Preparation and characterization of Low molecular weight chitosan by cellulase digestion

Qi Yin, Fang Yang, Xiaoling Lu, Xuehan Wu, Liya Chen, Lin Zhang, Kang Yang, Hao Li, Zeng Yang, You Long, Chao Shen, Bo Yao, Chenghong Huang *

Chongqing college of science and technology, Chongqing, China, 401331

* 2013008@cqust.edu.cn

Abstract. Chitosan is up to present the only discovered cationic natural sugar. Moreover, small molecule chitosan is essentially valued as it is extraordinary suitable for fabrication of nanosize particle encapsulated medicine delivery system. Therefore, preparation of small-molecule chitosan has significant applications. 10% concentration cellulase to digest raw CS (Mw=1.5×105) resulting of 4.9×104 of LWCS, which are successively confirmed by FT-IR characterization, XRD analysis, DD testing and viscosity comparison. It verified enzyme digestion is an effective tool for LWCS preparation.

Keywords: Chitosan；Cellulase.

1. Introduction

Nanoparticles based on chitosan for drug delivery are now received more attentions because the chitosan have some unique characteristics1. Chitosan is a positively charged linear PS with a random arrangement of β-(1-4)-linked d-glucosamine and N-acetyl-d-glucosamine2. The presence of massive number of amino groups on the surface of chitosan enable them generating positive zeta potentials3. As the pKa of amino groups on the chitosan is around 6.5, they tend to remain protonation at acidic and neutral pH and result in stabilizing the aggregation nanoparticles4. The presence of amino functional groups on the surface of the chitosan helps them chemically or physically interact with the nanoparticles, drugs, polymers and cells. Chitosan has been one of the most widely explored polysaccharide in the pharmaceutical and biomedical sectors. Orally administered chitosan can reduce the absorption of dietary fat and cholesterol. Their ability to prevent the loaded therapeutic molecules from GI and enzymatic degradations and facilitate mucoadhesion of the loaded therapeutic molecules to the secreted mucus aids in prolonging their residual time in the small intestines[5].

Chitosan has been extensively used as an absorption enhancer for macromolecules and as gene delivery vehicle6. Both properties are molecular weight (MW) dependent7. Natural chitosan is high degree of polymerization with large molecular weight. The nanosize of chitosan in view of the above is relative bigger. Generally, the ranges are approximately 100-800nm level, but this not conducive to efficiency of drug absorbance in intestinal tract after oral administration8. Thereby, it essentially to depolymerization. Currently, a large number of methods has been applied for the depolymerization of chitosan. The most popular methods are involved in drying9,autoclaving process10, chemical treatment11, enzymatic digestion12 and so on. Generally speaking, the products of autoclaving process and chemical treatment are heterogeneous leading to poor drug particle uniformity. The enzymatic digestion of chitosan products can maintain the original structure and the corresponding developed drug carrier particles have good homogeneity. CS can be truncate by several methods13-15. This research aims to adopt chitinase to depolymerize chitosan with optimal physical and chemical conditions and further evaluate the properties of the products.

2. Materials and methods

2.1 Reagent and apparatus

Chitosan (Mw=150,000) and N-acetyl-D-dextrose were both bought from Shanghai McLean Biochemical Technology Co., Ltd.; Cellulase (10000U/g) were purchased from Shanghai Aladdin Biochemical Technology Co., Ltd.; Potassium ferricyanide and glacial acetic acid were from Chengdu Kelong Chemical Ping Co., Ltd.; N-acetyl-D-glucosamine, biotechnology grade, were obtained from Shanghai McLean Biochemical Technology Co., Ltd.; Potassium ferricyanide, sodium hydroxide, anhydrous sodium acetate(AR), anhydrous sodium carbonate, hydrochloric acid, were all obtained from Chengdu Kelong Chemical Co., Ltd. pH meter(PBS-3E), Shanghai Instrument and Electronics Science Instrument Co., Ltd.; Ultraviolet visible spectrophotometer T6, Beijing spectral analysis General Instrument Co., Ltd.; infrared spectrometer (tensor-27), German electronic balance Shanghai Yueping Scientific Instrument Co., Ltd.

