ПРИКЛАДНАЯ БИОХИМИЯ И МИКРОБИОЛОГИЯ, 2012, том 48, № 2, с. 175–182

UDC 547.99

ENZYMATIC MODIFICATION OF CHITOSAN WITH QUERCETIN AND ITS APPLICATION AS ANTIOXIDANT EDIBLE FILMS

© 2012 E. Torres*, V. Marín*, J. Aburto**, H. I. Beltrán***, K. Shirai****, S. Villanueva*****, G. Sandoval*****

*Center of Chemistry-ICUAP, University of Puebla, Edificio 103G. Puebla 72570 México

**Mexican Institute of Petroleum, Eje Central Lázaro Cárdenas Norte 152, Col. San Bartolo Atepehuacan, México, D. F., 07730

*** Metropolitan Autonomous University-Cuajimalpa, Artificios 40-sexto piso, Col. Hidalgo, México, D. F., 01120.

**** Metropolitan Autonomous University-Iztapalapa, Av San Rafael Atlixco No.186, Col.Vicentina Mexico DF 09340

***** Center of Investigation and Technological Assistance and design of Jalisco. Guadalajara, Jalisco. Mexico 44270

e-mail: eduardo.torres@correo.buap.mx

Received August 15, 2011

Quercetin, rutin, naringin, hesperidin and chrysin were tested as substrates for cloroperoxidase to produce reactive quinones to graft onto chitosan. Quercetin and rutin quinones were successfully chemically attached to low molecular weight chitosan. The quercetin-modified chitosan showed an enhancement of plastic, antioxidant and antimicrobial properties as well as of thermal degradability. Finally, chitosan-quercetin films visibly decreased enzymatic oxidation when applied to *Opuntia ficus indica* cladodes.

Chitosan, poly((1 → 4)-2-amino-2-deoxy-β-D-glucose), is a product of the deacetylation of chitin, which is ranked, by prevalence, as the second polysaccharide in nature, just after cellulose. Chitosan has received increased attention for its commercial applications in the biomedical, food and chemical industries due to its biodegradability, biocompatibility and biological activities such as antimicrobial, antitumor, antioxidant and hypocholesterolemic functions. Chitosan contains a large number of hydroxyl and amino groups, which are two functional and strategic groups in organic synthesis; these groups provide several possibilities for derivatization or grafting. Modification with several reactive groups has produced chitosans with improved properties such as increased hydrophobicity, higher solubility in both water and organic media and improved antimicrobial properties [1–3] or with new properties, such as photosensitizing activity [4]. Enzymatic modification of chitosan has already been reported in the literature [5–8]. By using oxidative enzymes, chitosan has been grafted with phenol derivatives to confer higher hydrophobicity and viscosity [5, 9] or new functionalities, such as the ability to adsorb cationic dyes [7].

These experimental contributions make evident the importance and the potential of the functionalization of chitosan with specific molecules to provide biopolymers with improved properties [10]. Our first improvement of chitosan macromolecules is centered on the inclusion of natural flavonoids with known biological and chemical potential [11, 12] in their polymeric backbones. Therefore, in this report, we carried out the synthesis of chitosan-flavonoid conjugates by enzymatic treatment with chloroperoxidase (CPO, EC 1.11.1.10). Flavonoids are found in fruits, vegetables and a variety of other dietary sources with anticancer, antiviral, antimutagenic and lipid peroxidation inhibitory activities. Here, by oxidizing flavonoids with CPO in the presence of chitosan, we expect to produce adducts through the reaction of the catechol (in its *ortho*-quinonic form) moiety of flavonoids and the amino groups of the chitosan. With this modification, some properties of chitosan were improved. The modified polymer was used to diminish browning on *Opuntia ficus indica* cladodes, applying the chitosan-quercetin bioconjugates as an edible film.

MATERIALS AND METHODS

CPO from the marine fungus *Caldaromyces fumago* was a gift from Dr. M. A. Pickard from the University of Alberta, Canada. The enzyme has a specific activity of 22.000 U min^{-1} for the halogenation of monochlorodimedone. Quercetin, rutin, hesperidin, chrysin and naringin, chitosans of low molecular weight 75–85% deacetylated with 20–200 cP of viscosity, hydrogen peroxide, buffer salts and acids, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals were purchased from the Sigma-Aldrich Chemical Company (USA). HPLC organic solvents, isopropanol and acetonitrile, were purchased from J.T. Baker (USA).

Flavonoid oxidation by CPO. The enzymatic oxidation of flavonoids was carried out in a reaction mixture containing 20% isopropanol and 80% acetate buffer (60 mM, pH 3.0), 3.3 mM flavonoid, 100 pM CPO, 1.0 mM H_2O_2 and 20 mM KCl. The temperature was

kept constant at 25°C under stirring. The reaction progress was monitored by the decrease in absorbance in the range of 200–600 nm. All assays were performed 3 times to ensure repeatability.

