ELSEVIER

www.elsevier.nl/locate/carres

Carbohydrate Research 320 (1999) 257–260

CARBOHYDRATE
RESEARCH

Note

# Preparation of chitooligosaccharides from chitosan by a complex enzyme

Hu Zhang [a], Yuguang Du [a,*], Xingju Yu [a], Masaru Mitsutomi [b], Sei-ichi Aiba [c]

[a] *Department of Biochemical Engineering, Dalian Institute of Chemical Physics, Chinese Academy of Science, Dalian 116023, People's Republic of China*

[b] *Department of Applied Biological Sciences, Saga University, Saga 840, Japan*

[c] *Functional Polymer Section, Osaka National Research Institute, Osaka 653, Japan*

Received 8 January 1999; accepted 18 May 1999

---

**Abstract**

Chitosan of 24% degree of acetylation was depolymerized by a mixture of cellulase, alpha amylase, and proteinase to give the title oligosaccharides. The removal of products by membrane separation permitted yield maximization of products having degree of polymerization in the 3–10 range. © 1999 Elsevier Science Ltd. All rights reserved.

*Keywords:* Chitooligosaccharides; Enzyme mixture; Hydrolysis; Chitosan

---

*N*-Acetylchitooligosaccharides, from tetramer to heptamer, display strong responses to peritoneal exudate cells in BALB/c mice [1], and *N*-acetylchitohexaose as well as chitohexaose have significant growth-inhibitory effects against sarcoma-180 [2], MM-46 [2] and Meth-A solid tumors [3] in mice. Furthermore, *N*-acetylchitohexaose and *N*-acetylchitoheptaose are elicitors [4] and antibacterial agents [5]. These oligosaccharides have been prepared by chemical [6–8], fermentation [9,10] and enzymatic methods [11–17]. In this note, we report the preparation of chitooligosaccharides, from the tetramer to octamer, by an enzyme mixture.

After chitosan had been digested by the enzyme mixture at 40 °C for 40 min, a portion of the mixture was taken out and mixed with concentrated alkali. No precipitate was produced, indicating that the chitosan had been depolymerized into small oligomers. The hydrolysis rate by the enzyme mixture is higher than that of such unspecific enzymes as cellulase, lipase, and bromelain, but lower than that of the chitosanase from *Streptomyces griseus* (donated by Dr Masaru Mitsutomi; the specific activity was 2.6 units). In the mass spectrum of the hydrolyzates (Fig. 1) peaks corresponding to the mass numbers of $(M+Na)^+$ of trimer to heptadecasaccharide were detected. The products were composed mainly of chitooligosaccharides, especially of degree of polymerization (DP) 5 to 17.

In order to obtain the chitooligosaccharides of DP greater than three but less than 10 (which are more physiologically active) the reaction was coupled with product removal. By using membrane separation from the reaction mixture, the products of higher DP were obtained in good yield. The mass spectrum

---

* Corresponding author. Tel.: +86-411-4671991-858.
*E-mail address:* ygdu@rose.dicp.ac.cn (Y. Du)

PII: S0008-6215(99)00154-8

shown in Fig. 2 indicates peaks mainly for heterooligosaccharides from trimer to decamer.

Chitinase, chitosanase, and lysozyme can hydrolyze partially *N*-acetylated chitosans, but N-acetylation of the enzymatic hydrolyza-