2.2 Establishment of standard curve for sugar determination

Weigh 5.3 anhydrous sodium carbonate and prepare an aqueous sodium carbonate solution of 0.5 mol/mL. Weigh 10.0 g dried potassium ferricyanide and dissolved in sodium carbonate solution to make 0.5 mol/mL work solution. Prepare 2.0 mg/mL N-acetyl-D-dextrose standard solution for later use. 0.0, 0.1, 0.2, 0.3, 0.4 and 0.5mL N-acetyl-D-dextrose standard solution were firstly placed in tubes, then 2.0 mL (10.0 g/L)were successively added into the above solutions. In a boiling water bath for 10 min, remove the cuvette, cool to room temperature and settle to 25 m/L volume. The blank control was treated with deionized water to test the absorbance of the samples at 420 nm, and each sample was tested three times and averaged. Add 1.0 mL of subject into 25 mL test tube together with 2.0 mL afore-prepared potassium ferricyanide for 10 min incubation. The reaction solution regulated to constant volume 25.0mL. The blank control was run with deionized water to test absorbance at 420 nm. Each sample was repeated in triplicate and averaged.

2.3 Condition optimization of cellulase digestion

0.5g chitosan was dissolved in 50mL acetic acid sodium/acetate buffer solution with different pH values (pH 3.0, pH 3.5, pH 4.0, pH 5.0, pH 5.5, pH 6.0), respectively. After swelling for 2 hours, 0.05g cellulase (10% concentration) was added at 50 ℃ by magnetic stirring, and then heated to different temperature 100℃ for 10 min to inactivate the unreacted ones after 1 hour digestion. Then adopt aforementioned method procedure to determine reducing sugar concentration. Above reaction scheme was analogously applied to investigate optimal temperature and digestion for chitosan degradation.

2.4 Characterization

2.4.1 Determination of deacetylation degree

Deacetylation degree of chitosan and low molecular weight chitosan was determined by double jump potentiometric titration[16]. The calculation formula is as follows.

$$DD = \frac{C_{NaOH}(V_2 - V_1) \times 10^{-3} \times 16}{m \times 0.0994} \times 100\% \quad (1)$$

Where DD is deacetylation degree, CNaOH is concentration of NaOH (mol/L), The v1 and v2 are consumed NaOH (mol/L) volume of first and second jump point, m is sample weight, 16 and 0.0994 are relative molecular mass and theoretical molecular mass of amino group.

2.4.2 Viscosity measurement

The Ubbelohde viscometer was used to test the characteristic viscosity of the chitosan and LWCS. Record times of the prepared 0.25g/mL, 0.50g/mL, 0.75g/mL, 1.00g/mL and 1.25 g/mL

chitosan or LWCS solution passing through scale line a and scale line b, the calculated their characteristic viscosity by following formulas (2),(3) and (4).

$$\eta_r = \frac{T}{T_0} \quad (2)$$

$$\eta_{sp} = \eta_r - 1 \quad (3)$$

$$[\eta] = \lim_{C \to 0} \frac{\eta_{SP}}{C} = \lim_{C \to 0} \frac{\ln \eta_r}{C} \quad (4)$$

In which, T is outflow time of the solution (s), T0 is the outflow time of the pure solvent, ηr is relative viscosity, ηsp is Increase ratio viscosity and [η] is characteristic viscosity.

2.4.3 Determination of molecular weight

A gel penetration chromatograph (GPC)[17] was utilized to determine the molecular weight of CS and LWCS with test conditions of 30℃ temperature of Ultrahydrogel-2000 column, 0.1 mol/L acetic acid/sodium acetate buffer, 1.0 mL/min flow rate and polyethylene glycol as standard for 0.45 μm film filtered samples for measurement.

