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Production of chitooligosaccharides using an ultrafiltration membrane reactor and their antibacterial activity

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Abstract

To increase the solubility of chitosan in an aqueous solution and to facilitate its utilization, the enzymatic production of chitooligosaccharides with a high degree of polymerization (DP) was carried out using an ultrafiltration (UF) membrane reactor system. 80% of the oligosaccharides produced were in the range DP3–6. Compared with a batch reactor, in the UF membrane reactor system, at least 11 batches of substrate could be hydrolysed for the same quantity of chitosanuse. Oligosaccharides obtained using the reactor system showed antibacterial activity and a 0.5% concentration completely inhibited the growth of *Escherichia coli*.

Keywords: Chitooligosaccharides; Enzymatic hydrolysis; Ultrafiltration membrane reactor; Antibacterial activity

1. Introduction

Chitooligosaccharides and their *N*-acetylated analogues are useful for applications in various fields because they have specific biological activities such as antifungal activity (Hirano & Nagao, 1989; Kendra & Hadwiger, 1984; Kendra, Christian & Hadwiger, 1989; Uchida, Izume & Ohtakara, 1989;), antibacterial activity (Hirano & Nagao, 1989; Uchida et al., 1989; Ueno, Yamaguchi, Sakairi, Nishi & Tokura, 1997), antitumor activity (Suzuki, 1996; Suzuki, Matsumoto, Tsukada, Aizawa & Suzuki, 1989; Suzuki, Mikami, Okawa, Tokoro, Suzuki & Suzuki, 1986; Tokoro, Tatewaki, Suzuki, Mikami, Suzuki & Suzuki, 1988; Tsukada et al., 1990), immuno-enhancing effects (Suzuki, 1996; Suzuki et al., 1986; Suzuki, Watanabe, Mikami, Matsumo & Suzuki, 1992; Tokoro et al., 1988), and protective effects against infection with some pathogens in mice (Suzuki, Tokoro, Okawa, Suzuki & Suzuki, 1985; Suzuki, Okawa, Suzuki & Suzuki, 1987; Tokoro, Kobayashi, Tatekawa, Suzuki & Suzuki, 1989; Yamada, Shibuya, Kodama & Akatsuka, 1993).

Chitooligosaccharides can be obtained by either chemical (Defaye, Gadelle & Pedersen, 1994; Horowitz, Roseman & Blumenthal, 1957; Sakai, Nanjo & Usui, 1990; Tsukada and Inoue, 1981) or enzymatic hydrolysis (Izume & Ohtakara, 1987; Sakai et al., 1990; Uchida et al., 1989) of chitosan. In the case of chemical hydrolysis, chitooligosaccharides were prepared by partial hydrolysis of chitosan with concentrated HCl (Horowitz et al., 1957). However, acidic hydrolysis produced lower yields of oligosaccharides and a large amount of monomeric D-glucosamine. In addition, the oligosaccharides prepared by the acidic hydrolysis might be toxic because of chemical changes during conversion. In the case of enzymatic hydrolysis, Uchida et al. (1989) have reported that chitosanase from *Bacillus* sp. produced mainly the oligosaccharides (DP 2–6) and a small amount of D-glucosamine after prolonged incubation. Therefore, enzymatic hydrolysis of chitosan is attracting growing interest. Chitosanases which have a pH optimum in the range 4.0–6.8, however, are unavailable in bulk quantities for commercial application, partly because of their narrow substrate specificity (Pantaleone, Yalpani & Scollar, 1992a).

Recently, the susceptibility of chitosan to a number of different enzymes has been investigated. Aiba (1993; 1994a; 1994b) has carried out the hydrolysis of partially *N*-acetylated chitosans with chitinase and lysozyme, since these enzymes recognise *N*-acetyl glucosamine residues in chitosan. Pantaleone, Yalpani and Scollar (1992a; 1992b) reported the hydrolytic susceptibility of chitosan to a wide range of enzymes, including 10 kinds of glycanases, 21 kinds of protease, five lipases and a tannase, which were derived from various bacterial, fungal, mammalian and plant sources. Muzzarelli, Xia, Tomasetti and Ilari (1995;

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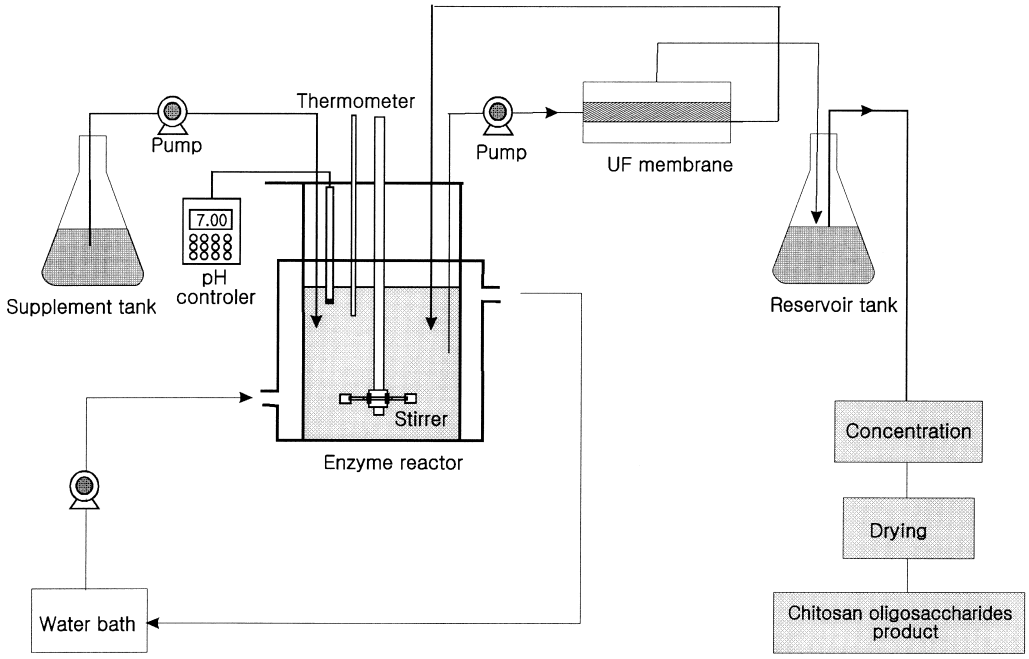