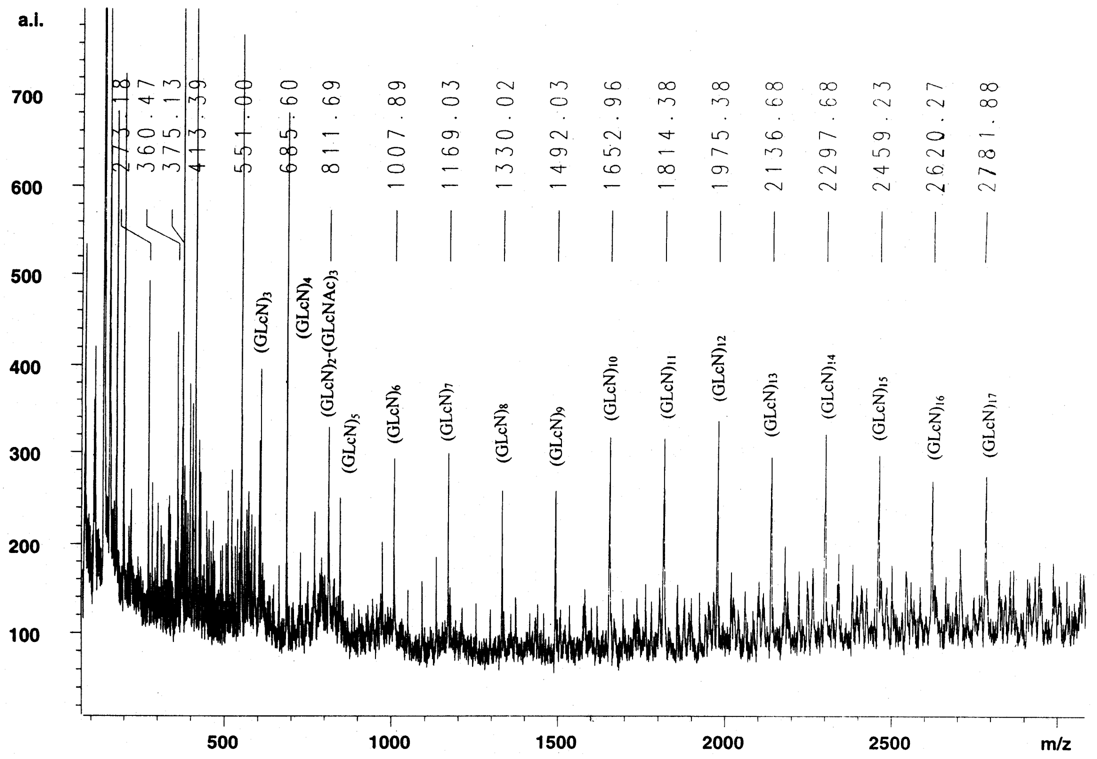

Fig. 1. TOF-MS of products from hydrolysis of chitosan by an enzyme mixture.

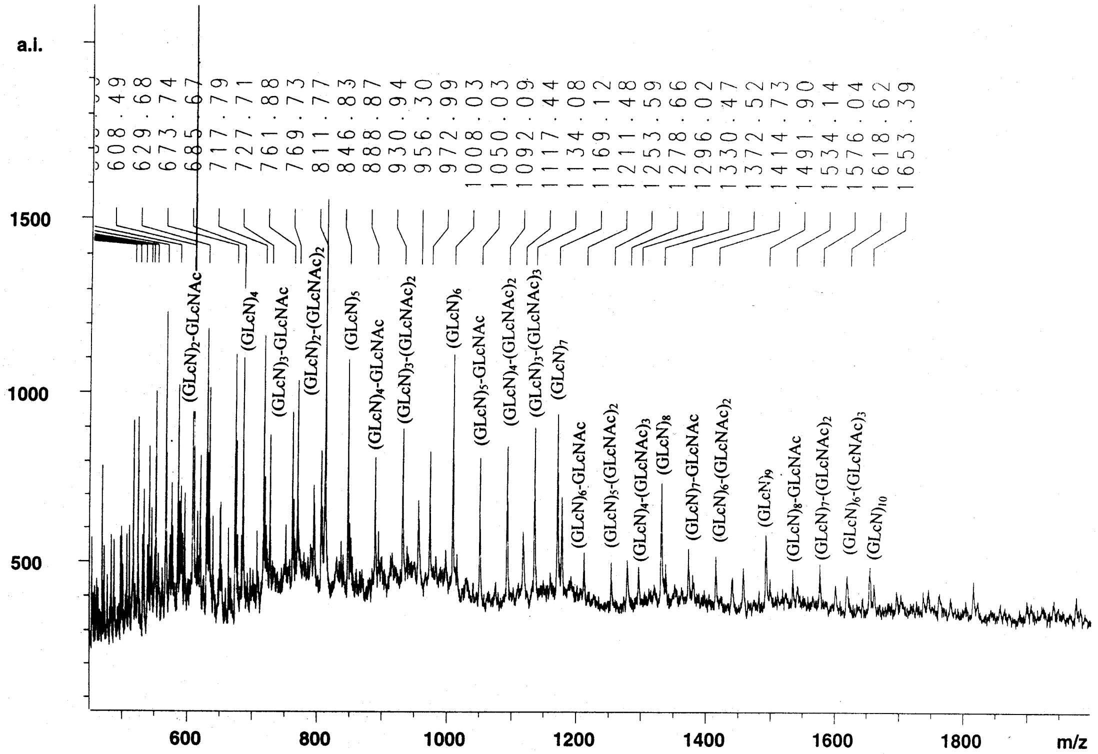

Fig. 2. TOF-MS of products separated from the reaction mixture by use of a membrane.

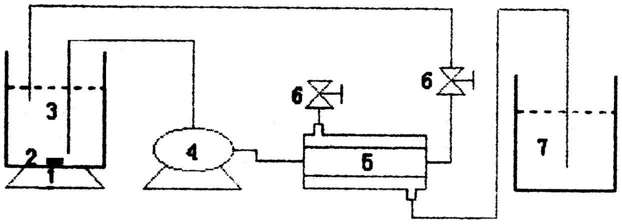

Fig. 3. Scheme for the reaction with in situ removal of products. 1, Magnetic stirrer; 2, magnetic stir bar; 3, reaction vessel; 4, pump; 5, hollow fiber membrane; 6, flow control valve; 7, products vessel.

tes produces mainly the dimer, trimer and tetramer of GlcNAc [11–13]. Chitosan can also be degraded by such unspecific enzymes as cellulase, lipase, and bromelain [14–17]. The rate of decrease in viscosity of chitosan is fast, but the average molecular weight of the products is about 10,000. These results show that the enzyme mixture can hydrolyze chitosan very rapidly, and it is cheaper than chitosanase.