2.4.4 FT-IR characterization

Fourier transform-infrared spectroscopy (FT-IR). Samples were mixed with KBr (m/m=1:10) and delivered for tablet compressing. The Nicolet 10 (Thermo Nicolet) was used for measurement. The scanning wavelength is from 4000 to 400 cm-1.

2.4.5 X-ray diffraction (XRD) characterization

Crystal structures were determined by an X-ray diffraction analyzer (Shimadzu, Japan XRD-70000). Scanning range is 5~60, the speed is 5/min

3. Results and discussions

3.1 Effect of pH, temperature and digestion time for CS degradation

According to created standard curve Y=-21.99x + 2.5866, the reducing sugar content was calculated and the optimum pH was plotted. In the pH range of 3.0-6.0. Cellulase activity firstly heightened with the increase of pH value, and then decreased with the increase of pH value. The optimum pH of cellulase for CS digestion was designated 5.0 for later study(fig 1a). The effect of degradation temperature on the degradation rate of CS by cellulase is shown in fig 1b. At 30-50 ℃, with the increase of temperature, the degradation rate of CS is accelerated, and at 50-80 ℃ with the increase of temperature, the rate become weak, so the optimal temperature of digestion is selected as 50 ℃. In the process of degradation, with the increase of time, the content of reducing sugar also gradually increased within 0-60 minutes, which indicated that the LWCS produced by digestion accordingly increased. After that time, the content of reducing sugar did not change with the increase(fig 1c), indicating that the production of reducing sugar had reached the maximum and then tended to balance.

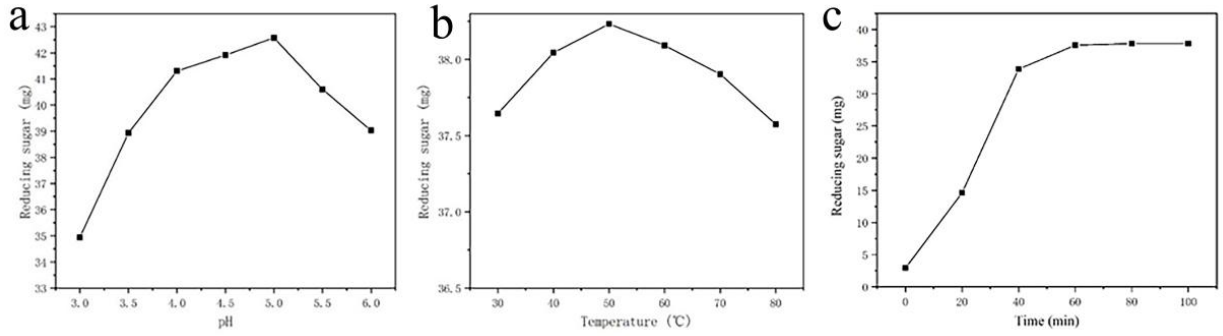

Figure 1 Reaction conditions of (a) pH,(b) temperature and (c) digestion time for CS degradation evaluated by reducing sugar determination.

3.2 Characterization of CS and LWCS

The FT-IR test result shows in fig2A, in which peak at 3420 cm-1 can be ascribed to -OH and -NH2 stretching vibration, peak at 2879 cm-1 can be attributed to stretching vibration of -CH2- group, but peaks at 1641 cm-1 and 1616 cm-1characteristic absorption peak should be caused amide I-band. While symmetrically deformed vibrations at 1419 cm-1 may be resulted from -CH2- and -CH3. Peak at 1380 cm-1 should be from expansion vibration of -CH3. Characteristic absorption peak at 1151 cm-1 and 1080 cm-1 rooted in stretching vibration of secondary hydroxyl group. Above typical absorption peaks including 3419 cm-1,2887 cm-1,1641 cm-1,1616 cm-1,1151 cm-1 and 1080 cm-1 of LWCS (fig 2B) are basically consistent with that of CS, which illustrated that the digestion will not destroy side chain structure but only truncate the length Main chain. XRD characterized results are shown in fig 2B, in which there is a strong diffraction peak at 20.3° of 2θ and it was supposed that they should be formed by stable and ordered hydrogen bonds network. After digestion, additional diffraction peaks appeared at 9.8° in LWCS and the corresponding one at 20.3° was attenuated. It believe that it is weakened by the hydrogen bond formation by digestion but the truncated LWCS will locally form new hydrogen bonds.