Enzymatic grafting of quercetin to chitosan. A total of 2.0 g of chitosan was added after 5 min to 250 ml of the reaction mixture described in the previous section. Chitosan was dissolved by the addition of acetic acid to reach a concentration of 1% w/v. After 12 h of stirring at room temperature, the pH was raised by adding 1.0 M NaOH to precipitate the modified chitosan. This solid was washed several times with 50% isopropanol solution to remove the non-reacted oxidized product until the elution did not show any oxidized quercetin or rutin, as measured by the UV-VIS spectra between 200–600 nm. Finally, the modified chitosan was dried under a vacuum in a phosphorus pentoxide atmosphere.

Calorimetric studies. Thermograms were obtained in a differential scanning calorimeter (DSC) Shimadzu DSC60 (Japan). In a typical determination, chitosan or its derivative (~15 mg) was placed in an aluminum cell and the temperature ramp was raised up from 25 to 400°C at 10°C/min with a nitrogen flux of 20 ml/min. The enthalpy of thermal decomposition for every sample was calculated using the software TA-60WS.

Antioxidant activity. The antioxidant activity of neat and modified chitosan was determined using the DPPH (diphenylpicrylhydrazyl) and the ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) method. For the former, 40 μM of DPPH radical were placed in a cuvette containing different concentrations of chitosan, 50% methanol and 1% acetic acid, in a total volume of 4 ml. The change in absorbance was measured at 515 nm after a 1-h incubation at room temperature.

The antioxidant capacity measured as the ABTS free radical-scavenging activity was determined according to the method described previously [13]. Chitosan samples previously dissolved in 1% acetic acid and properly diluted were added to the $ABTS^{\cdot+}$ solution, and the decrease of absorbance was measured at 734 nm after 15 min in the dark. A previous time scan was performed to check the stability of the $ABTS^{\cdot+}$ solution.

For both methods the antioxidant capacity was calculated according to the formula:

$$\text{Antioxidant capacity} = (A_0 - A_1)/A_0 \times 100\%,$$

where A_0 is the absorbance of the sample measured at time 0 and A_1 is that measured at 1 h of incubation.

Antimicrobial activity. To determine the antimicrobial activity of neat and modified chitosans seven pathogen microorganisms were selected and their growth in the presence and absence of the biopolymers was measured. The microorganisms assayed were: *Pseudomonas aeruginosa* PAO1T, *Staphylococcus aureus* ATTC 29789T, *Raoultella* (*Klebsiella*) *planticola* ATCC 33531T, *Ustilago maydis* 521 T, *Candida albicans* ATCC 10231T, *Pantoea ananatis* LMG 2665T and *Escherichia coli* 62348-69. The microorganisms were grown in TESMA and Luria-Bertani (LB) broth for 24 h at 30°C and 150 rpm.

To quantify the microbial populations, the preinoculum of each strain was performed in liquid medium TESMA (g/l):yeast extract – 2.7, glucose – 2.7, mannitol 1.8, K_2HPO_4 – 4.8, KH_2PO_4 – 0.65, agar – 16.0, and bromothymol blue – 50 (mg/l) one day before. Then the cells were washed with 10 mM $MgSO_4 \cdot 7H_2O$, followed by the population adjustment to an optical density of 0.05 at 450 nm. Four assays were tested: 1) inoculation of strains in the presence of neat chitosan at 2.5 mg/ml; 2) inoculation of strains in the presence of modified chitosan at 2.5 mg/ml, 3) inoculation of strains in the presence of acetic acid (0.25%), 4) inoculation of strains in culture broth as control. The type strains were inoculated in test tubes with 4 ml LB or TESMA broth and incubated at 30°C/150 rpm for 21 h. Finally, the account of microbial populations was done with serial dilutions using the "drop-plate" method [14, 15].

Preparation and application of antioxidant edible films on *Opuntia ficus indica* stems. Fresh cactus (*Opuntia ficus*) were purchased at the local market of Guadalajara Jalisco, México. The whole paddles were washed with water and dried on paper towels, and then the spines were removed manually using a kitchen knife. Five different batches of 11 paddles each (970 g per batch) were prepared for the assays. The samples were submerged for 5 min in 1.0% acetic acid containing no chitosan (batch 1), quercetin-modified chitosan at 0.3% (batch 2) and neat chitosan at 0.3% (batch 3). Two additional controls were carried out containing traditional antioxidants such as EDTA (0.08%) plus citric (0.5%) and ascorbic (1.5%) acid (batch 4) and citric (0.5%) and ascorbic (1.5%) acid (batch 5).

All of the batches were placed on open trays and kept at 23° ± 1°C relative humidity 48%. After treatment, the cactus batches were placed in a mesh to remove the excess water and were allowed to drain for 2 h; after that time, the cactus batches were placed on sheets of absorbent paper for half an hour, and, finally, put on trays for observation. Changes in the color of the cactus were followed by daily measurements of lightness (L), green-red dye (a) and yellow-blue dye (b) using a Hunter Lab (USA) colorimeter.

