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Antitumor Activity of Chitosan Oligosaccharides Produced in Ultrafiltration Membrane Reactor System

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Abstract Chitosan oligosaccharides (COSs) were prepared and fractionated into three groups of COS [a high molecular weight COS (HMWCOS), medium molecular weight COS (MMWCOS), and low molecular weight COS (LMWCOS)] according to their molecular weight, using an ultrafiltration membrane enzymatic bioreactor designed earlier [8]. Antitumor activity of these COSs was then examined against Sarcoma 180 solid (S180) or Uterine cervix carcinoma No. 14 (U14) tumor cell-bearing mice. Among these COSs, MMWCOS with molecular weight range from 1.5 to 5.5 kDa effectively inhibited the growth of both tumor cells in the mice. In addition, the administration of MMWCOS resulted in increased thymus weight among lymphoid organs. The mice treated with MMWCOS showed improved survival rate and larger number of survivors after 40 days of feeding. The most effective of MMWCOS for antitumor activity in the S180- or U14-bearing mice was 20 mg/kg/day or more.

Key words: Chitosan, oligosaccharide, antitumor, ultrafiltration, membrane

Chitin, a polymer of *N*-acetylglucosamine (β-1,4 linked 2-acetamido-D-glucose), is a cellulose-like biopolymer richly present in the exoskeleton of crustaceans and in the cell walls of fungi, insects, and yeast. Chitosan is derived from chitin by deacetylation, to different degrees, in the presence of alkali. Therefore, chitosan is a copolymer consisting of β-(1→4)-2-acetamido-D-glucose and β-(1→4)-2-amino-D-glucose units with the latter usually exceeding 80% [2]. Recently, studies of chitin and chitosan have been concentrated on their bioactivities, such as antitumor [15, 21, 23], immuno-enhancing [11, 15, 19], enhanced protection against certain pathogens infection in mice [5, 20], antifungal [2, 6, 10], and antimicrobial activition [7, 9, 16, 17, 22].

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Unlike usual functional polysaccharides, chitin and chitosan of smaller molecular weight exhibit the activities in an *in vivo* system [5, 6, 9, 11, 15-18, 20], which are produced by hydrolysis with enzymes such as chitinases or chitosanases [12, 13, 14]. The antitumor effects of *N*-acetylchitohexaose (hexamer of chitin oligosaccharide) and chitohexaose (hexamer of chitosan oligosaccharide) were observed by Suzuki *et al.* [18], Tokoro *et al.* [21], and Tsukada *et al.* [23], while the protective effect of *N*-acetylchitohexaose against *Listeria monocytogenes* infection in mice was examined by Tokoro *et al.* [20]. Furthermore, studies on the antitumor activity of chitin, chitosan, and their derivatives revealed that partially deacetylated chitin (especially 70% deacetylated chitin) and carboxymethyl chitin (CM-chitin) with an adequate degree of substitute (especially 0.80) were effective towards various tumor cells [15]. Some reports [18, 21, 23] further revealed that oligomers with small molecular sizes (at least hexamer or higher) possess antitumor activity, because of their potential immunological activities such as activation of peritoneal macrophages and stimulation of nonspecific host resistance.

In our previous study, three kinds of chitosan oligosaccharides (COSs) with different molecular weight ranges were prepared from chitosan (degree of deacetylation, 89%; viscosity 20 cps) donated from Kitto Life Co. (Seoul, Korea) by chitosanolytic hydrolysis and fractionated by ultrafiltration membrane (Millipore Co., Bedford, U.S.A.) enzymatic bioreactor system [8] [a high molecular weight COS (HMWCOS) ranging from 6.5 to 12.0 kDa; a medium molecular weight COS (MMWCOS) ranging from 1.5 to 5.5 kDa; a low molecular weight COS (LMWCOS) ranging from 0.5 to 1.4 kDa], and their antibacterial activity was examined [6, 7, 9]. In the present study, the antitumor activities of these COSs against Sarcoma 180 solid (S180) tumor cells and Uterine cervix carcinoma No. 14 (U14) tumor cells in BALB/c (4 weeks old, average body weight: 20±2 g) mice were examined, and their relationship between the molecular weights and antitumor activity was compared.

Table 1. Effect of COS on tumor growth in BALB/c mice implanted with Sarcoma 180 (S180) tumor cells by peritoneal administration.

Sample	Dose (mg/kg/day)	Number of mice	Body weight (g)		Tumor weight (mg)	Inhibition rate (%)
			Initial	Final		
Control		11	20.1±1.30	21.5±3.08	1032.5±839.5	
HMWCOS	50	11	20.2±0.92	17.1±1.97**	1147.0±933.9	-
	20	11	20.3±1.10	21.1±2.66	901.0±741.7	12.7
	10	11	20.3±1.27	22.8±3.43	395.5±284.8*	61.7
MMWCOS	50	11	20.5±1.18	23.6±2.37	345.2±218.6*	66.6
	20	11	20.5±1.04	23.7±2.69	665.6±340.1	35.5
	10	11	20.0±1.34	21.1±3.67	739.5±351.8	28.4
LMWCOS	50	11	20.1±1.45	21.7±3.98	904.0±510.3	12.4
	20	11	20.1±1.14	21.0±3.16	874.8±516.6	15.3
	10	11	20.0±1.26	23.3±2.20	973.5±417.1	5.7

*p<0.05; **p<0.01.

