Chitosan-Coated Paper: Effects of Nisin and Different Acids on the Antimicrobial Activity

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ABSTRACT: Chitosan coatings prominently improved both the gloss and oxygen barrier properties of paper. The gloss value in the machine direction was increased as a function of added chitosan. An oxygen-permeability value of 1.1 cm^3/m^2 d was obtained when 6.9 g/m^2 chitosan was applied to 80 g/m^2 copy paper. In addition, the effects on the mechanical properties were positive, but not significant. The water-vapor permeability of the paper increased as a result of the chitosan coating. Chitosan dissolved in 1.6, 3.2, and 6.4% lactic acid showed antimicrobial activity against *Bacillus subtilis*, whereas acetic and propionic acids (1.6, 3.2, and 6.4%) did not produce any notable activity. Nisin (0.08 g/L) did not enhance the antimicrobial activity of coatings prepared from chitosan dissolved in different acids. © 2004 Wiley Periodicals, Inc. J Appl Polym Sci 94: 986–993, 2004

Key words: biopolymers; coatings; mechanical properties; permeability; antimicrobial activity

INTRODUCTION

Antimicrobial packaging materials are interesting and promising applications of advanced active food packaging concepts. They can effectively control the microbial contamination of various solid and semisolid foodstuffs by inhibiting the growth of microorganisms on the surface of the food, which normally comes into direct contact with the packaging material. In recent years, research into environmentally friendly packaging materials and production methods has increased considerably.

Biopolymers are largely based on renewable resources such as starch and cellulose and polyhydroxyalcanoates produced by microbes. Other polymers, such as proteins and pectins, also have the potential to be developed for biodegradable plastics and polymers. Polylactides, that is, aliphatic polyesters formed through the polymerization of lactic acid, are usually included in this category since the monomers can be produced by fermentation.

Biobased polymers can be applied to paper with three methods: surface sizing, dispersion coating, and extrusion coating. The main principle behind all three methods is the application of a continuous, nonporous film on top of the base paper used. In surface sizing, water-based solutions are used. The solid content of the coating is limited and is typically lower than 10–15%. Dispersion coating allows much higher solid contents, but the polymer needs to be produced in a dispersion form. A high solid content allows better coat-weight control and reduces the amount of drying needed. Extrusion coating is possible as long as the polymer is thermoplastic and the polymer melt is stable. The runnability of an extrusion-coating line is, however, poor in comparison with that of dispersion coating and surface sizing.

Because of their origin, biobased coatings are typically hydrophilic and have limited liquid-water and water-vapor barrier properties. Their gas-barrier properties and resistance to oil and grease can be relatively good.

Chitosan, the β-1-4-linked polymer of 2-amino-2-deoxy-β-D-glucose, is prepared by the N-deacetylation of chitin, the second most abundant natural biopolymer after cellulose. Chitosan is an edible and biodegradable material that also has antimicrobial activity against different groups of microorganisms, including bacteria, yeasts, and molds.[1] As chitosan is positively charged below pH 6, it has better antimicrobial activity than chitin and many other biopolymers.[2] Chitosan disrupts the barrier properties of the outer membranes of gram-negative bacteria, and this makes it a potentially useful indirect antimicrobial for food protection.[3] Chitosan can also act as a chelating agent that selectively binds trace metals and thereby inhibits the production of toxins and microbial growth.[4] Because

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of its good film-forming properties, chitosan has been successfully used in food-packaging materials.[5] The use of *N,O*-carboxymethylchitin films to preserve fruits over long periods has been approved in both Canada and the United States.[6] The preparation of chitosan films[7–12] and chitosan laminated with pectin[13] or polyethylene[14] or mixed with lipids[15] has been reported. Chitosan has been mixed with methylcellulose and 4% sodium benzoate or potassium sorbate to form antimicrobial films. Such films possess significant antimicrobial properties against *Penicillium notatum* and *Rhodotorula rubra*.[8] Films have been formed through the dissolution of chitosan in hydrochloric, formic, acetic, lactic, and citric acid solutions.[16] The diffusivity of acetic and propionic acids incorporated into packaging films for processed meats has been determined,[17] and the strongest inhibition against *Enterobacteriaceae* and *Serratia liquefaciens* was observed when the acid release rates from the chitosan matrix were slower.[18] The browning and water loss in cut apple slices have been inhibited by coatings of chitosan and lauric acid.[19] Chitosan has also been used as an edible, invisible film for the shelf-life extension of seafoods.[20]