RESULTS AND DISCUSSION

Enzymatic oxidation of flavonoids. Quercetin and rutin were easily oxidized by CPO to produce a brown product, characteristic of *o*-quinones, which are generated by peroxidases and polyphenol oxidases [16]. Hesperidin and naringine were also oxidized according to UV-Vis spectra. Chrysin was not a substrate for

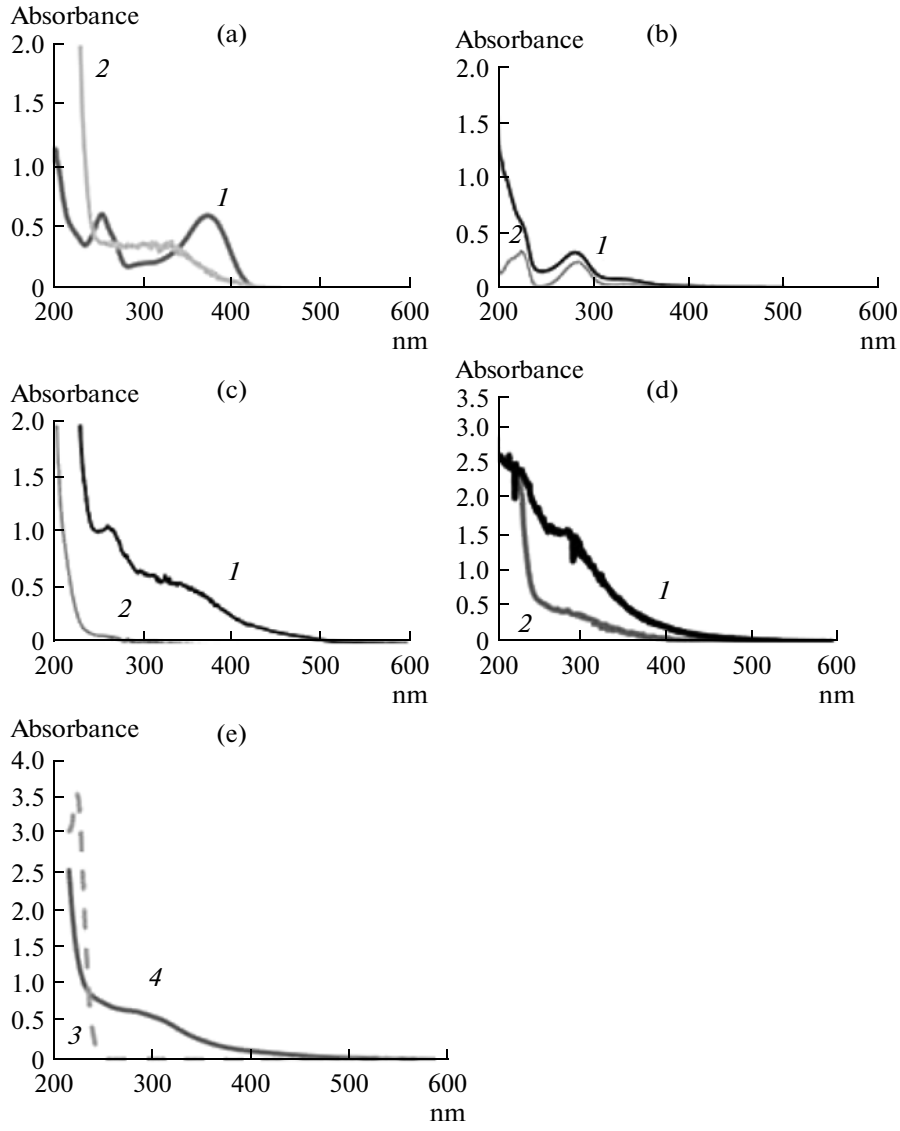

Fig. 1. Electronic absorption spectra of native (*1*) and oxidized (*2*) flavonoids (a–d) and native (*3*) and modified (*4*) chitosan by 1.0 mM H_2O_2 and 100 pM CPO (e). a – quercetin; b – naringin; c – rutin; d – hesperidin, e – chitosans.

CPO (data not shown). Fig. 1 displays the electronic absorption spectra of the assayed flavonoids before and after 1 min of the enzymatic action. As can be seen, the two bands at 250 and 350 nm of quercetin, hesperidin and rutin decreased significantly after enzymatic modification, and a new band appeared around 300 nm for quercetin.

Characterization of modified chitosan. It is well known that *o*-quinones produced from the oxidation of phenols can undergo subsequent non-enzymatic reactions [17] such as electrophilic attacks to nucleophilic moieties, i.e., amino groups from chitosan (Fig. 2). From all assayed flavonoids, only oxidized products from quercetin were able to be attached to chitosan.