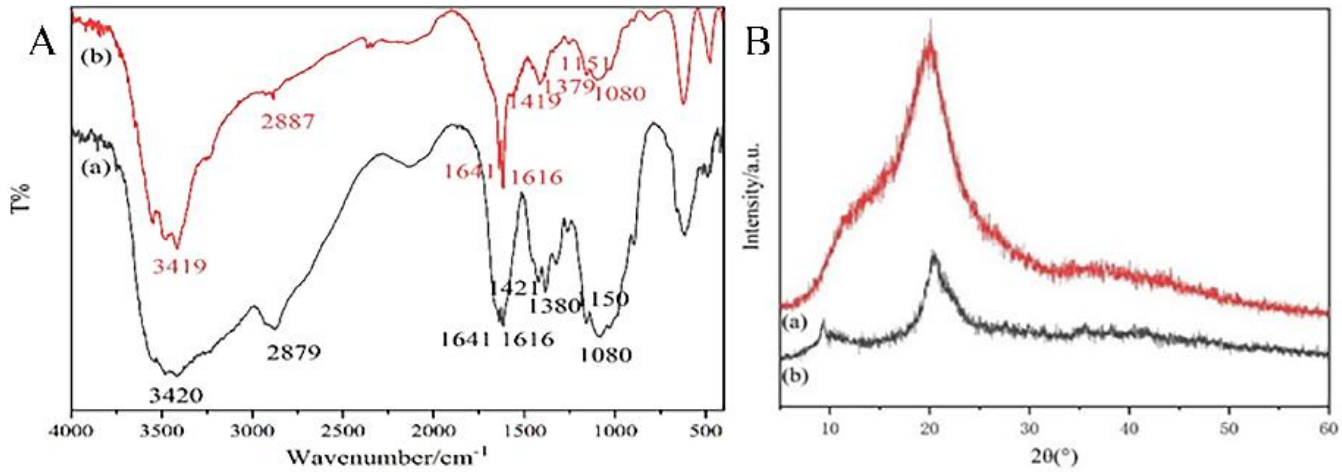

Figure 2 FT-IR and XRD analysis of original CS (a) and LWCS(b)

3.3 Measurement of DD

The pH~VNaOH relationship curve of the CS and LWCS are showed in figure 3. It can be seen that first and second jump of titration appear at pH=4.30 and 9.74 for CS as well as 3.39 and 7.52 for LWCS. The first jump may be resulted from neutralization reaction between NaOH and excess HCl but the second bring about neutralization of protonated amino group by NaOH.

Table 1 Deacetylation degree of CS and LWCS

Samples	m(g)	V_2-V_1(mL)	pH at first jump	pH at second jump	DD(%)
CS	0.2033	12	4.30	9.74	95
LWCS	0.2001	10	3.39	7.52	80