The idea of using chitosan combined with paper is not new. It has been used as a papermaking additive and for the surface treatment of paper for decades. Chitosan graft copolymers have been exploited for making paper products of improved dry strength,[21] and chitosan has been added to α-cellulose and unbleached sulfite to increase the burst, dry-tensile, and wet-tensile properties of handsheets.[22] Its use has been recommended for the manufacture of electric insulation papers and various types of technical papers, particularly wet-strength papers.[23] Chitosan has been proved theoretically and practically to meet the criteria for wet-strength agents.[24] The effects of chitosan treatments on 17 varieties of paper have been studied, and a surface treatment with a 1% chitosan solution improves all strength properties of paper.[25] A treatment with a 0.05–0.3% solution enhances the mechanical and printing properties and reduces the consumption of sizing agents.[26] Soaking in a chitosan solution improves the surface strength, softness, and permeability of machine-made paper.[27] Chitosan dissolved in acetic acid has been mixed with pulp and converted into sheets, which could then be used for food wrapping.[28,29] Water-insoluble, biodegradable food-packaging composite materials of chitosan and cellulose have been developed and patented.[30,31] Cationic chitosan acetate blends with poly(vinyl alcohol) and gelated starch have been used as fillers for paper.[32] Chitosan has also been precipitated onto wood pulp and glass fibers to be formed into paper sheets.[33]

Nisin is one of the most studied bacteriocins (antimicrobial peptides produced by bacteria, which inhibit other closely related bacteria). It belongs to a class of bacteriocins known as *lantibiotics*, which are small peptides defined by antimicrobial activity and the presence of lanthionine and uncommon amino acids. Nisin is the only lantibiotic that has been cleared in many countries for use with foodstuffs. For example, in Europe, according to Directive 95/2/EC, nisin (E 234) is on the list of additives allowed to be used in certain foodstuffs, such as cheese spread. On the other hand, reasonable amounts of nisin in meats have been proved to be ineffective, as some meat components interfere with its activity.[34,35] Nisin exhibits antimicrobial activity against many gram-positive bacteria, including some pathogenic bacteria, such as *Clostridium botulinum* and *Bacillus cereus*. Furthermore, nisin inhibits a range of spore-forming spoilage organisms, such as *Bacillus sporothermodurans*, *Bacillus stearothermophilus*, *Clostridium sporogenes*, and *Clostridium thermosaccharolyticum*, and acid-resistant spoilage organism such as *Bacillus coagulans*. However, it does not inhibit gram-negative bacteria, yeasts, or molds. Some studies suggest that nisin interferes successfully with cell-wall functions and the synthesis of cell-wall components in gram-positive organisms, but it is ineffective against outer cell membranes of gram-negative organisms. Nisin shows increased antimicrobial activity only at low pHs and is more soluble and stable under acidic conditions. At pH 2.5 and lower, solutions can be boiled without a loss of activity. Nisin reportedly can stand heating to 121°C, but it loses activity above pH 4 and below 20°C. Under neutral conditions, nisin is quickly inactivated. The introduction of lysine residues considerably enhances the solubility of nisin in the neutral pH range.[36] Thus, nisin may be effectively used as an antimicrobial substance only for acidic foodstuffs.[37]

