J. Chitin Chitosan 23(2), 69-75 (2018)
DOI http://dx.doi.org/10.17642/jcc.23.2.1

Preparation of Water-Soluble Chitosan and *Aloe vera* Mucilage and Their Use for Assembly of Polyelectrolyte Complexes

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ABSTRACT

In this work, the preparation and characterization of water-soluble polysaccharides from chitosan and *Aloe vera* was studied. The water-soluble polysaccharides were used to study polyelectrolyte complexes. The reaction time effect for chitosan hydrolysis by endo-chitosanase was studied at 40ºC and pH 5.00 to produce water soluble chitosan (WSCh). The physico-chemical characteristics of chitosan hydrolysates, water-soluble *A. vera* polysaccharides and polyelectrolyte complexes were determined. After 3 h of chitosan processing, a viscosity reduction of 90%, while only 2.3% of reducing sugars were released. A WSCh was recover by ultrafiltration (1 kDa) from chitosan hydrolysate after 12 h and was spray-dried with a yield of 9.7%. Cold-water extraction of *A. vera* mucilage from pulp gives a crude polysaccharide yield of 0.81 g/kg (wet basis) based on whole leaf weight. The water isolated mucilage is composed of a mixture of protein and mannan rich-polysaccharide. The results show WSCh's association capacity with *A. vera* mucilage by electrostatic interaction.

Keywords: Acemannan, Pectic substances, Chitosanase, Remazol brilliant blue, Spray-dyer

Introduction

Water-soluble chitosan (WSCh) is a low molecular weight polysaccharide derived from chitosan by controlled depolymerization, using chemical (1), enzymatic (2,3) or physical (4,5) methods. Chemically, WSCh is an amino-polysaccharide that consists of linear chains of D-glucosamine units linked by β-(1→4) glycosidic bonds. Various enzymatic methods to prepare WSCh, using crude or purified chitosanases, have been described by numerous researchers (6-10) scientists. Endo-chitosanases catalyze chitosan hydrolysis to yield low molecular weight derivatives such as WSCh or chitosan-oligosaccharides (COS). The production WSCh or COS from chitosan in continuous systems by immobilized enzymes has been reported earlier as well (3,11-13). The low molecular weight derivatives from chitosan are considered a specialty, evoking interest in the agriculture, cosmetic, feed, foods, medicine, pharmacy, polymer, and pharmaceutical industries. Presently, the production of chitosan hydrolyzates is still limited, therefore it is necessary to develop biotechnological processing to diversify the market in nutraceuticals, glycotechnology, and functional foods.

The polycationic characteristic of WSCh allows the ionic interaction with anionic biopolymers for the preparation of polyelectrolyte complexes. Low molecular weight chitosan has been used for preparation of polyelectrolyte complexes with carboxy-methylcellulose (14), alginate (15,16), heparin (17) and fucoidan (18).

Aloe vera is a succulent plant with a tremendous application in functional foods and medicine. *A. vera* gel contains a mixture of active nutriments such as mucilage, polysaccharides, proteins, organic acids, vitamins, and minerals (19). Purified mucilage can contain acemannan, pectic substances, and glycoproteins (20). The pectic substances and proteins could be conferring the polyanionic characteristics to form polyelectrolyte complexes. Until now, there is little information about polyelectrolyte complex formation between WSCh and *A. vera* mucilage.

In this study, we focused our work on enzymatic WSCh preparation, characterization, and further use for polyelectrolyte formation with *A. vera* mucilage. Infrared spectroscopy and colorimetric techniques were employed to investigate the interaction between WSCh with *A. vera* mucilage by using remazol brilliant blue, an anionic dye. The results found in this research may be important for the generation of polyelectrolyte assembled biomaterials using WSCh and

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A. vera polysaccharide.

Materials and Methods

Chemicals and biologicals

Commercial chitosan powder, purchased from Industrias Poseidon (Mexico City, Mexico). Purified endo-chitosanase (35000 U/g) from *Bacillus* sp. and exo- and endo-chitosanase (5000 U exo-chitosanase/g) powder from *Trichoderma viride,* purchased from Amicogen (Jinju, Korea). Remazol brilliant blue, D-glucosamine, galacturonic acid, and bovine serum albumin were purchased from Sigma-Aldrich (Mexico City, Mexico). Genipin (98%) from *Gardenia jasminoides* was obtained from Challenge Bioproducts Co. Ltd. (Yun-Lin Hsien, Taiwan, Republic of China). Lactic acid and isopropanol were purchased from Jalmex (San Nicolas de los Garza City, Nuevo Leon State, Mexico). *A. vera* plants were purchased from local dealer (Agua Nueva village, Saltillo City, Coahuila State, Mexico). Blue dextran 2000 was purchased from Amersham Bioscience (Uppsala, Sweden). All other chemical reagents were of reagent grade.