The antitumor activity of COSs were assayed as follows. The S180 cells (7.2×10^5 cells/mice) and U14 cells (1.0×10^6 cells/mice) were subcutaneously transplanted into the right groins of the mice. Twenty-four hours after the transplantation, the COS dissolved in physiological saline was intraperitoneally injected once a day for 24 days, while 0.9% saline was injected into the control group. After the final day, all mice were killed to evaluate the inhibitory effect of the COSs on the tumor growth. The tumors were excised and weighed, along with the spleen and thymus. The tumor growth inhibition rate was calculated using the following equation: Inhibition rate (%)=[(C-T)/C]×100, where C is the average tumor weight of the control group and T is that of the tested sample group. To determine the survival prolongation rate after the treatment, the mice were observed everyday for 40 days and the prolonged life (days) was counted from the day of tumor cell inoculation.

The results in Tables 1 and 2 show that the COSs exhibited an antitumor effect in both the S180- and U14-bearing mice, when treated with a single peritoneal injection per day for 24 days after tumor implantation. A relationship existed between the antitumor activity and the molecular weights. Among the three kinds of COSs, MMWCOS (medium molecular weights ranging from 1.5 to 5.5 kDa) most effectively inhibited the growth of both types of tumor. The other COSs, HMWCOS and LMWCOS, which had higher and lower molecular weight ranges, respectively, exhibited lower inhibitory rates against both types of tumor cell-bearing mice. In particular, no increase of inhibitory rate was observed with a high dose (50 mg/kg/day) of HMWCOS against both types of tumor cell-bearing mice. The mice in these groups also showed a slight decrease in body weight, even though no decrease was observed in any of the other groups. The optimal dose of MMWCOS was approximately 20 mg/kg/day or higher. As such, 50 and 20 mg/kg/day administrations of MMWCOS produced 66.6% and 35.5% inhibition rates of tumor growth in the S180-bearing mice, and 73.6% and 61.4% inhibitory effects in the U14-bearing mice, respectively.

Table 2. Effect of COS on tumor growth in BALB/c mice implanted with Uterine cervix carcinoma (U14) tumor cells by peritoneal administration.

Sample	Dose (mg/kg/day)	Number of mice	Body weight (g)		Tumor weight (mg)	Inhibition rate (%)
			Initial	Final		
Control		11	20.2±1.41	27.4±5.98	912.2±612.1	
HMWCOS	50	11	20.2±0.45	20.2±1.30*	965.2±839.7	-
	20	11	19.6±1.69	26.3±8.45	803.3±641.8	11.9
	10	11	19.7±1.37	31.0±3.41	772.7±592.2	15.3
MMWCOS	50	11	19.8±1.40	30.5±2.66	240.5±202.5**	73.6
	20	11	19.8±1.22	31.2±4.80	352.3±331.1**	61.4
	10	11	20.1±1.30	30.3±4.38	669.5±562.3	26.6
LMWCOS	50	11	19.9±1.00	31.6±3.40	665.0±477.9	27.1
	20	11	19.9±1.04	25.9±3.73	841.1±602.1	7.8
	10	11	19.9±0.94	30.8±3.57	879.9±650.3	3.5

*p<0.05; **p<0.01.

Table 3. Effect of COS on immune organs in BALB/c mice implanted with S180 or U14 tumor cells by peritoneal administration.

Sample	Dose (mg/kg/day)	Number of mice	S180		U14	
			Spleen (mg/10 g)	Thymus (mg/10 g)	Spleen (mg/10 g)	Thymus (mg/10 g)
Control		11	74.5±16.2	29.2±9.5	84.2±34.6	25.4±11.5
HMWCOS	50	11	66.4±28.3	6.5±4.5	108.5±68.3	14.5±4.8
	20	11	81.9±35.5	31.4±15.9	91.4±51.3	21.6±11.9
	10	11	84.3±21.8	31.6±8.5	76.2±27.2	25.9±9.0
MMWCOS	50	11	75.0±16.3	41.4±5.5	72.8±13.4	36.7±14.0
	20	11	73.8±24.9	37.1±9.7	81.3±19.3	33.8±8.4
	10	11	77.5±35.0	34.4±16.7	88.23±37.0	28.0±10.0
LMWCOS	50	11	66.1±18.6	31.9±6.7	79.9±19.5	27.8±6.7
	20	11	67.5±19.5	34.1±6.6	69.0±18.8	31.6±17.5
	10	11	74.6±25.2	33.2±10.5	91.4±34.8	30.9±7.5

These results revealed that the COSs produced a significant antitumor activity against the S180 or U14 tumor cell-bearing mice, and this activity was clearly dependent on molecular weight. The most effective molecular size of the COSs for their antitumor activity appeared to be around 5 kDa.

