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# Absorption Behavior in the Body of Chitosan Oligosaccharide according to Molecular Weight; An *In vitro* and *In vivo* Study

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**Abstract** Chitosan has a wide range of applications in biomedical materials as well as in dietary supplements. Chitosan oligosaccharide with free-amine group (COFa) is an improvement over traditional chitosan that lacks the usual impurities and materials detrimental to the body. Based on a previous study of water soluble chitosan (WSC, chitosan lactate), we investigated the molecular weight (Mw) - dependent absorption phenomena of COFa *in vitro* and *in vivo* with various Mws. The absorption of COFa was significantly influenced by its molecular weight. As Mw increases, the absorption decreases. The absorption profiles for 5 K COFa (Mw=5 kDa) were observed to be more than 10 times higher than those of high molecular weight chitosan (100 K HWSC Mw=100 kDa) in both *in vitro* and *in vivo* transport experiments. Furthermore, the *in vitro* transport experiment suggested that transcellular transport of the COFa (Mw <10 kDa) through Caco-2 cell layer could occur with a negligible cytotoxic effect. The COFas showed a cytotoxic effect on Caco-2 cells that was dependent on dose and Mw. COFa could be transported transcellularly through the Caco-2 cell layer.

**Keywords:** COFa, oral absorption, TEER value, molecular weight, Caco-2 cell

## Introduction

Chitosan [α(1→4) 2-amino 2-deoxy β-D glucan], a deacetylated form of chitin, is a mucopolysaccharide with structural features similar to cellulose. Chitosan has specific properties such as chelation that can be applied to the areas of health care, water-quality improvement, pharmaceuticals, protein binding, inhibition of tumor cells (1-4), and microbial cell growth (5-8). Chitosan is biocompatible, biodegradable, and nontoxic for mammals (9, 10), which makes it suitable for use as a food additive (11, 12) and as a hydrating agent in cosmetics. It has been more recently found to be a pharmaceutical agent in biomedicine (13-15). Since the US Food and Drug Administration approved chitosan as a feed additive in 1933 (16), chitosan has been used as a food additive to reduce blood glucose, cholesterol, and triglyceride levels in normal mice and neonatal mice with streptozotocin-induced diabetes (17, 18). In modern society, one of the most interesting effects of chitosan is the reduction of obesity caused by overconsumption of fatty foods. The body weight gain resulting from such eating habits causes most of the geriatric diseases. As a dietary fiber, chitosan has highly characteristic properties (19). Chitosan has been reported to reduce lipid absorption in the intestine by binding fatty acids and bile acids and by increasing their excretion. Therefore, oral administration of chitosan inhibits the development of atherosclerosis in individuals with hypercholesterolemia by lowering their serum cholesterol levels (20). Chitosan also has the novel characteristic of mucoadhesion and enhances paracellular drug transport via transient opening of the tight junction between epithelial cells (21-26). Chitosan has been considered one of the safest and most effective drug absorption enhancers for oral, buccal, nasal, gastrointestinal, and other mucosal drug delivery systems (20).

In our previous study (20), we evaluated the effect of Mw on oral absorption of water soluble chitosan (WSC). In this study, we investigated the Mw-dependent absorption phenomena of chitosan oligosaccharide with free-amine groups (COFas) *in vitro* and *in vivo* to apply this knowledge to the Food and Pharmaceutics industry. COFa (5 K:Mw=5 kDa, 10 K:Mw=10 kDa, and 20 K:Mw=20 kDa) was prepared by salt removal method (27) and compared to HWSC 100 K and WSC to investigate *in vitro* and *in vivo* oral absorption phenomena. Cellular transport of various fractions of COFa through the Caco-2 cell layer was investigated *in vitro*. The relationship between Mw and *in vivo* absorption phenomena of COFa was also investigated after oral administration of fluorescence- labeled COFa.