Chitosan hydrolysates preparation by endo-chitosanase

Chitosan enzymatic hydrolysis (3.4%, w/v) took place in 4 M sodium lactate buffer (pH 5.0) in duplicate, in a 250 mL Erlenmayer flask containing 120 mL of chitosan solution and endo-chitosanase solution (5.7 U). Flasks were incubated at 40ºC, on a rotary shaker (Innova44, New Brunswick Scientific, Edison, New Jersey, USA) at 150 rpm for 3, 6, 12, 24, 36, 48, and 60 h. A blank of sodium lactate buffer, instead of enzyme was kept. After incubation time, the samples were assayed for viscosity. Subsequently, the entire content of the flasks were boiled for 10 min, cooled to 24ºC and filtered through 0.45 mm cellulose microfilters (Whatman, England). Filtered chitosan hydrolysates were stored at 4°C until further analysis.

Enzymatically chitosan hydrolysates fractionation

Chitosan hydrolysates (1.4 mL) were subjected to fractionation on Fast Protein Liquid Chromatography system (FPLC, Akta Prime, Amersham Biosciences, Uppsala, Sweden). For this test a gel permeation column Sephadex™ G-25 Hi-Trap Desalting (5 mL, GE Healthcare, USA), equilibrated with 20 mM lactate sodium buffer (pH 5.45) with NaN_3 0.02% (w/v) was used. Each hydrolysate was eluted with 15 mL of the same buffer at a flow rate of 1 mL/min and 2 mL fractions were collected. The obtained fractions were analyzed for reducing sugars.

WSCh recovery

For recovery of WSCh, the 12 h hydrolysate (190 mL) was diluted with distilled water to 850 mL volume, concentrated by ultra filtration with a 1 kDa cutoff membrane on a QuixStand system (GE Healthcare, USA), and diafiltered fourteen times with 500 mL of distilled water at 20ºC. The diafiltered chitosan hydrolysate was sprayed dried (0.5 mm nozzle diameter; Mini Spray Dryer 290, Buchi, Flawil, Wil, Switzerland). The conditions of spray-drying were as follows: inlet air temperature was 180°C, outlet air temperate 57°C, atomization air flow rate of 15 mL/min, atomization air pressure of 2 bar, and aspirator level 100%.

Aloe vera mucilage preparation

The *A. vera* mucilage was prepared according to the method of Rangel-Rodriguez (26). The whole leaves were washed with distilled water, cut at the ends, and the side spines were removed with a knife. The peel was removed from leaves and the pulp was retrieved. Pulp was submerged in isopropanol in ratio 1:2 (w/v), boiled for 10 min and equilibrated for 12 h at room temperature. The blanched pulp was dried by water solvent-exchange and oven dried 40°C to a constant weight, the material was ground in a mortar and stored in glass containers. For mucilage extraction, dried pulp (25 g) was dispersed in distilled water (1 L) and stirred in a rotary shaker (100 rpm) at 40°C for 3.5 h. After incubation, the whole content was filtered through muslin cloth. The filtrate was precipitated by addition of 2 volumes of 98% isopropanol and centrifuged for 30 min at 8,000 rpm. The precipitate was washed with isopropanol (1,000 mL), centrifuged again, and collected, dried at 50ºC to a constant weight. The mucilage was ground in a mortar and stored in glass container. The mucilage yield is reported in dry weight of extracted material/wet weight of leave. The experiments were made in duplicate.

Viscosity

Viscosities of *A. vera* mucilage solution (1%, w/v), enzyme hydrolysates of chitosan, and their blanks were determined with a vibrational portable viscosimeter (Viscolite 700, Hydramotion Ltd, Malton, York, England). Measurements were made at 37°C and values were reported in centipoise (cP) units.

Polyelectrolyte interaction between aloe mucilage and WSCh

A. vera mucilage was dissolved in water at 1.25, 2.50 and 5.00 mg/mL and adjusted to pH 6.5 with 1 M NaOH. Four hundred μL of mucilage solution were added separately in micro tubes. Two hundred μL of WSCh at 1.25, 2.50 and 5.00 mg/mL (pH 5.0) were also added to each tube and incubated for 10 min at 4°C. After incubation, distilled water was added to bring the total volume up to 1200 μL in each tube and immediately centrifuged at 5000 rpm for 20 min at

4°C. After centrifugation, 200 μL remazol brilliant blue dye (0.1%, w/v) was added to each tube and further maintained for 20 min at 4°C, and centrifuged again at 5000 rpm for 20 min at 4°C. The supernatant was read at 536 nm using spectrophotometer. A control was performed in the same manner using distilled water instead of biopolymer solutions. The analysis was performed in quintuplicate.