Fig. 1 shows the absorption spectra of neat and quercetin-modified chitosan in a 1% acetic acid solution after being modified, washed and dried. As shown, the appearance of a band around 300 nm indicates the presence of the quercetin moiety in the structure of chitosan. As a control, we carried out the whole process in the absence of the enzyme and in the presence of all other components. As can be inferred from the figure, the control sample did not show any absorption band, meaning that there are not physical interactions between the two components. Additional spectroscopic evidences (FTIR and RMN) indicated that the chitosan was successfully modified with quercetin (data not shown). Among the most attractive properties of renewable polymers are their degradability, and antioxidant activity, which are especially important for the food industry to produce packaging or coatings to maintain important properties in food such as texture, taste and mouth feel [18, 19]. Therefore, we

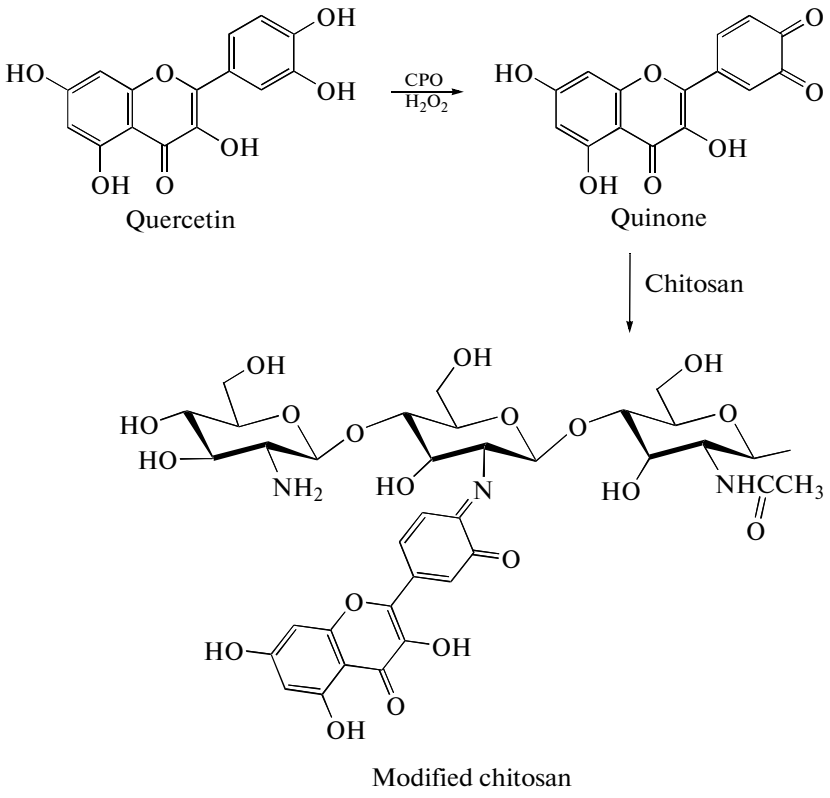

Fig. 2. Chloroperoxidase-catalyzed oxidation of quercetin, and the subsequent nucleoplilic modification of chitosan.

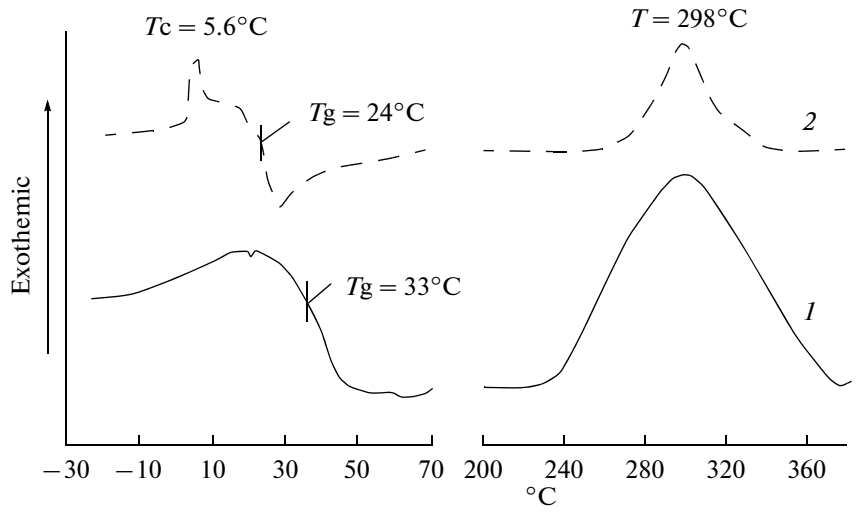

Fig. 3. Thermograms of unmodified chitosan (*1*) and modified chitosan (*2*).

conducted several experimental assays to quantify the changes in these properties as a first approximation for developing a food package or coating based on quercetin-modified chitosan.