The antitumor activity of polysaccharides seems to be dependent on their molecular size rather than on their chemical structure. For example, schizophyllan and lentinan, polysaccharides of both β-glucan type, exhibit most activity within a range of 100 to 200 kDa, and no activity below 10 kDa [3, 4]. However, oligosaccharides as well as high molecular weight chitin or chitosan have been known to have antitumor activity. Suzuki *et al.* [18] and Tsukada *et al.* [23] reported that hexa-*N*-acetylchitohexaose and chitohexaose, chitin and chitosan oligosaccharides composed of a hexamer, had strong antitumor immunopotentiating function. They suggest that the antitumor mechanism of these hexose oligomers was to involve increased production of lymphokines, including interleukins 1 and 2. However, their observations with the antitumor assay were rather restricted, since they used oligosaccharides with a limited molecular weight range. In the current study, however, chitosan oligosaccharides with diverse molecular weights were employed, particularly COSs with relatively higher molecular weights (comparing hexamers of chitin or chitosan). As a result, LMWCOSs with decamers or smaller molecular size showed relatively weak inhibitory rate. This was most likely due to large number of pentamers or smallers, as evidenced in the molecular weight distribution of LMWCOSs [8], which are known to have no activity [19, 21].

In contrast, it has been established that most polysaccharides do not have direct antitumor effect by chemotherapy, but rather exhibit an indirect effect via activation of the immune system. In particular, the hexamer of chitin or chitosan expresses an antitumor effect by stimulating the immune system, including mechanisms such as the defense functions of macrophages, polymorphonuclear leukocytes, cytotoxic T, and natural killer cell activities through the induction of interleukins 1 and 2 [19, 21]. Therefore, in the present study, to investigate the influence of the COSs on the enhancement of the immune system, lymphoid organs (immune organs: spleen and thymus) from the S180- and U14-bearing mice that received COSs were examined. Table 3 shows that there were no significant differences in the weight of spleen, yet slight increase in the weight of thymus with MMWCOS treatment. That is, the weights of thymus of both the S180- and U14-bearing mice treated with COS concentration of 20 mg/kg/day or higher exhibited remarkable increases. This increase in the thymus weight implied an improvement in the immune system, in particular, an activation of T lymphocytes. Consequently, this result suggests that MMWCOS inhibited tumor cells through the activation of T lymphocytes following stimulation of thymus organ.

The average survival period for the S180-bearing mice treated with the three kinds of COSs was at least 30 days or longer. In particular, the groups treated with MMWCOS at concentrations of 50 or 20 mg/kg/day exhibited extremely high survival prolongation rates (88.5% and 77.0%, respectively). In addition, there were no survivors in the control group after 40 days of feeding, and most mice died before 40 days, when administered with HMWCOS. Five mice survived when administered with LMWCOS at 50 or 20 mg/kg/day, while the group treated with MMWCOS at 50 mg/kg and 20 mg/kg/day had 7 and 6 survivors, respectively. Accordingly, the result on the survival prolongation rate and the number of survivors after 40 days of feeding suggested that MMWCOS was an excellent antitumor agent (Table 4).

In conclusion, the administration of MMWCOS at doses of 50 or 20 mg/kg effectively inhibited the growth of both types of tumor cells (S180 and U14) in mice and

Table 4. Effect of COS on survival prolongation of BALB/c mice implanted subcutaneously with Sarcoma 180 tumor cells.

Sample	Dose (mg/kg)	Number of mice	Mean survival days	Survival prolongation rate (%)	Survivors after 40 days
Control		15	23.17	-	0
HMWCOS	50	15	30.21	30.4*	0
	20	15	33.33	43.8*	2
	10	15	29.35	26.7*	0
MMWCOS	50	15	43.67	88.5**	7
	20	15	41.02	77.0**	6
	10	15	37.79	63.1**	4
LMWCOS	50	15	39.33	69.7**	5
	20	15	36.25	56.5**	5
	10	15	37.69	62.7**	3

The tumor-bearing mice were administrated peritoneally with COSs once a day for 24 days. *$p<0.05$; **$p<0.01$ versus the control group.

significant increment of thymus weights was observed. These results suggest that the antitumor effect produced by MMWCOS might have been induced by stimulating the thymus, which is related to the immune system. In addition, the survival prolongation rate and number of survivors after 40 days of feeding were also improved by treatment with MMWCOS. Further studies using MMWCOS are planned to clarify the antitumor mechanism involving the immune system.

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