Spectrophotometer assays

Total uronic acids present in mucilage were determined by colorimetry at 520 nm from a standard curve of galacturonic acid by the method of Blumenkrantz and Asboe-Hansen (21). Protein content in mucilage was evaluated at 590 nm by genipin assay (22), using glycine as standard. Reducing sugars present in mucilage and chitosan hydrolysates were determined at 660 nm by the method of Somogyi-Nelson (23,24). Glucose and glucosamine were used as standards in the assay. Total sugars from chitosan hydrolysates were determined by the method of Dubois *et al.* (25) and enzymatic-colorimetric assay using purified exo- and endo-chitosanase (26). Briefly, the enzymatic-colorimetric method consists in the total depolymerization of chitosan using exo and endo-chitosanase and further monosaccharide analysis. The analysis used 25 μL of sample and 200 μL of 20 mM sodium lactate buffer (pH 5.45). The samples were added to the 25 μL of exo- and endo-chitosanase (2.5 U/mL), incubated for 1 h at 37°C, and then assay for sugar, using Somogyi-Nelson assay (23,24). The relative degree of polymerization (DP) was determined by quantifying the relationship between total sugars and reducing sugars present in each hydrolysate. Glucosamine was used as standard in the assay. The color change (browning index) of chitosan hydrolysates was determined by spectrophotometry at 440 nm.

Infrared spectroscopy

A. vera mucilage, WSCh and polyelectrolyte complexes were analyzed in powder form by Fourier transform infrared attenuated total reflectance (FT-IR/ATR) spectroscopy (Perkin-Elmer, USA), operating at 4 cm^{-1} resolutions. The mirror velocity was 0.08 cm^{-1} and 35 interferograms were co-added before Fourier transformation. Spectra were collected from 4000 to 650 cm^{-1} and normalized to the absorption band at ~1008 cm^{-1} equaled 1. Normalization did not alter the proportion of signals in the original spectra.

Gas chromatography of *A. vera* mucilage

The individual neutral sugars from *A. vera* mucilage (2.0 mg) was hydrolyzed with 2 N trifluoroacetic acid (TFA) (500 μL) at 121°C for 1 h and derivatized to alditol acetates with $NaBH_4$ using the method of Albersheim *et al.* (27) and were analyzed (1.5 μL) using a gas chromatograph (Varian CP-3800, Palo Alto, California, USA) fitted with a flame-ionization detector and DB-225 capillary column (30 m × 0.25 mm i.d., 0.15 μm film thickness, Agilent Technologies/J&W Scientific, Santa Clara, California, USA). Column was maintained at 250ºC and helium was used as a carrier gas (3.0 mL min/1; 41.5 psi). The experiments were carried out in duplicates.

Results and Discussion

Production and characterization of chitosan hydrolysates obtained with endo-chitosanase

The endo-chitosanase was used for preparation of chitosan hydrolysates after a reaction time of 60 h. The chitosan hydrolysates were characterized for viscosity, reducing sugars, total sugars, color, and degree of polymerization. The viscosity profile of the chitosan hydrolysates obtained by endo-chitosanase is given in Fig. 1(a). The control samples showed a slight reduction in viscosity, indicating a negligible depolymerization of chitosan. The enzymatically treated chitosan recorded a significant decrease in viscosity after incubating the sample for 3 h with the endo-chitosanase. The viscosity of subsequent chitosan samples treated with enzyme slightly decreased after 6 h of incubation in comparison with the hydrolysate analyzed for 3 h. The use of purified enzyme can be useful to control the characteristics of the products formed after chitosan hydrolysis. The characteristics of this depolymerization pattern confirm the nature of endo-chitosanase.

The total sugar content present in chitosan hydrolysates (3, 6, 12, 24, 36, 48, and 60 h) were evaluated by enzymatic/colorimetric method and showed a content of 55.29 ± 2.86 g/L from 0 to 60 h. The phenol-sulfuric method for assay total sugars from chitosan hydrolysates showed a low sensitivity for total sugar content detection. Moreover, the phenol-sulfuric method for assay sugars in the presence of sodium azide at 0.02% (w/v) (28) also reported a low sensitivity. The time effect on the release of reducing sugars from chitosan by endo-chitosanase is given in Fig. 1(b). A linear pattern was obtained during 3 to 24 h of enzymatic hydrolysis and after that there was a minimal change in the content of reducing sugars. A breakdown of no more than 14% of total sugars (55.29 ± 2.86 g/L) was observed after 48 h of incubation.