Fig. 1. Schematic diagram for the production of chitooligosaccharides in the UF membrane reactor system.

1994) examined the action of papain and lipase in depolymerizing chitosan. From these results, a lot of commercial enzymes have been developed for efficient hydrolysis of chitosan. These enzymes, however, were added at relatively high concentrations, while chitosanase shows substantial activities at low concentrations.

In this study, we employed a UF membrane reactor system, which can repeatedly reuse the enzyme added and separate the enzyme from the final products, for enzymatic hydrolysis of chitosan with chitosanase. In addition, we investigated the production of relatively higher DP oligosaccharides and examined antibacterial activity of the oligosaccharides produced using this reactor system.

2. Materials and methods

2.1. Materials

Chitosan (degree of deacetylation, 89%) was obtained from Kitto Life Co. (Korea). The chitosanase (BN-262, 694 U/g protein) derived from *Bacillus pumilus* BN-262 and purified chitooligosaccharides from dimer to heptamer were purchased from Wako Chemical Industries, Ltd. (Japan), and D-glucosamine hydrochloride the monomer unit of chitosan structure, were from Sigma Chemical Co. (USA).

2.2. Preparation of chitosan solution

A 1% chitosan solution was prepared by dispersing 150 g of chitosan in 1 l of water, dissolving it and stirring by adding 400 ml of 1 M lactic acid and making up to 15 l with water. The pH was adjusted to be 5.5 with a saturated $NaHCO_3$ solution.

2.3. Analysis of the oligosaccharides by HPLC

The compositions of the oligosaccharides were analyzed by HPLC on TSK gel NH_2-60 column (4.6×250 mm, TOSOH Co. in Japan) at room temperature. One millilitre of the reaction mixture after incubation with chitosanase was mixed with 1 ml of acetonitrile. Then, 200 μl of the sample was chromatographed using 60% acetonitrile as the elution buffer and a flow rate 0.8 ml/min. The oligosaccharides were detected by monitoring the refractive index at eight times attenuation.

2.4. Enzymatic hydrolysis of chitosan in batch reactor

The optimum temperature for production of oligosaccharides was determined by treating 1% chitosan solution with chitosanase at temperatures ranging from 30 to 70°C in a batch reactor. The reaction mixture containing 100 ml of the chitosan solution and various units of chitosanase (5, 10, 15 and 25 U/g chitosan) was incubated for different times (10, 20, 40, 60, 90, 120, 180, and 240 min) at the optimum temperature. The reaction was stopped by boiling for 10 min. The degree of hydrolysis was expressed as the amount of reducing sugar (D-glucosamine) released from chitosan using Blix's method (Blix, 1948). The amount of reducing sugar was expressed as mg/g chitosan.