Several studies have been made to test the antimicrobial activity of nisin under various conditions and incorporated into various packaging materials. Nisin has been incorporated directly into a low-density polyethylene film; this resulted in the retention of the antimicrobial activity of nisin against *Lactobacillus helveticus* and *Brochothrix thermosphacta*.[38] Nisin has been dissolved in a binder solution (an acrylic polymer or a vinyl acetate/ethylene copolymer), which has been used as an antimicrobial coating material for paper against *Micrococcus flavus*.[39] It has also been dissolved in various zein solutions, which have been further formed into biodegradable films with activity against *Lactobacillus plantarum*[40,41] and *Listeria monocytogenes*.[42] Nisin incorporated into hydroxypropylmethylcellulose,[43] sorghum starch,[44] and polyvinylidene copolymer[45] films has been reported. Nisin-coated paper and plastic (70:30 polyethylene/polyamide) films,[46] as well as cellulose casings[47] and poly(vinyl chloride), linear low-density polyethylene, and nylon films,[48] have been studied as potential antimicrobial packaging materials. Recently, 0.5% nisin together with 5%

TABLE I
Tensile Properties of Uncoated and Chitosan-Coated Paper

Sample	Tensile index (Nm/g)	Strain (%)	Breaking length (km)
80 g/m^2 paper	33.49 ± 0.88	5.16 ± 0.44	3.41 ± 0.09
80 g/m^2 paper + 1.5 g/m^2 chitosan	35.63 ± 1.56	6.33 ± 0.54	3.63 ± 0.16
80 g/m^2 paper + 4.3 g/m^2 chitosan	38.49 ± 1.32	6.63 ± 0.28	3.92 ± 0.14
80 g/m^2 paper + 6.9 g/m^2 chitosan	37.96 ± 1.00	7.05 ± 0.33	3.87 ± 0.11

Means (n = 7) ± standard deviations are shown in the table.

ethylenediaminetetraacetic (EDTA) acid has been used as an additive in chitosan films, producing antimicrobial activity against *Bacillus subtilis*.[49] Nisin and chitosan have also been coated, in 3% concentrations, onto paper with a binder medium of a vinyl acetate/ethylene copolymer (pH 4.4) to provide antimicrobial activity against *Listeria monocytogenes* and/or *Escherichia coli*.[50]

EXPERIMENTAL

Mechanical and permeability properties

Coating solutions

The chitosan solution was made as follows.[11] A 2% chitosan solution was prepared with a 1% acetic acid solution. Chitosan (16 g; high-molecular-weight; Aldrich Chemical Co., Milwaukee, WI) was mixed with 800 mL of distilled water and 8 mL of glacial acetic acid; the solution was filtered and degassed to minimize the amounts of undissolved impurities and air bubbles.

Coating method

The chitosan solution was applied to the surface of the paper (size A3, 80 g/m^2 copy paper; Reflex, Australian Paper, Mount Waverly, Australia) with an RK Control K101 laboratory coater. The drying of the coated samples was performed in an air-circulation laboratory oven at 105°C. Three different coating levels were made with one, three, or five chitosan layers on the paper with Mayer bar no. 5 (RK Print Coat Instruments, Ltd., Litlington, United Kingdom), which produced a theoretical wet film deposit of 50 μm, and with drying for 1 min after every single layer. The speed of the coating bar was 2 m/min. The dried samples were conditioned and stored at least for 1 week in a climate room at 23°C and 50% relative humidity (RH) before the testing.

Tensile-property measurements

The tensile index, strain, and breaking length were determined with an Alwetron TH1 tensile tester (app. 65-F, type 1-2, AB Lorentzen & Wettre, Stockholm, Sweden). The preconditioned sheets were cut into 15 mm × 120 mm strips. The initial grip separation was set at 100 mm, and the crosshead speed was 10 mm/min. The environment was kept constant at 23°C and 50% RH during the testing.

Water-vapor-transmission-rate (WVTR) determination

WVTRs were determined gravimetrically with a modified ASTM E-96A procedure. Anhydrous calcium chloride (Damp Rid, Orlando, FL) was used as a desiccant and placed on the bottom of a circular aluminum dish, which had an inner mouth diameter of 8 cm and an inside depth of 2.2 cm. The samples were cut and mounted on the mouth area of the dish-coated side toward the high RH. Wax (50% beeswax and 50% paraffin wax) was used to seal the samples tightly against the dish surface. Briefly, wax was heated to 80°C and applied in a molten state around the samples with a metallic template, with a diameter of 8 cm, placed concentrically on the samples. After cooling, the template was carefully removed, and the dish was covered with a lid made from the same material as the dish; finally, it was weighed for the first time with an electronic scale (type 1602, Sartorius GMBH, Göttingen, Germany) and placed without its lid in place in an environmental test chamber (Thermoline Envirotherm LoHi 600, Scientific Equipment, Ltd., Melbourne, Australia) under testing conditions of 3.5°C and 95% RH. These are the typical storage conditions