The relative degree of depolymerization (DP) of chitosan hydrolysates by endo-chitosanase was evaluated by time, as shown in Fig. 2(a). The DP value of chitosan hydrolysates after 3 h of incubation was about 40 h. After 6 h, the DP was reduced by 50% compared to the first hydrolysate. After 24 h of hydrolysis, chitosan hydrolysates yielded a DP of 8. The pattern of chitosan hydrolysis confirmed in our results shows endo-chitosanase has a random attack mechanism on substrate (8,12,29). Color of chitosan hydrolysates increased

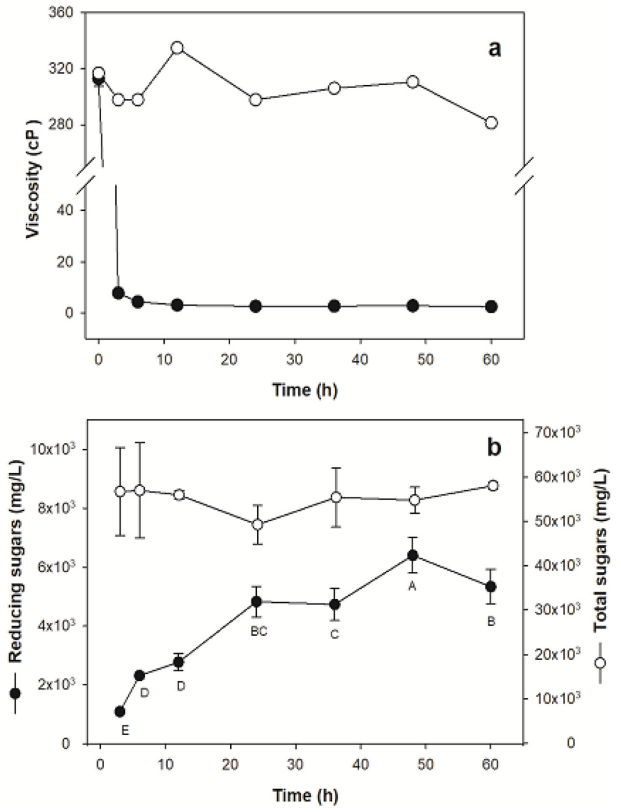

Fig. 1. Change of viscosity (a), reducing sugars and total reducing sugars (b) released during chitosan hydrolysis by endo-chitosanase. Symbols: black circles (control), white circles (enzyme). See text for details.

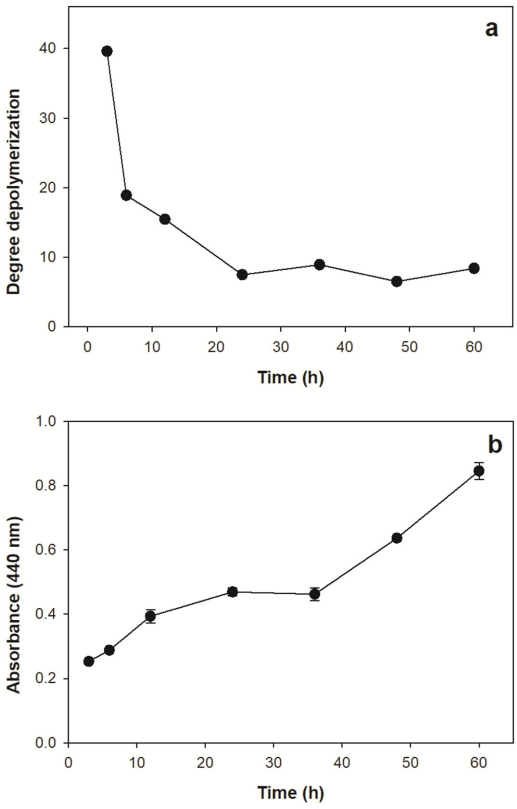

Fig. 2. Relative degree of polymerization (a) and color formation (b) of chitosan hydrolysates treated enzymatically.

as the reaction time increased (Fig. 2(b)). The color development in the samples is attributed to Maillard reactions after enzyme inactivation under acidic conditions. However, under our experimental conditions, chitosan was highly decreased, compared with the one obtained by using acetic acid (data not shown). The interaction of amino sugars, like glucosamine, with chitosan-oligosaccharides generates a brown color Chung *et al*. (30). The Maillard reaction of WSCh is thoroughly described in the literature and the browning reaction depends on time, temperature, and moisture (31).

The chitosan hydrolysates also were characterized by gel permeation chromatography, evaluating directly the reducing sugars in each fraction of the tube. The chromatographic profiles of each chitosan hydrolysate, obtained after endo-chitosanase treatment, and assayed as reducing sugars are given in Fig. 3. After 3 h treatment chitosan hydrolysates showed a heterogenous peak with reducing sugars of about 600 mg/L, and after 6 h enzymatic hydrolysis of the reducing sugars content increased twice. These results indicated that the enzyme can hydrolyze chitosan to WSCh or COS and not to glucosamine. The others hydrolysates showed the same elution pattern as the sample hydrolysates from 12 to 60 h.