## Materials and Methods

**Materials** COFa (5, 10, and 20 K) and high molecular weight chitosan (HWSC, 100 K) were supplied by Kittolife Co. (Seoul, Korea). Caco-2 cells were obtained from American Type Culture Collection (Rockville, MD, USA) at passage 20, and they were used between passage numbers of 30 to 50. Fluorescein isothiocyanate (FITC), Hank's balanced salt solution (HBSS), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), and collagen (from rat tail) were purchased from Sigma (St. Louis, MO, USA). Transwell cell culture chamber inserts (diameter=12 mm, pore size=3.0 μm) were purchased from Corning Inc. (Corning, NY, USA), and coated with collagen before use (8-10 μg/cm$^2$).

**Cell lines and cell cultures** Human intestinal Caco-2 cell lines were cultured in Delbecco's modified eagle

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medium (DMEM) supplemented with 1% nonessential amino acids (NEAA) and 10% fetal bovine serum (FBS) at 37°C under a humidified atmosphere with 5% $CO_2$. After reaching 90% confluence, the cells were harvested by trypsin-ethylenediamine tetraaceticacid (EDTA) treatment and seeded at a density of $3\times10^5$ cells/cm$^2$ on collagen-coated Transwell cell culture inserts. The cells were left to differentiate for 15-21 days after seeding. Transepithelial electric resistance (TEER) values were monitored using an EVOM TM epithelial voltohmmeter (World Precision Instruments, Germany), and the medium was regularly changed 3-4 times a week.

**Penentration through Caco-2 cell layer of COFa, *in vitro*** The COFa transport through Caco-2 cell monolayers was performed after 3 weeks of cell seeding. Transwell membranes were seeded with Caco-2 cells at a TEER value of over 500 Ωcm$^2$. Before the transport experiment, the cells were washed twice with phosphate buffered saline (PBS). They were then per-equilibrated for 1 hr with COFa- free transport media of pH 7.4 HBSS containing 10 mM 2-[4-(2-hydroxyethyl)-1-piperazynyl]-ethanesulfonic acid (HEPES). After removing the medium, the Caco-2 cell monolayers were treated with FITC labeled COFa solutions (10 mg/mL in HBSS) for 120 min in an apical compartment. During the treatment, samples for measuring the transported COFa were taken from the basolateral compartment while TEER values were monitored. After 2 hr of treatment, the cells were washed twice with PBS and incubated with fresh cell culture medium (DMEM). The recovery of TEER values was monitored for 22 hr after the treatment. The transported amount of COFa was measured by a fluorescence plate reader (Ex 490 nm and Em 520 nm, FL600 microplate fluorescence reader, Bio-Tek Ins., Winooski, VT, USA).

**Evaluation of cytotoxicity** The effect of molecular weight and concentration on COFas cytotoxicity were investigated using Caco-2 cell line in a modified MTT cytotoxicity assay. Cells were seeded at a density of $1\times10^4$ cell/well in 96-well cell culture plates and pre-incubated for 24 hr before chitosan treatment. The cells were then treated with chitosans of various concentrations (0.5-20 mg/mL in PBS 7.4 with 10% FBS) for 2 hr. The chitosan solutions were removed after treatment, and fresh cell culture media was added and incubated for 4 hr to stabilize the cells. Finally, the cells were incubated with MTT- containing media (0.5 mg/mL MTT in DMEM) for 4 hr. The medium was then removed, and the formazan crystals formed in living cells were dissolved in 100 μL dimethyl sulfoxide (DMSO). The relative cell viability (%) was calculated according to the following equation based on absorbance at 570 nm:

$$\text{Relative cell viability} = [(OD_{570,\,sample} - OD_{570,\,blank})/(OD_{570,\,control} - OD_{570,\,blank})] \times 100$$