By coupling the reaction with in situ removal of products, products of higher DP were separated out and not further cleaved by the enzyme action, and so the DP of the chitooligosaccharides is higher. Adding chitosan solution to the reaction vessel while the enzyme mixture was still active allowed continuous production to be realized. The hydrolysis of chitosan by the complex enzyme, coupled with membrane separation, is thus a useful procedure for preparing chitooligosaccharides (and *N*-acetylchitooligosaccharides by N-acetylation of the hydrolyzate) with DP values from 5 to 10.

The products of DP from 11 to 17 have not been hitherto reported. When the commercial chitosan was degraded for 40 min by the enzyme mixture, products with DP values up to 17 were obtained. The physiological importance of these is under study.

## 1. Experimental

*Materials.*—The chitosan sample was obtained from Qingdao Kading Co. in China. It was purified by AcOH. The molecular weight was $1.5 \times 10^6$ and the degree of N-acetylation was 24% (as analyzed by $^{13}$C NMR). The enzyme mixture included cellulase (0.8 U/mg), alpha amylase (500 U/mg), and proteinase (4 U/mg), all provided by The Oriental Reagent Co. in China.

*Hydrolysis of chitosan by the enzyme mixture.*—Chitosan (0.5 g) was dissolved in 2% AcOH (10 mL), and then the pH of the solution was adjusted with NaOH to $\sim$5.6. Enzyme mixture (5 mg) in 0.05 mol/L acetate buffer was added and the mixture was incubated for 40 min at 40 °C. The reaction was stopped by boiling for 10 min. The hydrolyzates were filtered on a hollow-fiber membrane (provided by Membrane Center, Dalian Institute of Chemical Physics, cutoff 50,000). The filtrate was concd to about one-twentieth with a rotary evaporator under diminished pressure. Alcohol was then added to form a precipitate, which was dried under diminished pressure.

*Reaction with in situ removal of external products.*—A scheme for the reaction with in situ external product removal is illustrated in Fig. 3. The surface area of the membrane was 134.8 cm$^2$ and the cutoff weight was 50,000. The osmotic flow rate was 10 mL/min, the recycle flow rate 7.5 mL/min and the volume in the reaction vessel was kept at 100 mL. After 40 min of reaction, the filtrate was collected and treated as before.

*Product analysis.*—Mass spectra were obtained with a matrix-assisted laser desorption–ionization time-of-flight mass spectrometer (Bruker, Germany).

## Acknowledgements

We thank Dr Xianglin Yuan and Professor Hanfa Zhou, Department of Chromatographic Analysis, for MS analysis. This study was financially supported by the National Science and Technology Ministry of China.

## References

[1] S. Suzuki, K. Suzuki, A. Tokoro, Y. Okawa, M. Suzuki, in R.A.A. Muzzarelli, C. Seuniaux, G.W. Gooday (Eds.), *Chitin in Nature and Technology*, Plenum, New York, 1985, pp. 485–492.
[2] K. Suzuki, T. Mikami, Y. Okawa, A. Tokoro, S. Suzuki, M. Suzuki, *Carbohydr. Res.*, 151 (1986) 403–408.
[3] A. Tokoro, N. Tatewaki, K. Suzuki, T. Mikami, S.

Suzuki, M. Suzuki, *Chem. Pharm. Bull.*, 36 (1988) 784–790.

[4] D. Roby, A. Gadelle, A. Toppan, *Biochim. Biophys. Res. Commun.*, 143 (1987) 885–892.

[5] S. Hirano, N. Nagao, *Agric. Biol. Chem.*, 53 (1989) 3065–3066.

[6] J.A. Rupley, *Biochim. Biophys. Acta*, 83 (1964) 245–255.

[7] S.A. Barker, A.B. Foster, M. Stacey, J.M. Webber, *J. Chem. Soc.*, *Chem. Commun.*, (1958) 2218–2227.

[8] C. Bosso, J. Defaye, H. Domard, A. Gadelle, C. Pedersen, *Carbohydr. Res.*, 156 (1986) 57–68.

[9] Y. Takiguchi, K. Shimahara, *Agric. Biol. Chem.*, 53 (1989) 1537–1541.

[10] Y. Takiguchi, K. Shimahara, *Nippon Nogei Kagaku Kaishi*, 65 (1991) 1655–1658.

[11] S. Aiba, *Carbohydr. Res.*, 265 (1994) 323–328.

[12] S. Aiba, *Carbohydr. Res.*, 261 (1994) 297–306.

[13] M. Izume, S. Nagae, H. Kawagishi, A. Ohtakara, *Biosci. Biotech. Biochem.*, 56 (1992) 1327–1328.

[14] E. Muraki, F. Yaku, H. Kojima, *Carbohydr. Res.*, 239 (1991) 227–237.

[15] R.A.A. Muzzarelli, M. Tomasetti, P. Ilari, *Enzyme Microb. Technol.*, 16 (1994) 110–114.

[16] R.A.A. Muzzarelli, W. Xia, M. Tomasetti, P. Ilari, *Enzyme Microb. Technol.*, 17 (1995) 541–545.

[17] M. Yalpani, D. Pantaleone, *Carbohydr. Res.*, 256 (1994) 159–175.