Based on the results, the chitosan hydrolysate obtained at 12 h was concentrated by ultra filtration with pore diameter of 1 kDa. The concentrated was diafiltered and spray-dried to obtain a light yellow powder, and its yield was 9.7% in relation with the total mass of chitosan. Other researchers who concentrated chitosan hydrolysates using Celluclast commercial enzyme as a source of chitosanase by ultrafiltration (9) have also reported these low yields. The powder showed an excellent solubility in water.

The FT-IR spectra derived from chitosan hydrolysates and depicted in Fig. 4. The spectrum was exhibited to broad absorption between 600-6000 cm^{-1}. According to FT-IR analysis, mainly show the absorption band corresponding to polysaccharides (700-1400 cm^{-1}). The major peaks detected in chitosan spectrum were absorbed around 1648 cm^{-1} (amide I), 1315-1325 cm^{-1} (amide III), 1520-1580 cm^{-1} (protonate amine group), characteristic band for chitosan and WSCh (32,33).

Characterization of *A. vera* mucilage

The isolated mucilage of *A. vera* obtained by cold-extraction method yielded 0.81 g/kg (wet basis) with respect to the whole leaf weight. The yield obtained in the present study

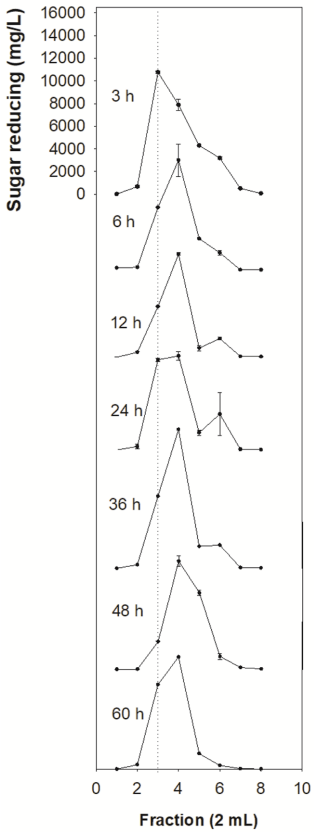

Fig. 3. Fractionation of chitosan hydrolysates by gel filtration chromatography and its characterization in terms of reducing sugars.

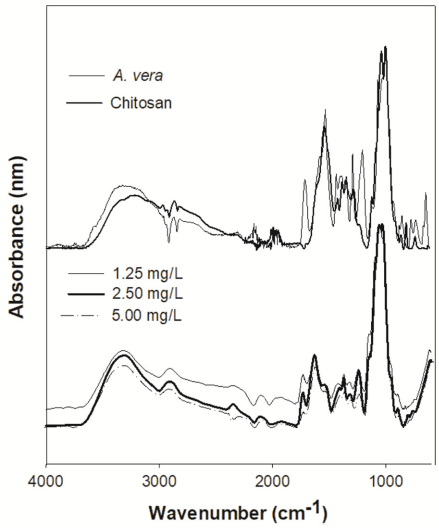

Fig. 4. Infrared spectroscopy of chitosan hydrolysate (12 h) obtained after spray drying, *A. vera* mucilage, complex polyelectrolyte WSCh (1.25; 2.50; 5.00 mg/mL) with *A. vera* mucilage.

was higher than the one from the hot-extraction process reported by Mandal and Das (34) for *A. vera*. Mucilage viscosity and pH were 15 ± 0.6 cP and 4.5, respectively. The pH result is similar to that reported in *A. vera* gel by Yaron (35). Total neutral sugars (48%) analyzed by gas chromatography of *A. vera* mucilage were: mannose (41%), glucose (2.99%), galactose (2.08%), xylose (1.22), arabinose (0.95%), rhamnose (0.12%) and fucose (0.10%). These results show that water-soluble mucilage isolated from Mexican *A. vera* contain mainly mannan and other sugars in minor content.

By colorimetric assays, the total sugar content and protein in dry basis were 43.9 ± 0.7% and 52.0 ± 3.3%, respectively. These results indicate that mucilage is a mixture of polysaccharides and glycoprotein. The total uronic acids (galacturonic/glucoronic acids) and reducing sugars were 8.3 ± 0.19% and 2.6 ± 0.33%, respectively. The anionic characteristics of isolated *A. vera* mucilage can be expected to be due to both the presence of the free carboxylic of uronic acids and the amino acids.