TABLE II
WVTRs of Uncoated and Chitosan-Coated Paper

Sample	WVTR (g/m^2 d)
80 g/m^2 paper	501.5 ± 3.4
80 g/m^2 paper + 1.5 g/m^2 chitosan	681.1 ± 54.3
80 g/m^2 paper + 4.3 g/m^2 chitosan	668.8 ± 52.1
80 g/m^2 paper + 6.9 g/m^2 chitosan	594.0 ± 57.9

Means (n = 4) ± standard deviations are shown in the table.

TABLE III
Oxygen Transmission Rates of Uncoated and Chitosan-Coated Paper

Sample	Oxygen transmission (cm^3/m^2 d)
80 g/m^2 paper	>10,000[a]
80 g/m^2 paper + 1.5 g/m^2 chitosan	>10,000[a]
80 g/m^2 paper + 4.3 g/m^2 chitosan	36.1 ± 28.2
80 g/m^2 paper + 6.9 g/m^2 chitosan	1.1 ± 1.3

Means (n = 3) ± standard deviations, are shown in the table.
[a] The detection limit of the method was about 10,000 cm^3/m^2 d.

TABLE V
Properties of the Coating Solutions

Chitosan solution	Viscosity (mPa s)	pH[a]
1.6% acetic acid	3220	2.6
3.2% acetic acid	2930	2.5
6.4% acetic acid	2360	2.3
1.6% propionic acid	2660	2.8
3.2% propionic acid	2680	2.7
6.4% propionic acid	2960	2.5
1.6% lactic acid	2080	2.2
3.2% lactic acid	3530	2.0
6.4% lactic acid	3450	1.8

[a] Measured before the addition of chitosan.

for apples and kiwis. Air inside the chamber was continuously circulated over the exposed surfaces of the samples. This was necessary to maintain uniform temperature and humidity conditions and to prevent the formation of a stagnant air layer above the samples. Weighings were repeated after 3, 6, 24, 27, and 30 h to determine by weight the amount of moisture transferred from the environmental chamber through the sample into the desiccant. WVTR was calculated from the line of a steady-state weight increase versus the time and with the following equation:

$$\mathrm{WVTR} = n/tA \quad (1)$$

where n is the amount of water vapor (g), t is the time (days), and A is the film area (m^2).

Oxygen-transmission determination

Measurements were performed with an Ox-Tran 2/20 oxygen-transmission rate system (Mocon, Modern Controls, Inc., Minneapolis, MI) with the method described in ASTM D 3985-81. Tests were carried out at 23°C and 0% RH with 20% oxygen as a test gas. Aluminum foil masks, with an inner diameter of 5 cm^2, were used to mount test pieces in the diffusion cell. The coated side of the paper faced the test gas. The results were expressed for 100% oxygen.

Gloss measurements

The gloss of chitosan-coated paper sheets was measured with a Glossgard II 75° gloss meter (Gardner/Neotec, Silver Spring, MD). The gloss was measured at five different positions, in both the machine (coating) and cross directions.

Antimicrobial properties

Coating solutions

Nisin (2.5%; Sigma Chemical Co., St. Louis, MO) was dissolved in 1.6, 3.2, and 6.4% acetic, propionic and lactic acids at a concentration of 0.08 g/L. Chitosan was added until it reached the concentration of 1.6% (w/v). The solutions were mixed with stirring on a magnetic stirrer and homogenized with a Bamix wand mixer (ESGE AG, Mettlen, Switzerland); then, they were allowed to stand for a couple of days, were filtered and degassed to minimize the amounts of undissolved impurities and air bubbles, and were finally stored at 4°C.