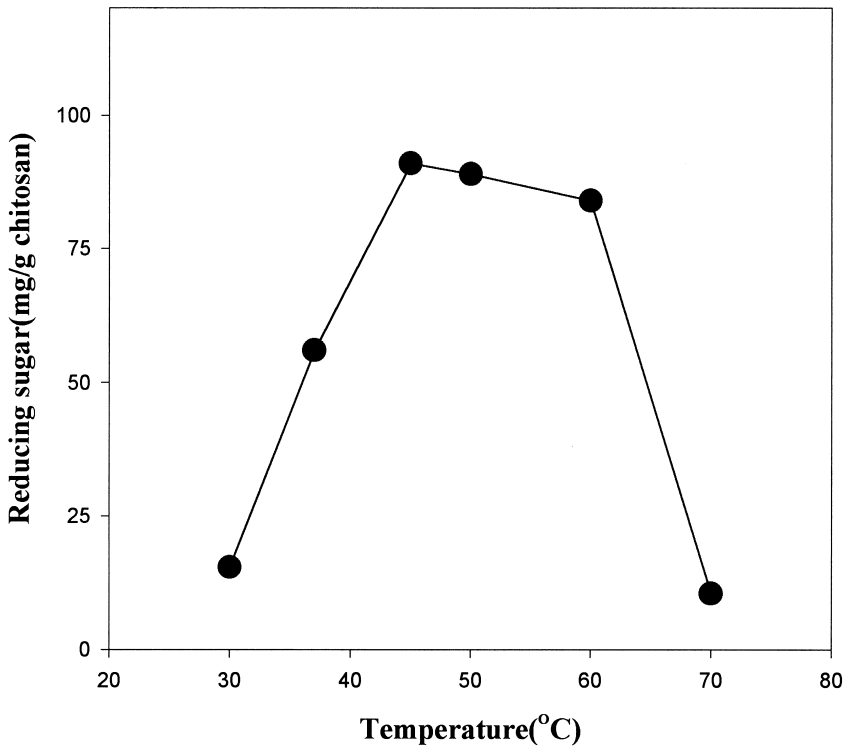

Fig. 2. Temperature dependence of the enzymatic hydrolysis of chitosan by chitosanese. The mixture (100 ml) of 1% chitosan solution (pH 5.5) and 5 U of enzyme was incubated for 60 min at various temperatures. The enzyme activity (mg/g chitosan) was expressed as the amount of the reducing sugar (D-glucosamine) released from chitosan.

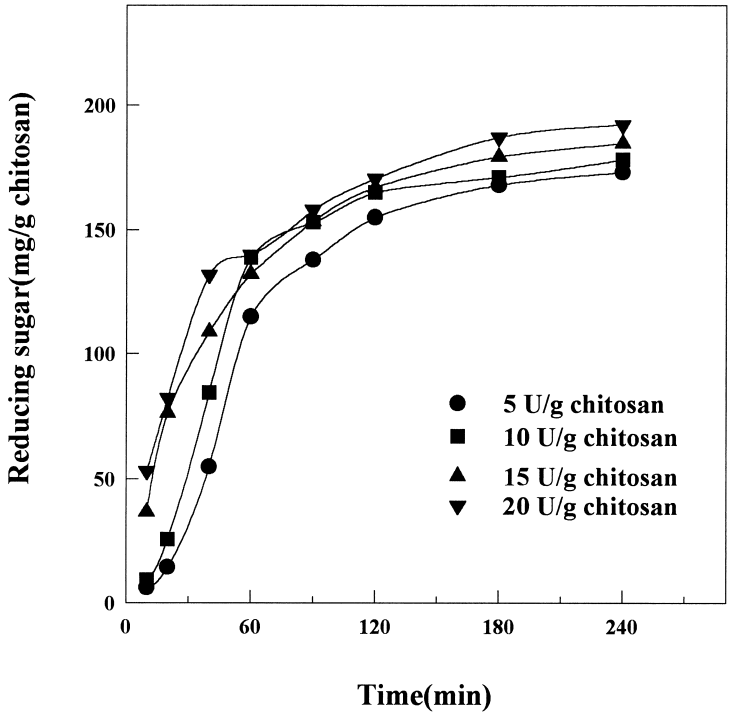

Fig. 3. Dependence of hydrolysis of chitosan on incubation time and enzyme added for the batch reaction. The experiment was carried out under the same conditions as Fig. 2 at 45°C.

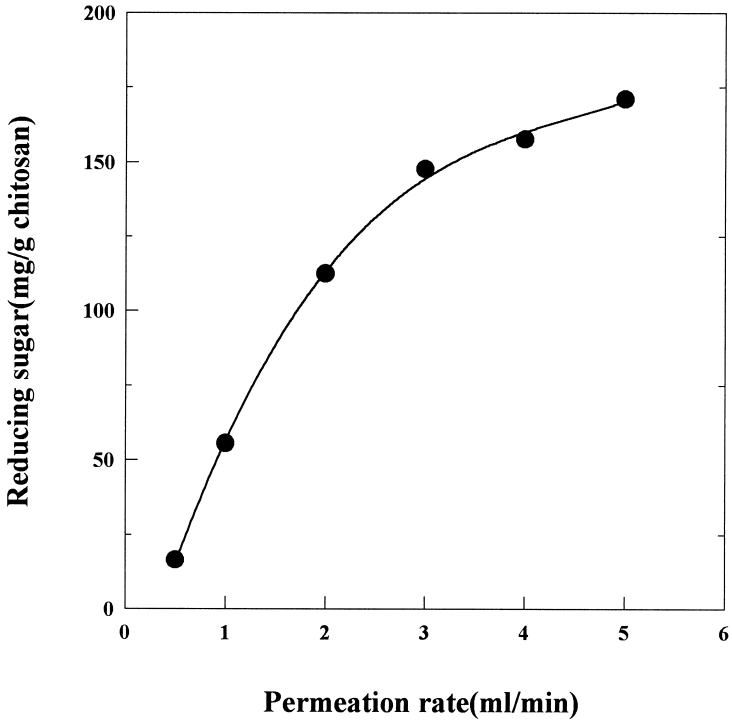

Fig. 4. Changes in the reducing sugar produced from chitosan at various permeation rates in the UF membrane reactor system. A 500 ml of 1% chitosan (5 g solid chitosan) containg 25 U of enzyme were incubated at 45° C for 180 min, and then passed through the UF membrane (MWCO 3000 Da) at various permeation rates.