The infrared spectroscopy peaks of mucilage powder's absorption band were mainly composed by signals corresponding to polysaccharides (Fig. 4). The major peaks detected in *A. vera* mucilage could be observed around 2820 cm^{-1} (O-H stretching), 2920 cm^{-1} (C-H stretching), 1738 cm^{-1} (C=O stretching), 1620 cm^{-1} (C=O stretching and C-C ring stretching), 1466 cm^{-1} (C-H bending), 1234 cm^{-1} (C-O-H bending), and 1008 cm^{-1} (C-O-C stretching). The assignment of peaks in the infrared spectra for *A. vera* mucilage was in agreement to the previous report from Xng *et al*. (36). The absorption band between 650 and 710 cm^{-1} are characteristic bands for mannans.

Polyelectrolyte interaction between Aloe vera mucilage and WSCh

Polyelectrolyte associations of anionic and cationic biopolymers were evaluated by mean of infrared spectroscopy and colorimetric methods. The FT-IR-ATR complex polyelectrolyte formation between WSCh and mucilage *A. vera* are showed in Figs. 4(c)-(d). The major peaks between 950 and 1200 cm^{-1} correspond to the carbohydrate mark in WSCh and mucilage *A. vera*. The spectra of 1500-1700 and 1750 cm^{-1} showed a decreased intensity, and it was attributed to the complex formation. The intensity of the esterified carboxyl groups at 1738 cm^{-1} decreased to a lower frequency due to the chitosan amino groups' interaction (1). The peak at 1738 cm^{-1} decreased as the WSCh concentration increased, because there are a great number of amino groups present that interact with carboxyl groups. The absence of the peak at 670 cm^{-1} corresponding to the mannan is attributed to the interaction with proteins present in mucilage *A. vera*. The absorption band between 950 and 1200 cm^{-1} showed an increased intensity and broad interaction for polysaccharides present in mucilage *A. vera*.

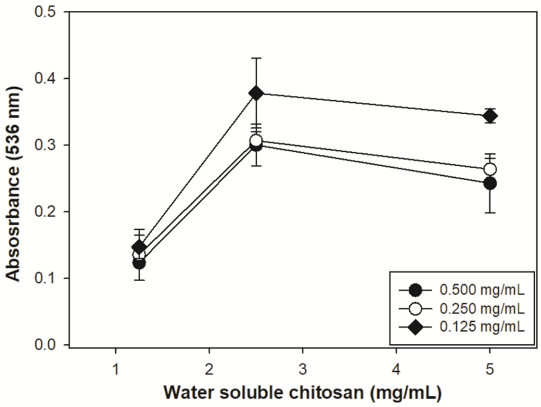

Fig. 5. Effect of concentration WSCh (1.25, 2.50 and 5.00 mg/mL) on polyelectrolyte complex formation with *A. vera* mucilage (1.25, 2.50 and 5.00 mg/mL), evaluated by use of remazol brilliant blue.

The effects of WSCh concentration on polyelectrolyte complex formation with *A. vera* mucilage was also studied by colorimetric assay-dye using remazol brilliant blue and infrared spectroscopy. The absorbance obtained as index of polyelectrolyte interaction between biopolymers is given in Fig. 5. By increasing the concentration of WSCh, the polyelectrolyte complex formation increased. The results showed WSCh's association capacity with *A. vera* mucilage by electrostatic interaction. The control sample, remazol brilliant blue dissolved in water, showed an absorbance of 0.566 ± 0.005. However, by using a concentration of WSCh higher than 5 mg/mL the formation of complexes did not improve. The polyelectrolyte interaction between two biomaterials, by using a low concentration to test the polyelectrolyte interaction, showed a lower capacity of interaction. Due to the presence of uronic acids in *A. vera* mucilage, the COO^- groups (37) allow the interaction with the NH_2 groups present in WSCh. In previous studies, Zamora-Iruegas (38) evaluated the formation of polyelectrolyte complex with WSCh and pectin using brilliant blue remazol dye. The degree of methyl-esterification in pectin has important effect for polyelectrolyte formation with WSCh. The formation of polyelectrolyte of *A. vera* gel-chitosan complex promotes the lipid lowering activity in Wistar rats (39).

Conclusion

The present study demonstrated that tailor-made WSCh are produced in a batch system using free chitosanase in presence of lactic acid buffer. The high solubility of WSCh is attributed to the diminution of molecular weight. The isolated mucilage of *A. vera* includes a fraction of mannan polysaccharide and protein. A system based on the polyelectrolyte complex between WSCh and *A. vera* mucilage can be expected to broaden technological applications.

Acknowledgements

A grant from the Mexican Council of Science and Technology (CONACYT) supported this research project, in addition to Fondos Mixtos from the state of Puebla, Mexico, and CONACYT-Fondos Mixtos, from the state Quintana Roo. Partially, CONACYT-National Science Foundation, USA also supported this study. A.M. Rangel-Rodriguez was supported by a graduate student scholarship from CONACYT.