***In vivo* absorption test in the intestine** Male Sprague-Dawley rats (240-260 g body weight) were fasted for 12 hr before the administration of COFas. Each rat was anesthetized with diethyl ether and force-fed FITC-labeled COFas solution through an oral gavage tube that was carefully passed down through the esophagus into the stomach. The FITC labeled COFa solutions were prepared in phosphate-buffered saline (PBS, pH 7.4) at a concentration of 10 mg/mL. The total volume of the COFa solution was 0.5 mL (dose=20 mg/kg). Blood samples (450 μL) were collected to measure the amount of COFas absorbed. These were drawn serially from a capillary in the retro-orbital plexus at each time interval (0.5, 1, 2, 3, and 4 hr) and directly mixed with 50 μL of 3.8% sodium citrate. The blood samples were immediately centrifuged at 2,400×g for 20 min to separate plasma from cellular blood components. A fluorescence plate reader was used to measure fluorescence intensity of 100 μL plasma samples. The fluorescence intensity values thus obtained were from total sample values to calculate FITC content. Seven rats were used for each type of COFa treatment.

Intestinal absorption of COFa was also investigated on a confocal laser microscope (Leica, Germany). Rats were given FITC-labeled COFas by oral gavage as described above. At 30 min after treatment, rats were anesthetized with diethyl ether, and were sacrificed by cutting their diaphragms. Duodenal and jejunal tissues were removed, washed with PBS, and fixed in buffered formalin (10% in PBS). The tissues were embedded in Tissue-Tek optimum cutting temperature (OCT) compound. The embedded specimens were cut into 20 μm sections with a microtome at -20°C, and placed on glass slides. The tissue sections were then washed to remove the OCT compound and mounted using glycerol. The slides were examined on a confocal laser microscope.

## Results and Discussion

To evaluate the oral absorption of 100 K HWSC and lactic acid-free COFa, we performed *in vitro* and *in vivo* experiments on celluar transport and intestinal absorption. In our previous study (20), we demonstrated a high permeation rate through Caco-2 cell layer, with negligible cytotoxic effects, using WSCs with lactic acid. In this study, 100 K HWSC and COFa with free amine group (27) were used to investigate the effect of molecular weight on intestinal absorption. Most of the experiments were conducted in a manner similar to the previous study. The FITC labeling of COFa achieved the same level of WSC (0.8-0.9% of FITC in the amine group of COFa) characterized in the previous study.

***In vitro* oral absorption according to Mw of COFa** The Mw of COFa is considered one of the most important factors in cellular transport and intestinal absorption. Various Mws of COFa were used to investigate permeability through differentiated Caco-2 cell layers grown on a permeable filter support. Penetration of COFa through the differentiated Caco-2 cell layer is shown in Fig. 1. This data shows that the Mw of COFa affected the movement of COFa through the Caco-2 cell layer. Increases in Mw (5, 10, 20 K) tended to decrease permeability through the Caco-2 cell layer. However, the penetration of 100 K HWSC was not observed because of its high Mw, and the concentration of COFa was maintained at the baseline level. We observed that the transport of 5 K COFa was more than 10 times that of high molecular weight WSC

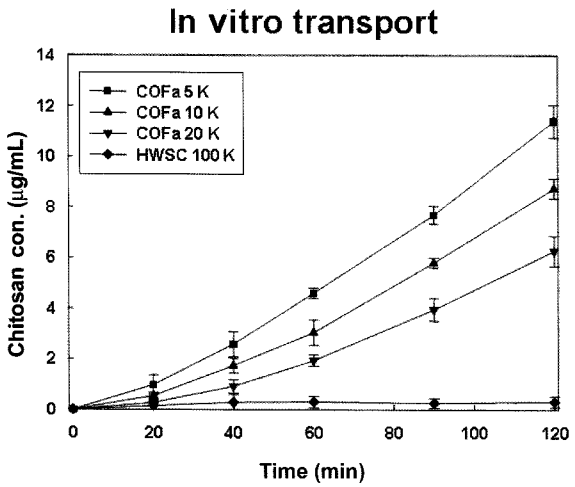


**Fig. 1. Penetration of COFas through differentiated Caco-2 cell layer** (The plotted data points are the means±SD, n=4).