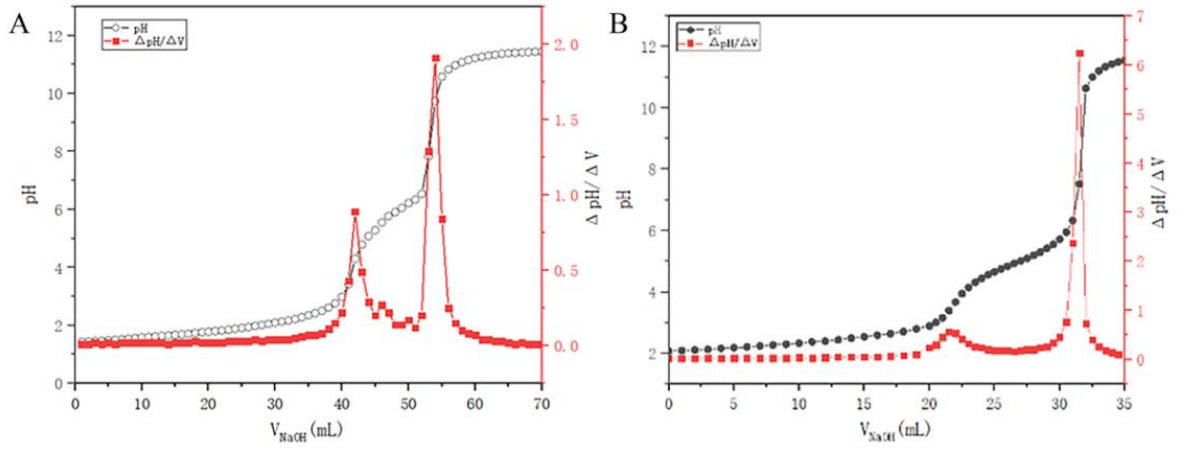

Figure 3 pH titration curves of CS (a) and LWCS (b).

3.4 Characteristic viscosity

lnηr/C~C and ηsp/C~C curves of CS and LWCS are shown in figure 4. Intrinsic viscosity [η] is the exapolated value of reduced viscosity or logarithmic viscosity by infinite dilution. As far as cs is concerned, linear relationship of lnηr/C and ηsp/C are Y=0.028X+0.51(R2=0.992) and Y=0.0564X+0.51 (R2=0.996). when the variable quantity is zero, the [η] of CS is 0.51 L/g. Accordingly, linear relationship of lnηr/C and ηsp/C are Y=0.0523X+0.28 (R2=0.995) and Y=0.0187X+0.33 (R2=0.9965) for LWCS determination with [η] value of 0.31 L/g. It is interpreted as the reduced viscosity of the truncated LWCS solution.

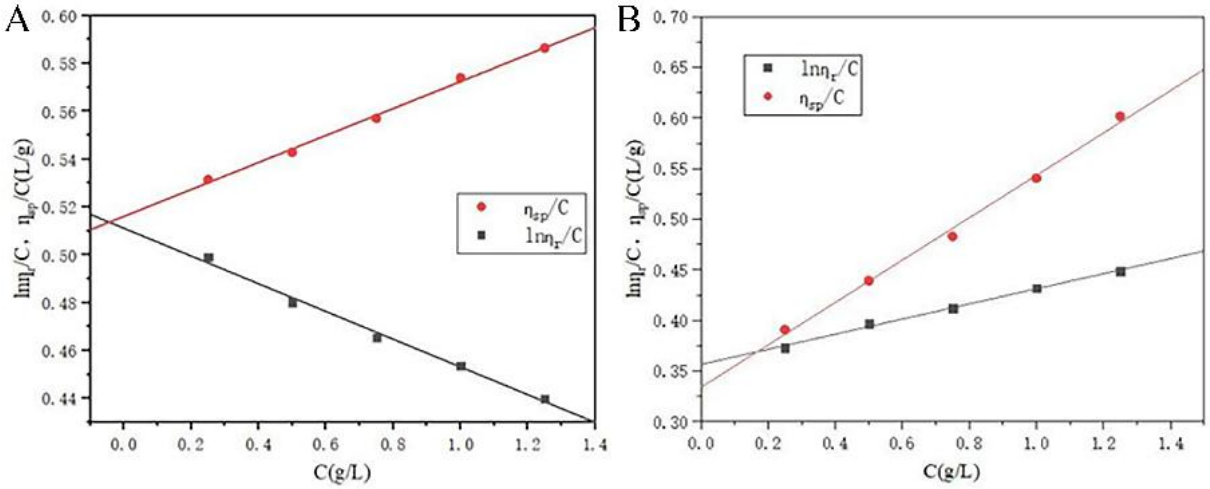

Figure 4 Intrinsic viscosity of CS(a) and LWCS (b) from their respective $\ln\eta_r/C$~C and η_{sp}/C~C curves.