Coating method

Solutions were applied to an uncoated board surface (A4 size, 230 g/m^2; Cupforma Classic 230, Stora Enso, Imatra, Finland) with a K Control K202 laboratory coater (RK Print Coat Instruments, Litlington, UK).

TABLE IV
Gloss Values of Uncoated and Chitosan-Coated Paper

Sample	Gloss (machine direction)[a]	Gloss (cross-direction)
80 g/m^2 paper	5.7 ± 0.2	5.7 ± 0.2
80 g/m^2 paper + 1.5 g/m^2 chitosan	8.3 ± 0.5	7.3 ± 0.2
80 g/m^2 paper + 4.3 g/m^2 chitosan	12.2 ± 0.3	10.3 ± 0.4
80 g/m^2 paper + 6.9 g/m^2 chitosan	16.6 ± 0.7	14.3 ± 0.8

Means (n = 5) ± standard deviations, are shown in the table.
[a] The coating was made in the machine direction.

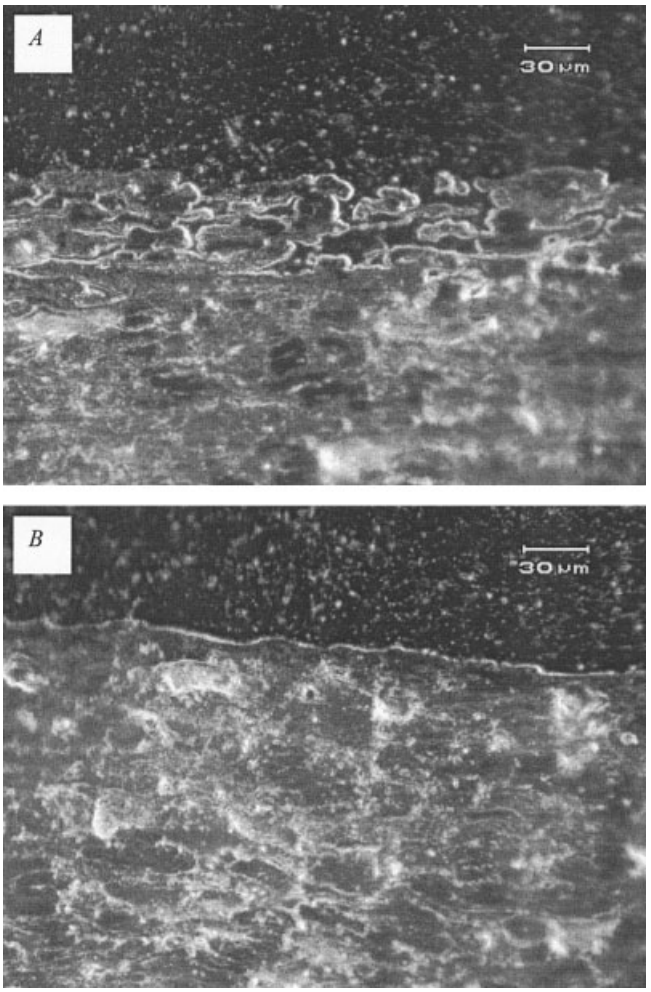

Figure 1 (A) Uncoated and (B) chitosan/6.4% propionic acid coated boards (taken with an Olympus BH-2 microscope equipped with an Olympus DP12 microscope digital camera system).

The drying of the coated sheets was performed under ambient conditions for 1 day. Standard coating bar no. 5, giving a theoretical wet film deposit of 50 μm, was used to prepare sheets with an approximately 5 g/m^2 dried coating. The speed of the coating bar was approximately 4 m/min. The dried samples were stored at 4°C before the testing.

Antimicrobial activity

Inhibition zone method. All the sheets were cut into disks (Ø 10 mm), which were used in the tests for antimicrobial activity. The antimicrobial activity was determined with a modified agar diffusion assay and *B. subtilis* as the test organism. A commercial spore suspension of *B. subtilis* (1.10649, Merck, Darmstadt, Germany) was diluted as described in EN Standard 1104 (1995). The plates were examined for possible inhibition zones after incubation at 30°C for 1, 3, and 6 days. The tests were performed with three parallel samples.