Chitosan degradability. Differential scanning calorimetry (DSC) was employed to study the polymer degradability and also to check on any variation in structural properties of chitosan after modification with quercetin. The thermal curves of chitosan and quercetin-modified chitosan are depicted in Fig. 3. As can be seen in the DSC curve, neat chitosan showed a typical broad exothermic peak (T_{onset} 239.14°C, T_{peak} 298.71°C, T_{endset} 367.44°C) that can be attributed to the degradation of the saccharide structure of the mol-

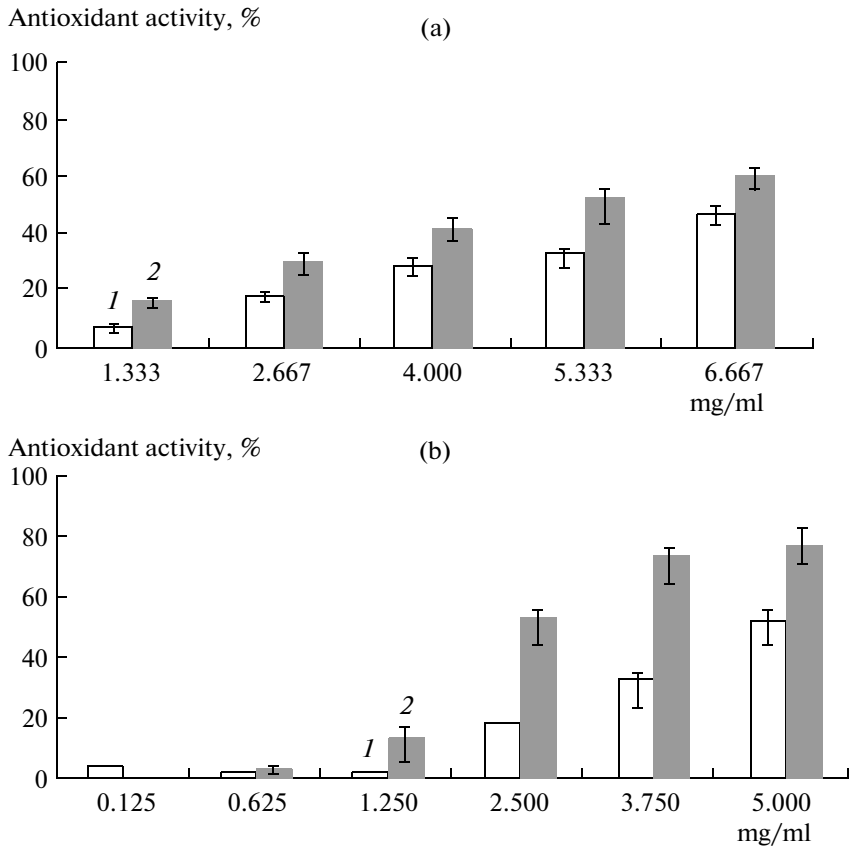

Fig. 4. Antioxidant activity of unmodified (*1*) and modified (*2*) chitosan, measured as the reduction of the (a) ABTS and (b) DPPH radicals. The 100% activity corresponds to the complete reduction of 7.0 mM ABTS radical or 6.5 mM DPPH radical.

ecule, including the dehydration of saccharide rings and the decomposition of the acetylated and deacetylated units of chitosan [20, 21]. Modified chitosan displayed the same exothermic peak (T_{peak} 297.82°C) but less wide (T_{onset} 273.05°C, T_{endset} 319.22°C). The associated heats were 212.82 J/g and 33.62 J/g, respectively. This result could suggest that the chitosan modified with quercetin is even more degradable compared to the unmodified counterpart, because 6 times less heat is necessary to degrade the modified biopolymer. The lower thermal stability could be due to a new arrangement of the modified chitosan, whereby the quercetin molecules are inserted between chitosan macromolecules, which weakens the interconnection between polysaccharide chains [20]. As a result, the chitosan part of the modified biopolymer is more susceptible to thermal degradation.

In addition, some changes were observed at lower temperatures. Neat chitosan displayed an endothermic signal, Tg, at 33°C, associated with the transition from a crystalline to an amorphous state (Fig. 3). Meanwhile, modified chitosan showed a Tg at a lower value, 24°C, and also showed an exothermic signal at 5.6°C, attributed to the crystallization of polymer fragments. The change in the Tg value indicates a transition from a crystalline to an amorphous state for the modified polymer at room temperature; however, the neat chitosan needs more heat to undertake the same transition. This relationship may indicate an increase in plasticity for the quercetin-chitosan polymer compared to the unmodified polymer.