References

1. Choi, G. J., Kim, H. S., Sim, S. J., Kim, Y. D., Woo, K., & Cho, Y. S.: Hydrolytic depolymerization of chitosan using organic acids to oligo-chitosaccharides. *J. Chitin Chitosan,* **1999**, *4*, 90-95.
2. Park, R. D., Rhee, C. O., Lee H. C., Cho, C. S., and Jo, D. H.: A comparative study of chito oligosaccharide production from chitosan. *J. Chitin Chitosan*, **1999**, *4*, 96-101.
3. Song, J. Y., Alnaeeli, M., and Park, J. K.: Efficient digestion of chitosan using chitosanase immobilized on silica-gel for the production of multisize chitooligosaccharides. *Process Biochem*. **2014**, *49*, 2107-2113.
4. Han, Y. S., Kim, K. S., Lee, Y. N., and Cho, S. H.: Ultrasonic degradation of chitosan. *J. Chitin Chitosan*, **1999**, *4*, 1-7.
5. No, H. K., Kim, S. D., Kim, D. S., Kim, S. J., and Meyers, S. P.: Effect of physical and chemical treatments on chitosan viscosity. *J. Chitin Chitosan*, **1999**, *4*, 177-183.
6. Choi, Y. J., Kim, E. J., Kim, Y. S., and Shin, Y. C.: Development of chitosanase production for the production of chitosan oligosaccharides. *J. Chitin Chitosan*, **1997**, *2*, 40-48.
7. Kim, S. K., Jeon, Y. J., and Zan, H. C.: Antibacterial effects of chitooligosaccharides with different molecular weights prepared using membrane reactor. *J. Chitin Chitosan*, **2000**, 5, 1-8.
8. Lee, K. S., Choi, M. R., and Lim, H. S.: Development of co-encapsulating technology for the production of chitosan oligosaccharides. *Biotechnol. Bioprocess Eng.* **2000**, *5*, 345-349.
9. Shin, S. S., Lee, Y. C., and Lee, C. (1997). The chitosanolytic activity of commercial food grade enzymes. *Food Sci. Biotechnol.* **1997**, *6*, 265-269.
10. Kuroiwa, T., Ichikawa, S., Hiruta, O., Sato, S., and Mukataka, S.: Factors affecting the composition of oligosaccharides produced in chitosan hydrolysis using immobilized chitosanase. *Biotechnol. Prog.* **2002**, *18*, 969-974.
11. Jeon, Y. J. and Kim, S. K.: Continuous production of chito-oligosaccharides using a dual reactor system. *Process Biochem.* **2000**, *35*, 623-632.
12. Jeon, Y. J., Park, P. J., Byun, H. G., Song, B. K., and Kim, S. K.: Production of chitosan oligosaccharides using chitin-immobilized enzyme. *Korean J. Biotechnol. Bioeng.* **1988**, *13*, 147-154.