3.5 Average molecular weight

GPC was used to determine average molecular weight and molecular weight distribution of CS and LWCS related parameters of Number average molecular weight (Mn) and Weight average molecular weight (Mw). In practice, Polydispersity index (PD) from Mw/Mn was usually to weigh the breadth of the polymer molecular weight distribution. PD=1 denote the subject is belong to Single-molecule distribution of high-polymer compounds, PD=1.0~1.2 signify High molecular compounds with a narrow molecular weight distribution, while PD=1.2~2.0 means High molecular compounds of moderate molecular weight and PD>2.0 indicates High molecular compounds with a wide molecular weight distribution. Measurement results of GPC displayed in figure 5 make clear that Mn and MW are 8.5×104 and 1.5×105 Da of CS but are 2.8×104 Da and 4.9×104 Da that of LWCS, which demonstrated there has a significantly lower cut down after digestion.

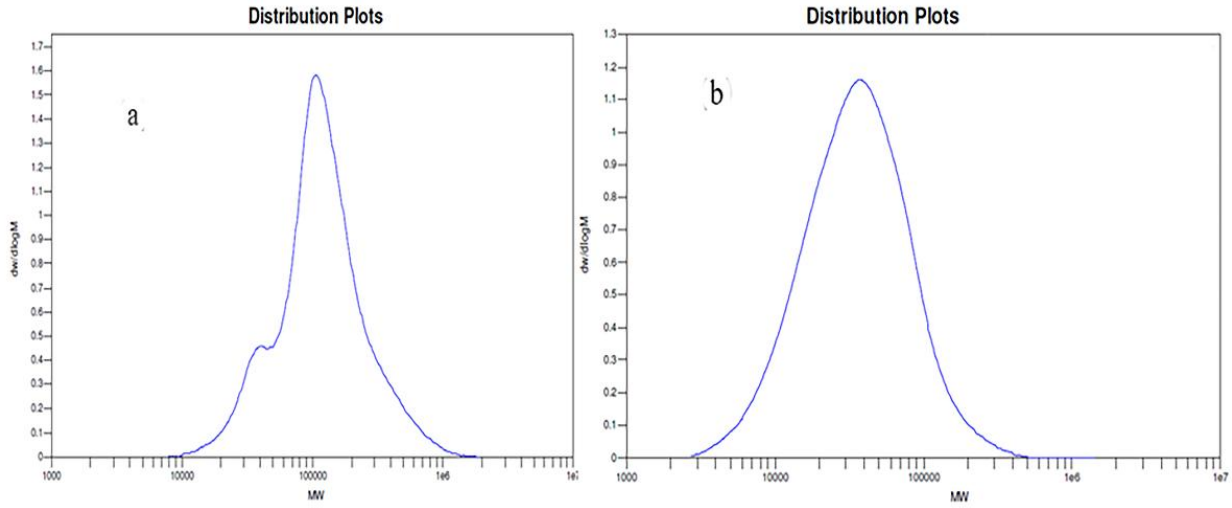

Figure 5 Average molecular weight distribution of CS (a) and LWCS (b) determined by CPC.

4. Summary

LWCS is very fit for preparation of nanosize drug delivery system and it is theoretically feasible to prepare small molecular CS by cellulase degradation. We employ 10% concentration cellulase to digest raw CS (Mw=1.5×105) resulting of 4.9×104 of LWCS, which are successively confirmed by FT-IR characterization, XRD analysis, DD testing and viscosity comparison. It verified enzyme digestion is an effective tool for LWCS preparation.

Acknowledgment

Thanks to Science and technology training program of CQUST(202311551010,202411551008).

References

[1] Fang, Y.; Wang, K.; Li, Q.; Huang, c., pH responsive release of paclitaxel by self-assembling Chitosan-Ethyl Vanillin@GNRs nanocomposites. Inernational journal of pharmecutics 2021, 607, 121047.