Antioxidant activity. Recently, the antioxidant activity of chitosan and its derivatives has attracted attention. The effects are similar to those of phenolic antioxidants [22]. Here, the measure of antioxidant activity may help us to understand the functional properties of the polymers. The antioxidant activity of the modified chitosan was enhanced throughout the experimental range when compared to the neat chitosan. Indeed, for the ABTS method, at a concentration of 1.3 of mg/ml of both polymers, showed 6.6% of the antioxidant capacity for neat chitosan and 17% of the antioxidant activity for modified chitosan a value 1.5 times higher for the modified biopolymer (Fig. 3a). At a concentration of 6.7 mg/l, 47% (3.3 mM) of the ABTS radical was reduced for the neat chitosan (Fig. 4a) and 62% (4.2 mM) of the ABTS radical was reduced for the modified chitosan. We then evaluated the percent reduction of the DPPH radical as a measure of the antioxidant activity of the polymers (Fig. 4b). A reduction of 50% of the DPPH radical was reached at 5 mg/ml for the neat chitosan,

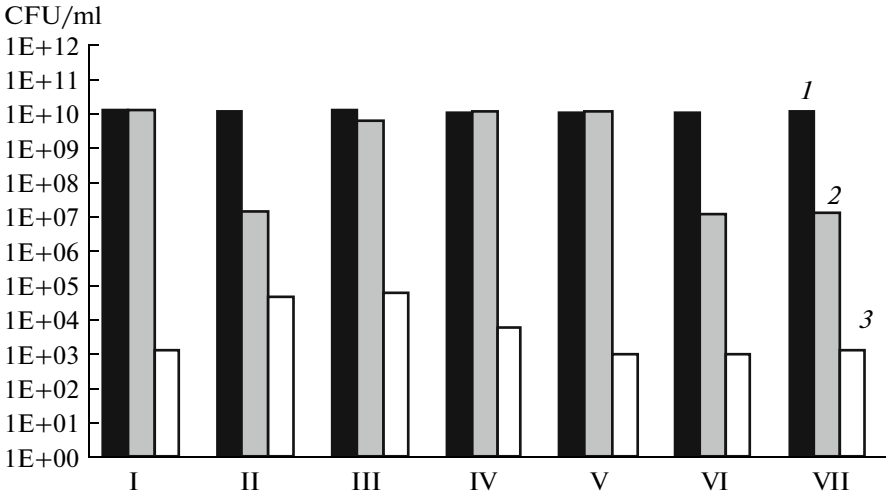

Fig. 5. Antimicrobial activity of control (*1*), unmodified (*2*) and modified chitosan (*3*) after 21 h growing at 30°C/150 rpm against seven different microorganism. Strains: 1 – *Candida albicans* ATCC 10231T; II – *Escherichia coli* 62348-69; III – *Raoultella planticola* ATCC 33531T; IV – *Pantoea ananatis* LMG 2665T; V – *Pseudomonas aeruginosa* PAO1T; VI – *Staphylococcus aureus* ATTC 29789T; VII – *Ustilago maydis* 521 T.

and at 2.5 mg/ml for the modified polymer. The maximal reductions of the DPPH radical were 32 μM (80%) and 20 μM (50%) for the modified and neat chitosan, respectively. A greater difference in antioxidant activity between the biopolymers was found at a concentration of 2.5 mg/ml, where the modified polymer showed a 50% antioxidant activity and the neat chitosan showed only a 17% antioxidant activity, an increment of about three times for the modified chitosan.

The increased antioxidant potential showed by the modified biopolymer could be explained in terms of some structural aspects provided for the oxidized quercetin moiety: a carbonyl function on the C-ring conjugated to two hydroxyl groups on C3 (C-ring) and C5 (A ring), and a double bond between C2 and C3. The hydroxyl groups may be dehydrogenized, deprotonated or oxidized. These structural features of quercetin have been reported as determinants for its free radical scavenging and/or its antioxidant activity [23, 24].

Antimicrobial activity. Chitosan has a natural antimicrobial activity well reported in the literature for a wide spectrum of microorganisms [25]. Although the mechanism of action is not yet fully elucidated and being highly dependent on the type of microorganism, the ability to kill bacteria has been correlated to its capacity to disrupt the outer and inner membrane mediated by its positive-charged amino group at C2 position, whereas it is related to direct interaction of the biopolymer with negatively charged fungal cells [25]. Immobilization of an antimicrobial agent like quercetin on chitosan must, in principle, change this capacity. To analyze this point, the effect of the chitosans on the growth of seven pathogen microorganisms was determined (Fig. 5). As it can be observed, control assays reached a cell population of 10×10^{10} CFU/ml, and the growth was not affected by the presence of 0.25% of acetic acid. Neat chitosan inhibited the growth of three microorganisms, *Escherichia coli*, *Ustilago maydis* and *Staphylococcus aureus* up to three orders of magnitude. On the other side, quercetin-modified chitosan was able to inhibit all microorganisms up to seven orders of magnitude, showing a higher and also a broader spectrum of antimicrobial activity than the neat chitosan. As mentioned before, the mechanism by which chitosan act is not completely elucidated, varying with the degree of acetylation, molecular weight (MW), distribution of the pendant acetyl groups as well as its conformational structure [26, 27]. For modified chitosan, the presence phenolic hydroxyl groups of quercetin could explain the better antimicrobial activity [28]. Since the antimicrobial capacity of chitosan oligomers [26] is higher we foresee that decreasing the molecular weight of chitosan, the modified biopolymer could show even higher antimicrobial activity. In addition, an increment in the deacetylation degree of chitosan up to 90–95% may enhance its antimicrobial activity. These determinations are currently carried out in our laboratory.