2.5. Enzymatic hydrolysis of chitosan in the UF membrane reactor

The UF membrane reactor (Millipore Minitan™ system, Millipore Co., USA) is shown in Fig. 1. It consisted of a supplement tank, a reservoir tank, a water bath for controlling reaction temperature, three peristaltic pumps, a membrane cartridge with molecular weight cut off (MWCO) 3000 Da and an enzyme reactor vessel. The amount of reducing sugar produced from chitosan by the UF membrane reactor at a given permeation rate was compared with the optimum conditions for the enzyme concentration, incubation time, and reaction temperature determined in the batch reactor. The oligosaccharides produced from chitosan at different permeation rates were analyzed by HPLC on the TSK gel NH_2-60 column. The reactor system for semi-continuous production of oligosaccharides was also operated under the optimum conditions. A new substrate was added to the reactor tank after enough incubation and recycling time had been allowed to hydrolyze the chitosan solution.

2.6. Assay for antibacterial activity

Antibacterial activities of chitosan and chitooligosaccharides were examined as the inhibitory effects against the growth of *E. coli*. A 0.5 ml of 1% sample solution in 0.05 M acetate buffer (pH 6.0) was added to the mixture of 0.5 ml of the cultured bacteria solution and 49 ml of tryptic soy broth medium, and incubated with shaking at 37°C. The inhibitory effects were estimated periodically by measuring the turbidity of the cultured medium at 640 nm. In a control group, 0.5 ml of 0.05 M acetate buffer in place of the oligosaccharides was added to the mixture.

3. Results

3.1. Production of chitooligosaccharides

The optimum temperature for hydrolyzing 1% chitosan solution with chitosanase was determined using the batch reactor at a pH of 5.5 for 60 min, and was found to be 45°C (Fig. 2). The enzyme was relatively stable in the temperature range from 45 to 60°C but was rapidly inactivated at higher temperatures.

Amounts of reducing sugar produced at various enzyme concentrations at the optimum temperature (45°C) was estimated at different incubation times. As shown in Fig. 3, the release of reducing sugar increased with the amount of the added enzyme and incubation time. At times greater than 60 min the rate of release slowed and the dependence on enzyme concentration reduced. The results suggest that only 5 U chitosanase for 1 g chitosan was necessary with the incubation times of more than 180 min at 45°C.

As schematically shown by the UF membrane reactor system in Fig. 1, the enzyme added in the reaction vessel for hydrolysis of chitosan is recycled and oligosaccharides

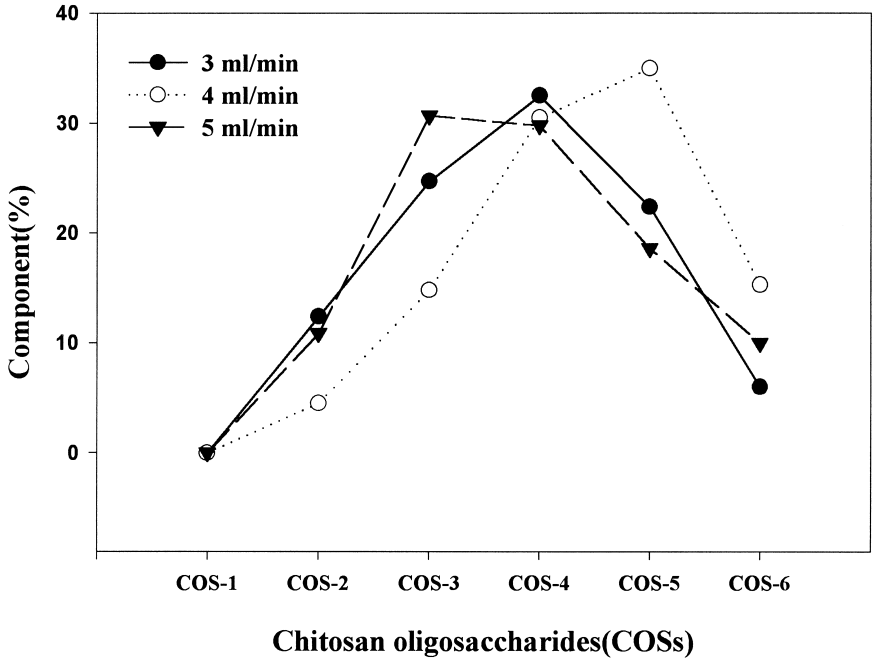

Fig. 5. Components of the oligosaccharides produced at different permeation rates in the UF membrane reactor system. The components of the oligosaccharides were separated and analyzed on HPLC at room temperature and detected by monitoring the refractive index at eight times attenuation.

smaller than the MWCO of the membrane used are separated from the substrate and the enzyme. The dependence of the amount of reducing sugar produced on the permeation rate in the UF membrane reactor is shown in Fig. 4. The production of reducing sugar progressively increased on increasing the permeation rate and reached a plateau at 3 ml/min. Thus a permeation rate of 3 ml/min or above was required for the production of the oligosaccharides. The components of the oligosaccharides produced after enzymatic hydrolysis of chitosan in the UF membrane reactor were analyzed by HPLC and are shown in Fig. 5. This system produced relatively large quantities of pentamers