Bacteria reduction method. The sheets were cut into 1.5 cm $\times$ 1.5 cm test pieces, and each piece was placed in a petri dish. A *B. subtilis* (0.1 mL; 1.10649; Merck) spore suspension diluted in sterile peptone saline to approximately 1×10^6 colony forming units/mL was placed on each test piece. The petri dishes were placed on a tray containing a wetted paper sheet, covered with a lid, and incubated at 30°C for 1–3 days. After the incubation, 5 mL of sterile peptone saline was added to the petri dishes, and the bacteria were washed from the test pieces with 5 min of shaking (at 100 rpm; AG CH 4103 orbital shaker, Infors, Bottmingen, Switzerland) at 25°C. The number of surviving bacteria in the test solution was measured via plating onto tryptic soy agar (TSA) plates and incubation for 24 h at 30°C.

RESULTS AND DISCUSSION

Mechanical and permeability properties

High-molecular-weight chitosan was easy to apply to paper with a laboratory coater. The coatings were even and homogeneous without any bubbles or defects. As the coating method simulates relatively well traditional coating lines, it is reasonable to believe that high-molecular-weight chitosan can be applied to paper on a commercial scale. However, preliminary tests (not included in this report) showed that this was not the case for solutions of lower molecular weight chitosan. The viscosity of these solutions was not high enough to be used with a laboratory coater; that is, the wetting of paper was a problem.

Chitosan coatings improved the tensile properties, as shown in Table I. As chitosan itself can form tough, flexible, and tear-resistant films, coatings could be expected to enhance both the tensile strength and elongation of paper. Although these improvements were

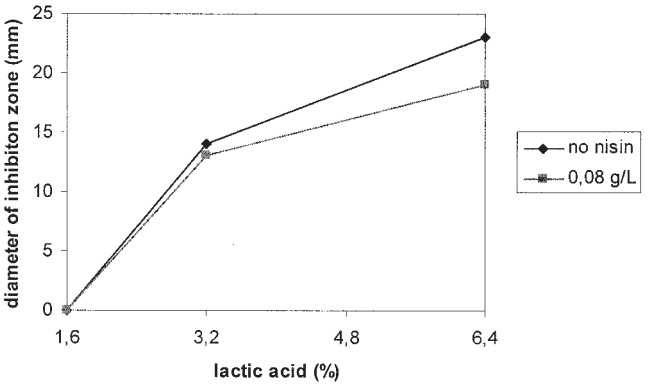

Figure 2 Activity of nisin as a function of the aqueous lactic acid concentration. Aqueous lactic acids were used as solvents for coating solutions containing 1.6% (w/v) chitosan and specified amounts of nisin.

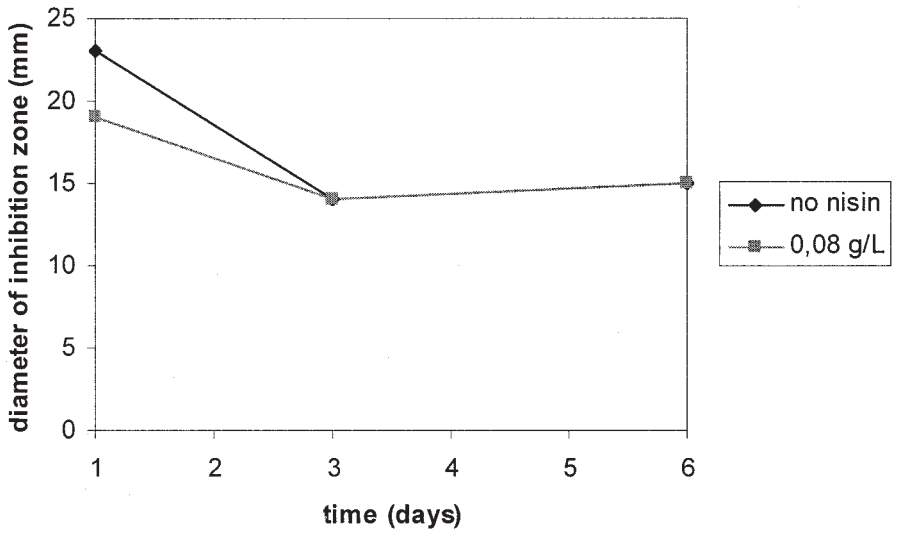

Figure 3 Activity of nisin dissolved in 6.4% aqueous lactic acid as a function of time. The coating solutions contained 1.6% (w/v) chitosan and specified amounts of nisin.