Inhibition of enzymatic browning of *Opuntia ficus indica* stems. *Opuntia ficus indica* has important nutritional and medical applications [29]. During storage of its stems, dark spots appear and the original bright green color turns into olive to brown shades; this browning is one of the main postharvest issues [30, 31]. According to our results, quercetin-modified chitosan is a better antioxidant, and in addition, we determine that it is still able to form films. Therefore, this bioconjugate could be used as an antioxidant edible film on fruits and vegetables. To test this property, stems of *Opuntia ficus indica* were coated with chitosan-quercetin, native chitosan and EDTA-ascorbic acid-citric

Color parameters of *Opuntia ficus* indica stems coated with edible films at day 0 and after 5 days of storage at 23°C

Batch**	*a**		*L**		*b**	
	0	5	0	5	0	5
1 – no treatment	−9 ± 1	−7 ± 1	58 ± 4	39 ± 4	36 ± 4	25 ± 4
2 – chitosan-quercetin	−11 ± 1	−9 ± 1	61 ± 4	45 ± 4	37 ± 4	28 ± 4
3 – neat chitosan,	−10 ± 1	−7 ± 1	58 ± 4	39 ± 4	33 ± 4	26 ± 4
4 – EDTA-ascorbic and citric acid	−9 ± 1	−7 ± 1	55 ± 4	34 ± 4	33 ± 4	25 ± 4
5 – ascorbic and citric acid	−10 ± 1	−8 ± 1	53 ± 4	39 ± 4	32 ± 4	23 ± 4

** The samples were submerged during 5 min in 1% acetic acid and placed in a mesh to remove the excess of water and letting it drain for 2 h; after that time, were placed on sheets of absorbent paper for half an hour to finally put them in trays for observation.

acid and ascorbic-citric acid. Also, a negative control without treatment was performed. The protective effect of the different coatings was measured by color conservation during controlled storage at ambient temperature (23°C).

Results of the color variation of the stems treated with different coatings are presented in the table. Statistical analysis showed that there was a significant effect between the different coatings on all of the color parameters (L^*, a^* and b^*). L^* measures the luminosity, ranging from black ($L^* = 0$) to white ($L^* = 100$). Values of a^* can be positive (red) or negative (green), while positive values of b^* are yellow and negative values of b^* are blue [32].

As can be observed in the table, a^* values of fresh stems are neatly negative, meaning that they have a green color. After five days of storage, the a^* values increase for all coatings, indicating a fading of the green color. It can be seen, however, that the paddles coated with chitosan-quercetin films showed the most negative a^* values after storage, indicating that they retain most of the green color compared to the stems treated with other antioxidants. The native chitosan treatment showed also a protective effect (although less than the quercetin doped chitosan), while the traditional antioxidant coatings and the stems without treatment showed a greater loss of green color (a^* values less negative).

High L^* values at day 0 reflect a luminous color and, after five days of storage, the luminosity of the stems decreased. Again, the stems coated with chitosan-quercetin films retained more luminosity compared to the stems coated with other treatments and the non-treated stems. Similarly, b^* values after storage were greater for the cladodes protected by chitosan-quercetin films, indicating a better retention of the yellow tone.

Therefore, it can be stated that enzymatic modification of chitosan has conferred to the biopolymer improved biological properties.

* * *

We demonstrate here the feasibility of covalent enzymatic chitosan modification with a representative flavonoid, quercetin. The quercetin-modified chitosan showed an enhancement of the antioxidant and antimicrobial properties and retained thermal degradability. Moreover, the quercetin moiety conferred a lower temperature of the crystalline-amorphous transition, i.e., an enhanced plasticity. In addition, the antioxidant activity of modified chitosan was improved by quercetin attachment. Finally, color conservation was improved during storage of *Opuntia ficus indica* stems coated with chitosan-quercetin films.

ACKNOWLEDGMENTS

The authors are thankful for the financial support given by SEP-CONACyT 80986, PROMEP/103.5/11/5880. Special thanks are given to M.S. Atilano Gutierrez for recording the 300 MHz solid state NMR spectra.

REFERENCES

1. Kim, C.H., Choi, J.W., Chun, H.J., and Choi, K.S., *Polym. Bull.*, 1997, vol. 38, no. 4, pp. 387–393.
2. Jang, M.-K. and Nah, J-W. *Bull. Korean Chem. Soc.*, 2003, vol. 24, no. 9, pp. 1303–1307.
3. Tangpasuthadol, V., Pongchaisirikul, N., and Hoven, V.P., *Carbohydr. Res.*, 2003; vol. 338, no. 9, pp. 937–942.
4. Nowakowska, M., Lukasz, M., and Szczubialka, K., *Biomacromolecules*, 2008. vol. 9, no. 6, pp. 1631–1636.
5. Chen, T., Kumar, G., Harris, M.T., Smith, P.J., and Payne, G.F. *Biotechnol. Bioeng.*, 2000, vol. 70, no. 5, pp. 564–573.
6. Vachoud, L., Chen, T., Payne, G.F., and Vazquez-Duhalt, R., *Enzyme Microb. Technol.*, 2001, vol. 29, no. 6–7, pp. 380–385.
7. Chao, A.-C., Shyu, S.-S., Lin, Y.-C., and Mi, F.-L., *Bioresour. Technol.*, 2004, vol. 91, no. 2, pp. 157–162.
8. Kaneko, Y., Matsuda, S.-I., and Kadokawa, *J. Biomacromolecules*, 2007, vol. 8, no. 12, pp. 3959–3964.
9. Wu, L.Q., Embree, H.D., Balgley, B.M., Smith, P.J., and Payne, G.F., *Environ. Sci. Technol.*, 2002, vol. 36, no. 15, pp. 3446–3454.