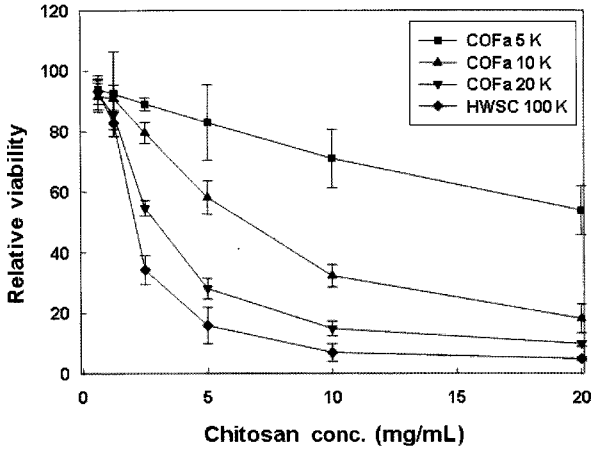


**Fig. 3. Caco-2 cell viability after 2 hr incubation with COFa solutions** (The data is expressed as mean±SD, n=4).

with lactic acid after a 120 min treatment. In our previous study, transport was observed to have been enhanced more than 23 times with low molecular weight WSC under the same conditions (20). Therefore, the penetration absorption of salt- free COFa was lower than that of WSC. The penetration rate of COFa through the Caco-2 cell layer seems to be lower because the salts from WSC were removed.

The different transport phenomena were evaluated to learn how to change the tight junction between Caco-2 cell layers. The penetration and/or interaction of the COFa with the Caco-2 cell layer also influenced the TEER values. The changes in TEER values during COFa treatment are shown in Fig. 2A. The tight junction between Caco-2 cell layers was loosened by treatment with COFa for 120 min. This opening increased with increases in the Mw. In the case of COFas with Mw lower than 10 K, the TEER values slightly increased at 20 min after treatment (about 40%). Subsequently, the TEER values decreased as treatment time increased. Figure 2B illustrates the recovery patterns of the TEER values of the Caco-2 cell layer. COFas (5 K and 10 K) showed a rapid recovery of TEER values after the treatment. After the removal of COFa solution and the addition of fresh culture medium, the TEER values continued to decrease for up to 200 min and then started to increase. At 22 hr after treatment, the TEER values had completely returned to their initial levels. However, the Caco-2 cell layers treated with 20 K COFa and 100 K HWSC showed a delayed onset and slower rate of recovery of their TEER values.

**Viability and morphology of Caco-2 cells according to Mw** Cytotoxicity of various COFas according to the Mw was investigated by MTT assay with Caco-2 cells, and the results are illustrated in Fig. 3. Caco-2 cell viability was significantly influenced by the concentration and the Mw of COFa. At low concentrations (<1 mg/mL), COFa did not show a cytotoxic effect on the intestinal Caco-2 cell line. In the case of COFa with Mw of 5 K and 10 K, cell viability slightly decreased as COFa concentration increased. However, the viability of cells treated with 20 K COFa and 100 K HWSC was dramatically decreased with increasing COFa concentration. The cell viability of lower than 50% was investigated by 5 mg/mL COFa treatment. Morphology of the Caco-2 cells was investigated after 2 hr of incubation with solutions of COFa (10 mg/mL) at various molecular weights. When treated with toxic materials, the Caco-2 cells separated from the surface of the culture dish. This result is shown in Fig. 4. When