not so significant, chitosan could be regarded as a reinforcement layer. However, coatings are very seldom used to improve tensile properties, which for paper are naturally good enough.

Chitosan is definitely not a good water-vapor-barrier material, as has been well known for years. In fact, the uncoated paper seemed to prevent water-vapor transmission more effectively than chitosan coatings (Table II). A possible reason for the relatively good barrier properties of the uncoated paper is that the paper in question was a copy paper, into which certain sizing agents are normally added to increase the internal strength and to slow down, for example, coating ink or perhaps, in this case, water-vapor penetration into the paper structure. The surface of the paper wetted by a chitosan/acetic acid solution, which possibly dissolved the sizing agent and definitely disrupted the fiber network, and could be expected to increase the roughness, porosity, and permeability. As the chitosan-coating level increased, the influence of wetting became smaller, and this improved the barrier properties.

Oxygen-permeability tests showed that the barrier properties against oxygen were improved as a function of the coating thickness (Table III). Apparently, one layer (1.5 g/m^2) of chitosan was not enough to provide a completely impermeable coating. As the chitosan-coating level increased, the gloss increased (Table IV). The chitosan coatings were completely transparent and glossy, so their positive effect on the gloss values was expected. The gloss values in the coating machine direction were higher than in the cross direction. This is a common phenomenon and is due to the continuous bar movement in one direction, which leaves behind a polished surface.

Antimicrobial properties

Nisin was easily dissolved in acetic, propionic, and lactic acids, whereas chitosan did not dissolve immediately. Two days of storage were necessary for the solutions, followed by filtering and degassing until clear and bubbleless solutions were ready for the coating trials.

High-molecular-weight chitosan formed viscous solutions, as shown in Table V. Typically, the viscosity of the coating solutions varied between 2000 and 3500 mPa s. The addition of nisin did not have any effects on the viscosity. All the solutions were easy to apply onto the board with a laboratory coater. The coatings were even and homogeneous without any bubbles or defects. As shown in Figure 1, the surfaces of uncoated and chitosan-coated boards were very different. The porous structure typical of fiber-based materials was tightly covered by the chitosan layer. As the oxygen-

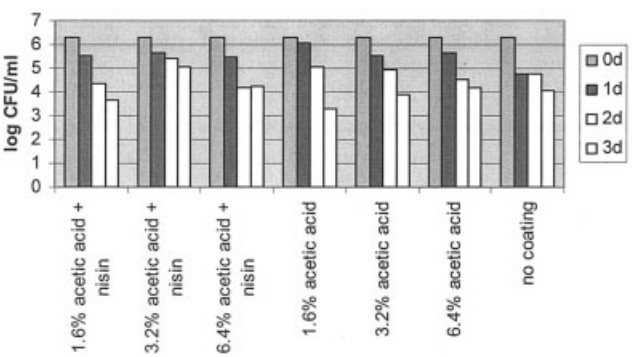

Figure 4 Effect of coatings dissolved in aqueous acetic acid on *B. subtilis* suspensions as a function of time. The coating solutions contained 1.6% (w/v) chitosan.

permeability tests showed, the chitosan coatings effectively reduced the oxygen-transmission rates of paper.