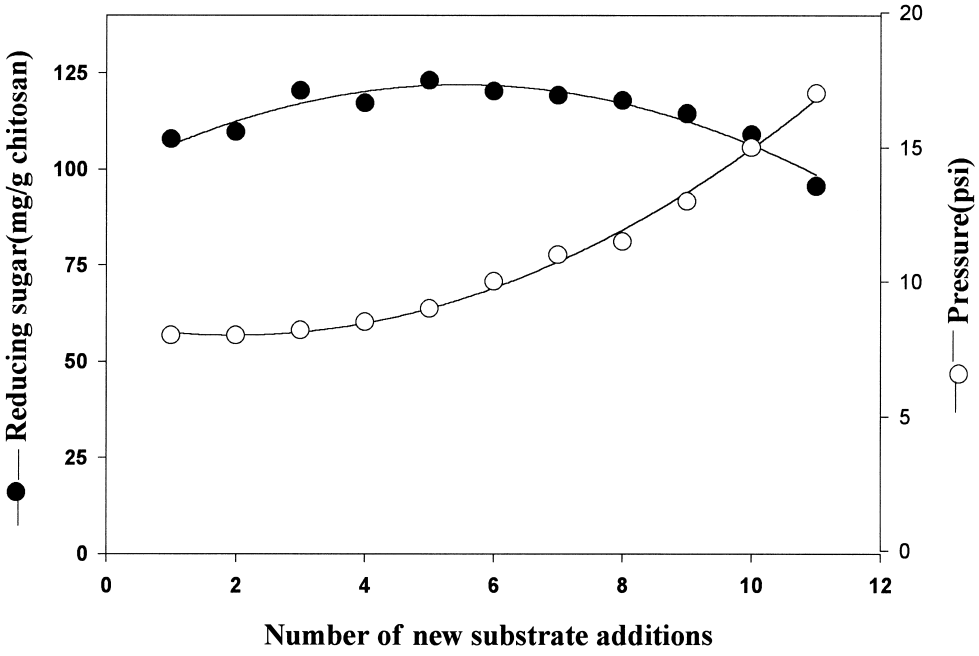

Fig. 6. Changes in the reducing sugar and transmembrane pressure (related to membrane fouling) in the UF membrane reactor system following serial addition of new chitosan substrate. The UF membrane reactor system after 180 min incubation was operated with 4 ml/min of permeation rate and the hydrolyzed chitosan solution was passed through the UF membrane.

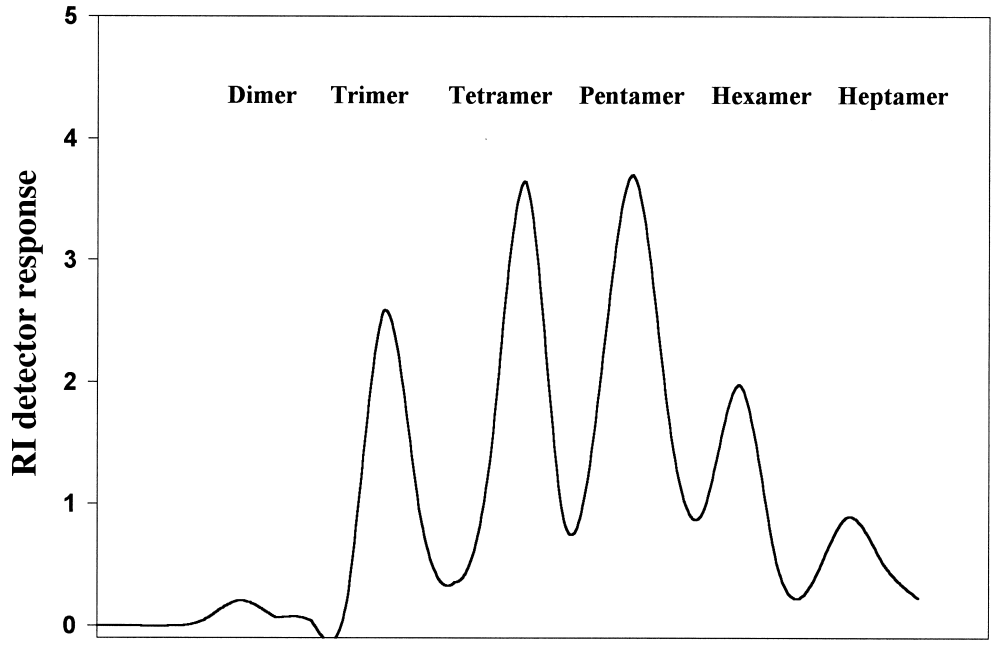

Fig. 7. HPLC patterns of the oligosaccharides obtained using the UF membrane reactor system. The analytical procedures were performed under the same conditions as Fig. 5.

and hexamers but no monomers. The composition of the oligosaccharides was dependent on the permeation rate in the reactor system (Fig. 5). The highest production of pentamer and hexamer was achieved at a permeation rate of 4 ml/min. At this permeation more than 80% of the oligosaccharides ranged from trimer to hexamer. Consequently, the UF membrane reactor system proved to be effective for the production of relatively larger oligosaccharides which might have various physiological functions.