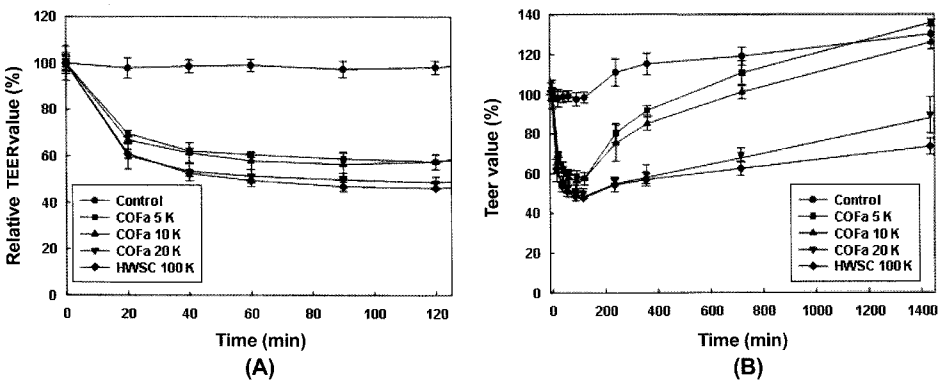


**Fig. 2. Change of TEER values of during COFa transport (A) and recovery patterns (B)** (The data is expressed as mean±SD, n=4).

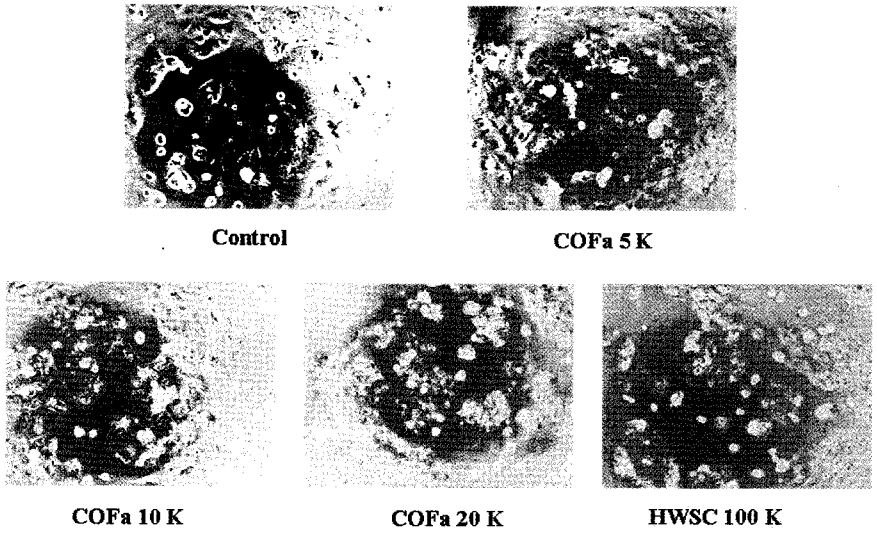


**Fig. 4. Caco-2 cell morphology after 2 hr incubation with COFa solutions** (10 mg/mL).

treated with low molecular weight COFa (5 K and 10 K), cells still retained their original shapes, but high Mw COFa (20 K) and 100 K HWSC severely damaged the cell surfaces. This result reveals that the cell cytotoxicity was significantly influenced by the Mw of COFas as well as by their concentration.

***In vivo*, intestinal absorption of COFa with various Mws** Several researchers have reported that chitosan has cholesterol lowering, antitumor, antidiabetic, and antimicrobial effects. High oral absorption of functional food containing chitosan is very important for these aforementioned bioactivities. To evaluate the ability of absorption in the intestine of COFa, an *in vivo* absorption test was performed. The oral absorption of the COFa used in this study was highly influenced by its Mw. The relationship between Mw and intestinal absorption is shown in Fig. 5. As seen in the *in vitro* Caco-2 cell layer penetration experiment, the amount of COFa absorbed in the intestine decreased with increasing Mw. COFa with Mws of 5 K, 10 K, and 20 K showed similar patterns of intestinal absorption. However, absorption of 100 K HWSC into the intestine was not observed. Plasma COFa concentrations,

