vaginalis*. *J. Antimicrob. Chemother.* **1:** 121–126.
19. Smith, B. F. and B. T. Stewart. 1966. Fine structure of *Trichomonas vaginalis*. *Exp. Parasitol.* **19:** 52–63.
20. Nielsen, M. H. 1976. *In vitro* effect of metronidazole on the ultrastructure of *Trichomonas vaginalis* Donné. *Acta Pathol. Microbiol. Scand. (B)* **84:** 93–100.
21. Carosi, G., G. Filice, F. Suter, and A. Dei Gas. 1977. *Trichomonas vaginalis*: Effect of trinidazole on ultrastructure *in vitro*. *Exp. Parasitol.* **43:** 315–325.
22. Müller, M. 1973. Biochemical cytology of trichomonal flagellates. *J. Cell Biol.* **57:** 453–474.
23. Lindmark, D. C. and M. Müller. 1973. Hydrogenosome, a cytoplasmic organelle of the anaerobic flagellate *Trichomonas foetus* and its role in pyruvate metabolism. *J. Biol. Chem.* **248:** 7724–7728.