The optimum conditions for the production of chitooligosaccharides in the UF membrane reactor system was a recycling time of 180 min, a permeation rate of 4 ml/min and a

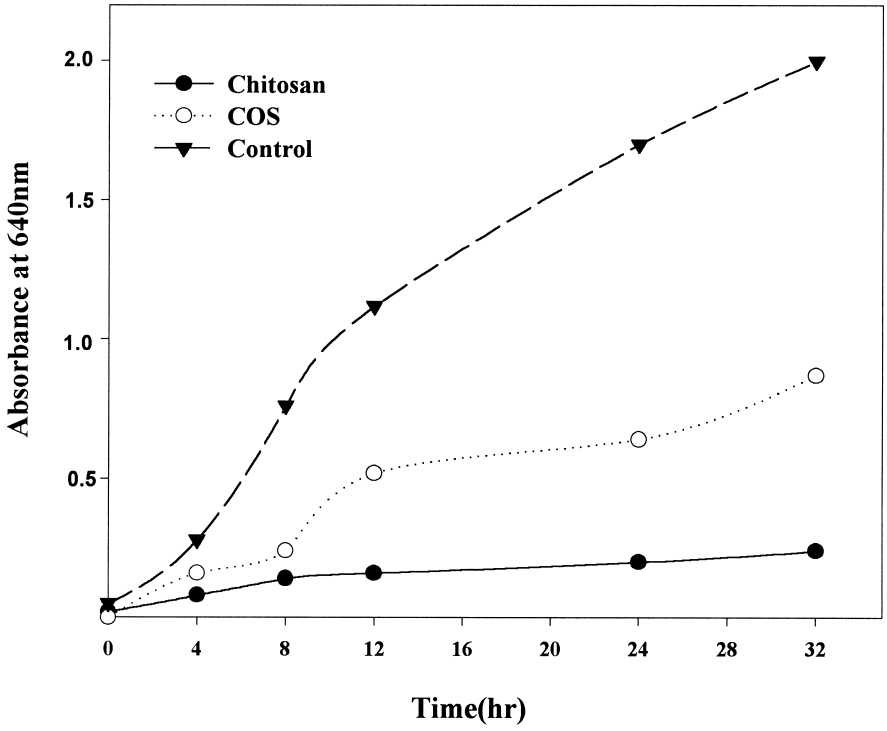

Fig. 8. The inhibitory effects of chitosan and chitooligosaccharides (COS) on the growth of *E. coli*.

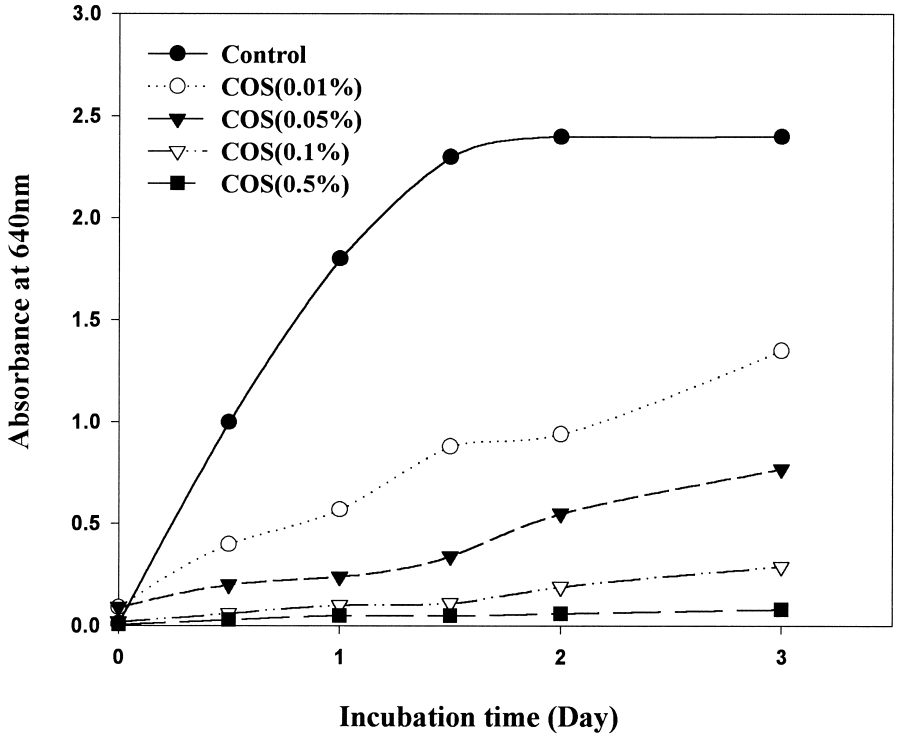

Fig. 9. The inhibitory effects of chitooligosaccharides at various concentrations on the growth of *E. coli*.

reation mixture in 5 U chitosanase per gram of chitosan. Under these conditions, the change in reducing sugar production and membrane pressure was measured with increasing additions of new substrate (Fig. 6). A decrease of enzyme activity and an increase of transmembrane pressure were detected after five cycles of new substrate addition. These might result from membrane fouling and accumulation of the substrate in the reaction vessel. However, 25 U of chitosanase was sufficient for the conversion of 3.5 l of 1% chitosan solution (35 g of solid chitosan) into the oligosaccharides.

The oligosaccharides produced from the UF membrane reactor system were separated and analyzed by HPLC (Fig. 7). The components of oligosaccharide were mainly trimers and pentamers, and their yields calculated from each peak area were about 72.5%. At a relatively higher DP oligosaccharides, hexamer and heptamer, were 14.2 and 11.3%, respectively. Dimers, however, were detected at only trace levels.

3.2. Antibacterial activity

The antibacterial activities of chitosan and the oligosaccharides produced in the UF membrane enzyme reactor system were examined against the growth of *E. coli.* As shown in Fig. 8, in the case of chitosan the growth of *E. coli* was completely inhibited, since the absorbance was below 0.2 after 32 h incubation. The growth of the bacterium was also considerably inhibited by the addition of the oligosaccharides, compared with the control.