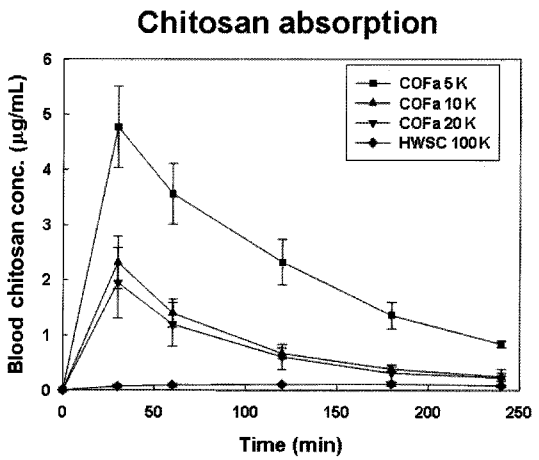


**Fig. 5. Plasma COFas concentration after oral administration** (The plotted data points are the means±SD, n=4).

calculated on the basis of fluorescence intensity, reached their maximum level at 30 min after oral administration of COFa and decreased continuously. The maximum plasma

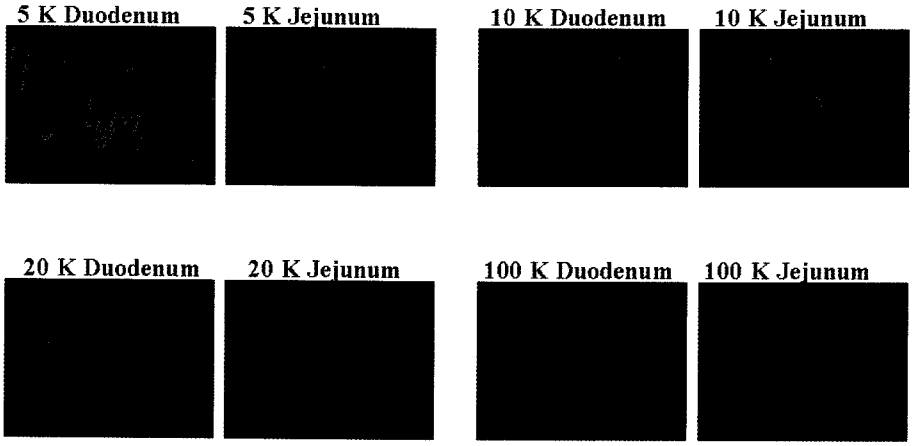


**Fig. 6. Histological studies of intestinal absorption through confocal laser microscopy of rat intestines.**

concentrations ($C_{max}$) of COFa were observed to be 4.8 μg/mL in rats fed 5K WSC, 2.4 μg/mL in rats fed 10 K WSC, and 2.0 μg/mL at dosages of 20 K WSC. Compared to the low molecular weight of WSC shown in the previous result (20), the maximum plasma COFa concentra-tions were lower than that of WSC. The high absorption of WSC is considered to be attributed to the opening effect of tight junctions by salts. At 100 K, the plasma HWSC levels were maintained at the baseline with negligible $C_{max}$ values (<0.5 μg/mL) because of poor absorption.

The same relation between plasma COFa level and Mw was also identified on a confocal laser microscope. In rats fed FITC-labeled 5 K COFa, a dense fluorescence intensity was observed from the epithelium of villi in the duodenum and jejunum (Fig. 6). The fluorescent images were also detected at submucosal levels with absorption of oligomeric chitosans (5 K COFa and 10 K COFa). This result shows that fluorescence intensities were inversely proportional to COFa Mw. In rats given 100 K HWSC, there were no detectable symptoms of COFa absorption. Thus we know that HWSC was not absorbed in the intestine. However, COFa with a low Mw could be considered safe for applications not only in the food industry but also in the pharmaceutical industry.

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