[2] Ke, C.-L.; Deng, F.-S.; Chuang, C.-Y.; Lin, C.-H., Antimicrobial Actions and Applications of Chitosan. Polymers 2021, 13, 1-24.

[3] Naveed; Muhammad; Phil; Lucas; Sohail; Muhammad, Chitosan oligosaccharide (COS):An overview. International Journal of Biological Macromolecules: Structure, Function and Interaction 2019, 2, 129827-843.

[4] Li, Z.; Yang, F.; Yang, R., Synthesis and characterization of chitosan derivatives with dual-antibacterial functional groups. International Journal of Biological Macromolecules 2015, 75, 378-387

[5] Chen, H.; Cui, S.; Zhao, Y.; Zhang, C.; Zhang, S.; Peng, X., Grafting Chitosan with Polyethylenimine in an Ionic Liquid for Efficient Gene Delivery. Plos One 2015, 1-17.

[6] Lodhi, G.; Yon-SukKim; Hwang, J.-W.; Kim, S.-K.; Jeon, Y.-J.; Je, J.-Y.; Ahn, C.-B.; Sang-HoMoon; Jeon, B.-T.; Park, P.-J., Chitooligosaccharide and Its Derivatives: Preparation and Biological Applications. BioMed Research International 2014, 2014, 1-13.

[7] Xia, W.; Liu, P.; Zhang, J.; Chen, J., Biological activities of chitosan and chitooligosaccharides. Food Hydrocolloids 2011, 25, 170-179.

[8] Yin, W.; Tian, L.; Wang, S.; Zhang, D.; Guo, S.; Lang, M., Co-delivery systems of paclitaxel prodrug for targeted synergistic therapy of breast cancer. Journal of Drug Delivery Science and Technology 2022, 69, 103157.

[9] Arantes, M. K.; Kugelmeier, C. L.; Cardozo-Filho, L.; Monteiro, M. R.; Oliveira, C. R.; Alves, H. J., Influence of the drying route on the depolymerization and properties of chitosan. Polumer engineering and science 2015, 1969-1976.

[10] Beyazita, N.; Çakran, H. S.; Ali Cabira, Y. A.; Demetgül, C., Synthesis, characterization and antioxidant activity of chitosan Schiff base derivatives bearing (−)-gossypol. Carbohydrate Polymers 2020, 240, 116333.

[11] A.MoRRIs, G.; KöK, M. s.; G.ADAMs, G.; HARDING, T. E., Polysaccharide drug delivery systems. Biotechnology and Genetic Engineering Reviews 2010, 27, 257-284

[12] SK, K.; N, R., Enzymatic production and biological activities of chitosan oligosaccharides(COS) : A review. Carbohydrate Polymers 2005, 62, 357-368.

[13] Li, J.; Du, Y.; Chen, P., Preparation and characterisation of low molecular weight chitosan and chito-oligomers by a commercial enzyme. Polymer Degradation and Stability 2005, 87, 441-448.

[14] Wu, Y.; Yao, P.; Wei, Y., Influence of ultraviolet-irradiated oxygen on depolymerization of chitosan. Polymer Degradation and Stability 2009, 94, 851-858.

[15] Cai, Q.; Gu, Z.; Yong, C.; Han, W.; Fu, T.; Song, H.; Li, F., Degradation of chitosan by an electrochemical process. Carbohydrate Polymers 2010, 79, 783-785.

[16] Kong, X., Simultaneous determination of degree of deacetylation, degree of substitution and distribution fraction of –COONa in carboxymethyl chitosan by potentiometric titration. Carbohydrate Polymers 2012, 88, 336-341.

[17] Nguyen, S.; Winnik, F. M.; Buschmann, M. D., Improved reproducibility in the determination of the molecular weight of chitosan by analytical size exclusion chromatography. Carbohydrate Polymers 2009, 528-533.