The inhibitory effects of the oligosaccharides were also investigated at different concentrations ranging from 0.01 to 0.5% (Fig. 9). The inhibitory effect increased with increased concentration. Adding 0.5% of the oligosaccharides completely inhibited the growth of *E. coli*. The antibacterial activity was similar to 0.1% chitosan. Further, the antibacterial activity of the oligosaccharides was detected even for a 0.01% concentration. Therefore, the inhibition rates were greatly dependent on the amount of the oligosaccharides added.

4. Discussion

In our previous study (Jeon, Park, Byun Song & Kim, 1998), we achieved the production of higher chitooligosaccharides ranging from tetramers to hexamers using an immobilized chitosanase physically adsorbed on chitin as a carrier. However, the yield was low because the immobilized enzyme showed a lower affinity and lower reaction rate than the free enzyme.

The production of chitooligosaccharides by enzymatic hydrolysis has so far been studied using batch reactors (Izume & Ohtakara, 1987). This batch method has some disadvantages such as low yields and use of large quantities of expensive chitosanase. This can be overcome by using a UF membrane enzymatic reactor system. The most important factor in the UF reactor system was control of the permeation rate because the components of the oligosaccharides obtained were dependent on the permeation rate.

At a permeation rate of 4 ml/min, 80% of the oligosaccharides ranged from trimer to hexamer (Fig. 5). This result shows that the residence time of the substrate was important for the production of oligosaccharides. This reactor system could hydrolyse at least 11 batches of substrate for the same amount of enzyme used in the batch reactor. However, it did not allow the oligosaccharides to be continuously produced because of the increase in transmembrane pressure (Fig. 6). This means membrane fouling occurs reducing the permeation flow rate and accumulating substrate. It seems to occur because of the high viscosity of chitosan. Therefore, reducing chitosan viscosity prior to treatment in the membrane system may allow membrane fouling to be alleviated.

It is well known that chitosan and its hydrolysates show antibacterial activity and their activity is greatly dependent on chain length (Hirano & Nagao, 1989; Kendra et al., 1989; Uchida et al., 1989). We also observed that the oligosaccharides obtained with enzymatic hydrolysis using the UF membrane reactor system inhibited the growth of *E. coli* and these inhibitory effects increased as concentration increased. This result was attributed to high DP of the oligosaccharides, mainly tetramer or more.

5. Conclusion

In this work, we showed that the UF membrane reactor system can be used to produce chitooligosaccharides by enzymatic hydrolysis. In addition, oligosaccharides with a relatively high DP (trimer to hexamer), were successfully obtained at a yield of above 80 % by adjusting the permeation rate. These oligosaccharides inhibited the growth of *E. coli* and these effects were increased as the concentration increased. 0.5 % of the oligosaccharides completely inhibited the growth of *E. coli*.

Acknowledgements

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References

Aiba, S. (1993). Studies on chitosan: 6. Relationship between *N*-acetyl group distribution pattern and chitinase digestibility of partially *N*-acetylated chitosans. *International Journal of Biology and Macromolecules, 15*, 241–245.

Aiba, S. (1994a). Preparation of *N*-acetylchitooligosaccharides by hydrolysis of chitosan with chitinase followed by *N*-acetylation. *Carbohydrates Research, 265*, 323–328.

Aiba, S. (1994b). Preparation of *N*-acetylchitooligosaccharides from lysozymic hydrolysates of partially *N*-acetylated chitosans. *Carbohydrates Research, 261*, 297–306.

Blix, G. (1948). The determination of glucosamine and galactosamine. *Acta Chemica Scandinavica, 2*, 467–469.

Defaye, J., Gadelle, A., & Pedersen, C. (1994). A convenient access to β-(1 → 4)-linked 2-amino-2-deoxy-D-glucopyranosyl fluoride oligosaccharides and β-(1 → 4)-linked 2-amino-2-deoxy-D-glucopyranosyl oligosaccharides by fluorolysis and fluorohydrolysis of chitosan. *Carbohydrates Research, 261*, 267–277.

Hirano, S., & Nagao, N. (1989). Effects of chitosan, pectic acid, lysozyme, and chitinase on the growth of several phytopathogens. *Agricultural and Biological Chemistry, 53*, 3065–3066.

Horowitz, S. T., Roseman, S., & Blumenthal, H. J. (1957). The preparation of glucosamine oligosaccharides. I separation. *Journal of the American Chemical Society, 709*, 5046–5049.

Izume, M., & Ohtakara, A. (1987). Preparation of D-glucosamine oligosaccharides by the enzymatic hydrolysis of chitosan. *Agricultural and Biological Chemistry, 51*, 1189–1191.

Jeon, Y. J., Park, P. J., Byun, H. G., Song, B. K., & Kim, S. K. (1998). Production of chitosan oligosaccharides using chitin-immobilized enzyme. *Korean Journal of Biotechnology and Bioengineering, 13*, 147–154.

Kendra, D. F., & Hadwiger, L. A. (1984). Characterization of the smallest chitosan oligomer that is maximally antifungal to *Fusarium solani* and elicits pisatin formation in *Pisum sativum. Experimental Mycology, 8*, 276–281.