13. Kim, S. K. and Rajapakse, N.: Enzymatic production and biological activities of chitosan oligosaccharides (COS): A review. *Carbohydr. Polym*. **2005**, 62, 357-368.
14. Ichikawa, S., Iwamoto, S., and Watanabe, J.: Formation of biocompatible nanoparticles by self-assembly of enzymatic hydrolytes of chitosan and carboxymethyl cellulose. *Biosci. Biotechnol. Biochem.* **2005**, *69*, 1637-1642.
15. Barkowiak, A. and Hunkeler, D.: Alginate-oligochitosan microcapsules: A mechanistic study relating membrane and capsule properties to reaction conditions. *Chem. Mater.* **1999**, *11*, 2486-2492.
16. Barkowiak, A. and Hunkeler, D.: Alginate-oligochitosan microcapsules. II. control and permeability of the membrane. *Chem. Mater.* **2000**, *12*, 206-212.
17. Kweon, D. K., Song, S. B., and Park, Y. Y.: Preparation of water-soluble chitosan/heparin complex and its application as wound healing accelerator. *Biomaterials* **2003**, *24*, 1595-1601.
18. Huang, Y. C. and Li, R. Y.: Preparation and characterization of antioxidant nanoparticles composed of chitosan and fucoidan for antibiotics delivery. *Mar. Drugs* **2014**, *12*, 4379-4398.
19. Hamman, J. H.: Review: Composition and applications of *Aloe vera* leaf gel. *Molecules* **2008**, *13*, 1599-1616.
20. Wozniewski, T., Blaschek, W., and Franz, G.: Isolation and structure analysis of a glucomannan from the leaves of *Aloe arborescens* var. Miller. *Carbohydr. Res.* **1990**, *198*, 387-391.
21. Blumenkrantz, N. and Asboe-Hansen, G.: New method for quantitative determination of uronic acids. *Anal. Biochem.* **1973**, *54*, 484-489.
22. Lee, S. W., Lim, J. M., Bhoo, S. H., Paik, Y. S., and Hahn, T. R.: Colorimetric determination of amino acids using genipin from *Gardenia jasmonides*. *Anal. Chim. Acta* **2003**, *480*, 267-274.
23. Nelson, N.: A photometric adaptation of the Somogyi method for the determination of glucose. *J. Biol. Chem.* **1944**, *153*, 375-380.
24. Somogyi, M.: Notes on sugar determination. *J. Biol. Chem.* **1952**, *195*, 19-23.
25. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F.: Colorimetric method for determination of sugars and related substances. *Anal. Chem.* **1956**, *28*, 350-356.
26. Rangel-Rodriguez, A. M.: Water soluble chitosan applications and anionic polymers on the formation complex polyelectrolyte, gels and microspheres (in Spanish). M.Sc. Thesis, Laboratory of Applied Glycobiology, Food Research Department. School of Chemistry, Universidad Autonoma de Coahuila, Saltillo City, Coahuila State, Mexico, **2008**.
27. Albersheim, P., Nevins, D. J., English, P. D., and Karr, A.: A method for the analysis of sugars in plant cell-wall polysaccharides by gas-liquid chromatography. *Carbohydr. Res*. **1967**, *5*, 340-345.
28. Linder, P. and Shomer, I.: Interfaces of azide in assays of carbohydrate. *Food Chem.* **1984**, *14*, 141-153.
29. Choi, Y. J., Kim, E. J., Piao, Z., Yun, Y. C., and Shin, Y. C.: Purification and characterization of chitosanase from *Bacillus* sp. strain KCTC 0377BP and its application for the production of chitosan oligosaccharides. *Appl. Environ. Microbiol.* **2004**, *70*, 4522-4531.
30. Chung, Y. C., Kuo, C. L., and Chen, C. C.: Preparation and important functional properties of water soluble chitosan produced through Maillard reaction. *Bioresour. Technol.* **2005**, *96*, 1473-1482.
31. Kerch, G., Rustichelli, F., and Ausili, P.: Effect of chitosan on physical and chemical processes during bread baking and staling, *Eur. Food Res. Technol.* **2008**, *226*, 1459-1464.
32. Mohammad, R. K.: Review: A review of several reported procedures to determine the degree of N-acetylation for chitin and chitosan using infrared spectroscopy. *Carbohydr. Polym.* **2007**, *71*, 497-508.
33. Seo, S. B. and Kajiuchi, T.: Preparation of water soluble chitosan blenders and their application to removal of heavy metal ions from wastewater. *Macromol. Res.* **2002**, *10*, 103-107.
34. Mandal, G. and Das, A.: Structure of the D-galactan isolated from *Aloe barbadensis* Miller. *Carbohydr. Res.* **1980**, *86*, 247-257.
35. Yaron, A.: Characterization of *Aloe vera* gel before and after autodegradation, and stabilization of the natural fresh gel, *Phytotherapy Res.* **1993**, *7*, S11-S13.
36. Xng, J. M., Li, F. F., Liang, Y. Z., and Liu, T. P.: Determination and extraction of Aloe polysaccharides by polyethylene glycol/ $(NH_4)_2SO_4$ two phase system. *China Pharmaceutical J.* **2007**, *42*, 1.45-5.45.
37. McConaughy, S. D., Kirkland, S. E., Treat, N. J., Stroud, P. A., and McCormick, C.L.: Tailoring the network properties of Ca^{2+} crosslinked *Aloe vera* polysaccharide hydrogels for *in situ* release of therapeutic agents. *Biomacromolecules* **2008**, *9*, 3277-3287.
38. Zamora-Iruegas, C. A.: Preparation and characterization of polyelectrolytic complexes with chitosan oligosaccharides and anionic polysaccharides (in Spanish), BSc. Thesis, Laboratory of Applied Glycobiology, Food Research Department, School of Chemistry, Universidad Autonoma de Coahuila, Saltillo City, Coahuila State, Mexico, **2008**.
39. Geremias, R., Pedrosa, R. C., Locatelli, C., de Favere, V. T., Coury-Pedrosa, R., and Laranjeira, M. C.: Lipid lowering activity of hydrosoluble chitosan and association with *Aloe vera* L. and *Brassica olearaceae* L. *Phytother. Res.* **2006**, *20*, 288-293.

Received	December	20	2017
Revised	March	14	2018
Accepted	March	19	2018