There are two types of mechanisms for antimicrobial activity of packaging materials treated with antimicrobial substances: release mechanisms and binding mechanisms. To determine the efficiency of the antimicrobial properties, both the inhibition zone method and bacterial reduction method can be used. In the inhibition zone test, an antimicrobial sample is placed on a solid agar medium containing the test strain. A clear zone surrounding the sample indicates the diffusion of antimicrobial substances from the sample material generating growth inhibition. This method is useful for testing releasable antimicrobial substances. In the case of nonreleasing substances, such as chitosan, the bacteria reduction test should be used. The liquid growth media is seeded with the test strain and placed into direct contact with the sample material. This method can be used to measure the reduction of surviving cells, presented as colony forming units as a function of time.

It has been reported[51] that the inhibition zone test is not suitable for chitosan films under all conditions; thus, the bacteria reduction method was used as a supplementary test for validating the final results. As the stability of the coated paper did not allow shaking methods in which the test pieces would be incubated in a bacterial suspension (e.g., ASTM E 2149-01), the tests were carried out through the incubation of a drop of a bacterial suspension on the paper surface. Indeed, the methods did not give fully comparable antimicrobial activities. According to both tests, chitosan that was dissolved in acetic and propionic acids did not have any activity against *B. subtilis*. Not even the addition of nisin generated any activity, and this was unexpected. The preliminary tests (not included in this report) showed that it should have certain antimicrobial activity against *B. subtilis*. The concentration of 0.08 g/L in this case possibly was too low. Chitosan that was dissolved in aqueous lactic acid, however, seemed to have strong antimicrobial activity according to both tests. The addition of nisin did not clearly enhance the activity. According to inhibition zone test results, the acidity (from 1.6 to 6.4% lactic acid) strengthened the activity during 6 days period of incubation (Figs. 2,3). However, this was not the case with the bacteria reduction test results, which did not indicate any major positive effects due to different lactic acid concentrations (Figs. 4–6). As the lactic acid containing samples were the only ones with antimicrobial activity, it is reasonable to believe that chitosan itself in this case did not generate any inhibition of growth of *B. subtilis*.

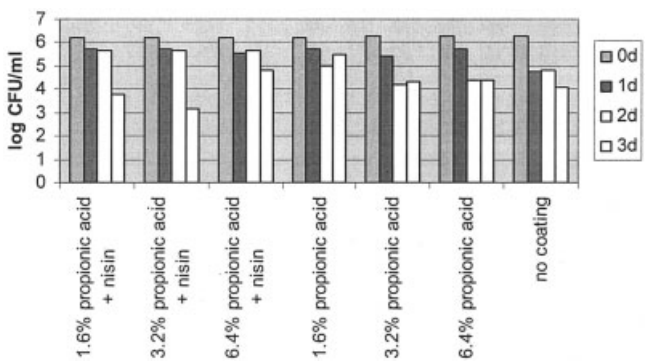

Figure 5 Effect of coatings dissolved in aqueous propionic acid on *B. subtilis* suspensions as a function of time. The coating solutions contained 1.6% (w/v) chitosan.

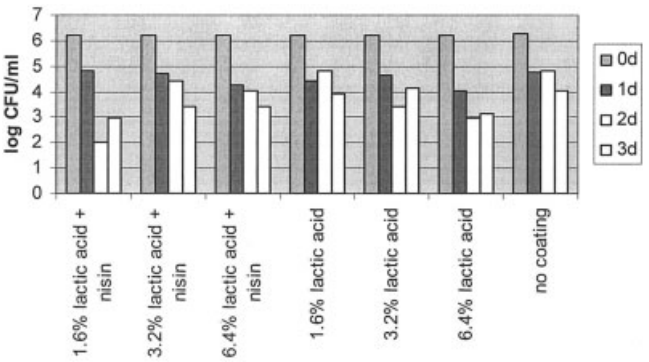

Figure 6 Effect of coatings dissolved in aqueous lactic acid on *B. subtilis* suspensions as a function of time. The coating solutions contained 1.6% (w/v) chitosan.

CONCLUSIONS

The viscosity of chitosan solutions can be modified with raw chitosan materials of different molecular weights. High-molecular-weight chitosan formed solutions that were suitable for small-scale coating trials. Chitosan coatings containing lactic acid had antimicrobial activity against *B. subtilis* and so could be used in paper packing for foods such as breakfast cereals, snack confectionery, and bread.

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