10. Nyanhongo, G.S., Prasetyo, E.N., Kudanga, T., and Guebitz, G. Grafting of Functional Molecules: Insights into Peroxidase-Derived Materials. *Biocatalysis Based on Heme Peroxidases,* Ed E.Torres & M. Ayala, Berlin, Heidelberg: Springer, 2010, pp. 155–177.
11. Kadereit, D. and Waldmann, H., *Chem. Rev.,* 2001, vol. 101, no. 11, pp. 3367–3396.
12. Levitzki, A.*, Acc. Chem. Res.*, 2003, vol. 36, no. 6, pp. 462–469.
13. Stratil, P., Klejdus, B., and Kuban, V., *J. Agric. Food Chem.*, 2006; vol. 54, no. 3, pp. 607–616.
14. Hoben, H.J. and Somasegaran, P., *Appl. Environ. Microbiol.*, 1982, vol. 44, no. 5, pp. 1246–1247.
15. Herigstad, B., Hamilton, M., and Heersink, J., *J Microbiol Methods*, 2001, vol. 44, no. 2, pp. 121–129.
16. Pourcel, L., Routaboul, J.-M., Cheynier, V., Lepiniec, L., and Debeaujon, I., *Trends Plant Sci*, 2007; vol. 12, no. 1, pp. 29–36.
17. Torres, E., Bustos-Jaimes, I., and Le Borgne, S., *Appl. Catal. B*, 2003; vol. 46, no. 1, pp. 1–15.
18. No, HK, Meyers, S.P., Prinyawiwatkul, W., and Xu, Z., *J. Food Sci*, 2007, vol. 72, no. 5, pp R87–R100.
19. Hansen, N.M.L. and Plackett, D., *Biomacromolecules*, 2008, vol. 9, no. 6, pp. 1493–1505.
20. Pawlak, A. and Mucha, M., *Thermochim Acta*, 2003, vol. 396, no. 1–2, pp. 153–166.
21. Paulino, A.T., Simionato, J.I., Garcia, J.C., and Nozaki, J., *Carbohydr Polym*, 2006, vol. 64, no. 1, pp. 98–103.
22. Je, J.-Y., Park, P.-J., and Kim, S.-K., *Food Chem Toxicol*, 2004, vol. 42, no. 3, pp. 381–387.
23. Ji, H.-F., Tang, G.-Y., and Zhang, H.-Y., *QSAR Comb. Sci.*, 2005, vol. 24, no. 7, pp. 826–830.
24. Fiorucci, S., Golebiowski, J., Cabrol-Bass, D., and Antonczak, S., *J. Agric Food Chem*, 2007, vol. 55, no. 3, pp. 903–911.
25. Pacheco, N., Larralde-Corona, C.P., Sepulveda, J., Trombotto, S., Domard, A., and Shirai, K., *Int. J. Biol. Macromol.*, 2008, vol. 43, no. 1, pp. 20–26.
26. No, H.K., Young Park, N., Ho Lee, S., and Meyers, S.P., *Int. J. Food Microbiol.*, 2002, vol. 74, no. 1–2, pp. 65–72.
27. Fernandez-Saiz, P., Ocio, M.J., and Lagaron, J.M., *Biopolymers*, 2006, vol. 83, no. 6, pp. 577–583.
28. Nonaka, T., Uemura, Y., Ohse, K., Jyono, K., and Kurihara, S., *J. Appl. Polym. Sci.*, 1997, vol. 66, no. 8, pp. 1621–1630.
29. Feugang, J.M., Konarski, P., Zou, D., Stintzing, F.C., and Zou, C., *Front Biosci.*, 2006, vol. 11, no. 1, pp. 2574–2589.
30. Cantwell, M., RodrÌguez-Felix, A., and Robles-Contreras, F., *Scientia Horticulturae*, 1992, vol. 50, no. 1–2, pp. 1–9.
31. Nerd, A., Dumoutier, M., and Mizrahi, Y., *Postharvest Biology and Technology*, 1997, vol. 10, no. 2, pp. 135–143.
32. McGuire, G.R., *HortScience*, 1992, vol. 27, no. 12, pp. 1254–1255.