Kendra, D. F., Christian, D., & Hadwiger, L. A. (1989). Chitosan oligomers from *Fusarium solani*/pea interactions, chitinase/β-glucanase digestion of sporelings and from fungal wall chitin actively inhibit fungal growth and enhance disease resistance. *Physiology and Molecular Plant Pathology, 35*, 215–230.

Muzzarelli, R. A. A., Tomasetti, M., & Ilari, P. (1994). Depolymerization of chitosan with the aid of papain. *Enzyme and Microbial Technology, 16*, 110–114.

Muzzarelli, R. A. A., Xia, W., Tomasetti, M., & Ilari, P. (1995). Depolymerization of chitosan and substituted chitosans with the aid of a wheat germ lipase preparation. *Enzyme and Microbial Technology, 17*, 541–545.

Pantaleone, D., Yalpani, M., & Scollar, M. (1992a). Unusual susceptibility of chitosan to enzymic hydrolysis. *Carbohydrates Research, 237*, 325–332.

Pantaleone, D., Yalpani, M., & Scollar, M. (1992b). Susceptibility of chitosan to enzymic hydrolysis. In C. J. Brine & P. A. Sandford & J. P. Zikakis (Eds.), *Advanced chitin and chitosan*, (pp. 292–303). Amsterdam: Elsevier.

Sakai, K., Nanjo, F., & Usui, T. (1990). Production and utilization of oligosacch-arides from chitin and chitosan. *Denpun Kagaku, 37*, 79–86.

Suzuki, K., Tokoro, A., Okawa, Y., Suzuki, S., & Suzuki, M. (1985). Enhancing effects of *N*-acetyl chitooligosaccharides on the active oxygen-generating and microbicidal activities of peritoneal exudate cells in mice. *Chemical and Pharmaceutical Bulletin, 33*, 886–888.

Suzuki, K., Mikami, T., Okawa, Y., Tokoro, A., Suzuki, S., & Suzuki, M. (1986). Antitumor effect of hexa-*N*-acetylchitohexaose and chitohexaose. *Carbohydrates Research, 151*, 403–408.

Suzuki, K., Okawa, Y., Suzuki, S., & Suzuki, M. (1987). Candidacidal effect of peritoneal exudate cells in mice administered with chitin or chitosan: the role of serine protease on the mechanism of oxygen-independent candidacidal effect. *Microbiology and Immunology, 31*, 375–379.

Suzuki, S. (1996). Studies on biological effects of water soluble lower homologous oligosaccharides of chitin and chitosan. *Fragrance Journal, 15*, 61–68.

Suzuki, S., Matsumoto, T., Tsukada, K., Aizawa, K., & Suzuki, M. (1989). Antimetastatic effect of *N*-acetyl chitohexaose on mouse bearing Lewis lung carcinoma. In G. Skjak-Braek & T. Anthonsen & P. Sandford (Eds.), *Chitin and chitosan*, (pp. 707–713). London: Elsevier.

Suzuki, S., Watanabe, T., Mikami, T., Matsumo, T., & Suzuki, M. (1992). Immuno-enhancing effects of *N*-acetyl chitohexaose. In C. J. Brine & P. A. Sandford & J. P. Zikakis (Eds.), *Advanced chitin and chitosan*, (pp. 96–105). Amsterdam: Elsevier.

Tokoro, A., Tatewaki, N., Suzuki, K., Mikami, T., Suzuki, S., & Suzuki, M. (1988). Growth-inhibitory effect of hexa-*N*-acetylchitohexaose and chitohexaose against Meth-A solid tumor. *Chemical and Pharmaceutical Bulletin, 36*, 784–790.

Tokoro, A., Kobayashi, M., Tatekawa, N., Suzuki, S., & Suzuki, M. (1989). Protective effect of *N*-acetyl chitohexaose on *Listeria monocytogens* infection in mice. *Microbiol. Immunol., 33*, 357–367.

Tsukada, S., & Inoue, Y. (1981). Conformational properties of chitooligosacchar-ides: titration, optical rotation, and carbon 13 NMR studies of chitooligosaccharides. *Carbohydrates Research, 88*, 19–38.

Tsukada, K., Matsumoto, T., Aizawa, K., Tokoro, A., Naruse, R., Suzuki, S., & Suzuki, M. (1990). Antimetastatic and growth-inhibitory effects of *N*-acetylchitohexaose in mice bearing Lewis lung carcinoma. *Japanese Journal of Cancer Research, 81*, 259–265.

Uchida, Y., Izume, M., & Ohtakara, A. (1989). Preparation of chitosan oligomers with purified chitosanase and its application. In G. Skjak-Braek & T. Anthonsen & P. Sandford (Eds.), *Chitin and chitosan*, (pp. 373–382). London: Elsevier.

Ueno, K., Yamaguchi, T., Sakairi, N., Nishi, N., & Tokura, S. (1997). Antimicrobial activity by fractionated chitosan oligomers. In A. Domard & G. A. F. Roberts & K. M. Varum (Eds.), (pp. 156–161). *Advances in chitin science*, 2. Lyon: Jacques Andre Publisher.

Yamada, A., Shibuya, N., Kodama, O., & Akatsuka, T. (1993). Induction of phytoalexin formation in suspension-cultured rice cells by *N*-acetyl chitooligosaccharides. *Bioscience, Biotechnology and Biochemistry, 57*, 405–409.