International Journal of Pharmaceutics 359 (2008) 70–78

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Improvement of intestinal absorption of insulin and water-soluble macromolecular compounds by chitosan oligomers in rats

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ARTICLE INFO

Article history:
Received 8 January 2008
Received in revised form 21 February 2008
Accepted 16 March 2008
Available online 22 March 2008

Keywords:
Chitosan oligomer
Intestinal absorption
Absorption enhancer
Insulin
Membrane toxicity
Tight junction

ABSTRACT

The effects of five chitosan oligomers on the intestinal absorption of fluorescein isothiocyanate-labeled dextrans (FDs) and insulin were studied by an *in situ* loop method. The absorption of FD4 from the jejunum was effectively improved in the presence of 0.5% (w/v) chitosan hexamer and dimer. However, chitosan hexamer did not improve the colonic absorption of FD4, although we found a moderate increase in the colonic absorption of FD4 in the presence of chitosan pentamer and dimer. The absorption enhancing effect of chitosan hexamer decreased as the molecular weights of FDs increased. In addition, we found a remarkable increase in plasma insulin levels and a significant hypoglycemic effect after jejunal administration of insulin with chitosan hexamer. In the toxicity studies of chitosan hexamer, we found no significant increase in the release of total protein and activity of lactate dehydrogenase (LDH) from the intestinal epithelium in the presence of chitosan hexamer (0.5%, w/v), indicating that this compound was a safe absorption enhancer for improving the intestinal absorption of poorly absorbable drugs. Finally, the transepithelial electrical resistance (TEER) and the permeability of FD4 in rat jejunal membranes with or without chitosan hexamer (0.5%, w/v) were examined by an *in vitro* diffusion chamber method. We observed a moderate decrease in the TEER values of rat jejunal membranes and a corresponding increase in the permeability of FD4 in the presence of chitosan hexamer (0.5%, w/v). These findings suggested that chitosan hexamer might loosen the tight junction of the intestinal epithelium, thereby improving the intestinal permeability of hydrophilic macromolecular compounds via a paracellular pathway.

1. Introduction

The intestinal absorption of hydrophilic macromolecules especially peptide and protein drugs after their oral administration is usually very poor. This has been attributed to their extensive hydrolysis by the proteolytic enzymes in the gastrointestinal tract or their poor membrane permeability characteristics (Lee and Yamamoto, 1989). Therefore, various approaches such as alternative routes of administration (Yamamoto et al., 1994b), absorption enhancers (Morishita et al., 1993; Uchiyama et al., 1999), protease inhibitors (Yamamoto et al., 1994a), chemical modification (Asada et al., 1994, 1995) and different dosage forms (Tozaki et al., 1997; Fetih et al., 2006) have been examined in attempts to overcome problems with the delivery of these peptides and proteins via the gastrointestinal tract. Of these approaches, absorption enhancers including surfactants, bile salts, chelating agents and fatty acids is one of the most promising methods. Recently, it was demonstrated that nitric oxide (NO) donors and polyamines were also effective for improving the intestinal absorption of poorly absorbable drugs (Utoguchi et al., 1998; Fetih et al., 2005; Miyake et al., 2006; Gao et al., 2008). However, some absorption enhancers can cause damage and irritate the intestinal mucosal membranes. This is a limiting factor for their clinical use. Indeed, our previous studies demonstrated that there existed almost linear relationship between the effectiveness of various absorption enhancers and their membrane toxicity (Yamamoto et al., 1996; Uchiyama et al., 1996). Therefore, an absorption enhancer with high effectiveness and low mucosal toxicity should be developed in clinical use.

Chitosan is the second most abundant natural occurring organic material after cellulose and can be obtained by the chemically deacetylation of chitin, a natural material found in crab and shrimp shells. This compound is very safe due to its biocompatibility and biodegradation characteristics (Hirano et al., 1988; Chandy and Sharma, 1990). Chitosan also has mucoadhesive properties, which are probably mediated through ionic interactions between positively charged amino groups in chitosan and negatively charged surface of the epithelial cell membrane (Lehr et

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doi:10.1016/j.ijpharm.2008.03.016

al., 1992). On the other hand, chitosan has also been employed as a pharmaceutical excipient in oral drug formulations in order to improve the dissolution of poorly absorbable drugs (Sawayanagi et al., 1982a, 1982b; Imai et al., 1991) or for sustained release of drugs by a process of slow erosion from a hydrated compressed matrix (Miyazaki et al., 1981, 1988; Takayama et al., 1990).

In the previous studies, chitosan has already been demonstrated as an absorption enhancer to increase the transport of a hydrophilic marker molecule across intestinal Caco-2 cell monolayers (Artursson et al., 1994), and to improve the absorption of insulin and a decapeptide across the nasal and intestinal mucosa, respectively (Illum et al., 1994). However, chitosans used in these previous studies were macromolecular compounds and they generally had very poor solubility in water at physiological pH, which brought much difficulty to use it in clinical application. To overcome these drawbacks of chitosans, we recently focused on chitosan oligomers, new types of chitosan molecules, which have remarkably water-soluble characteristics due to their low molecular weights. Therefore, these chitosan oligomers can be expected to become useful absorption enhancers rather than conventional chitosans. Furthermore, we recently found that these chitosan oligomers could improve the pulmonary absorption of interferon α without serious membrane damage to the lung tissues (Yamada et al., 2005). However, no studies have been carried out to examine the effects of chitosan oligomers on the intestinal absorption of poorly absorbable drugs including peptide and protein drugs. Moreover, the absorption enhancing mechanisms of chitosan oligomers have not been fully elucidated in the previous studies.

In this study, therefore, we selected five chitosan oligomers including chitosan dimer, trimer, tetramer, pentamer, and hexamer as absorption enhancers and effects of chitosan hexamer on the intestinal absorption of fluorescein isothiocyanate dextrans (FDs) (FD4, FD10 and FD70) and insulin were examined by an *in situ* closed loop method. In addition, we also examined the intestinal membrane toxicity of the chitosan hexamer by measuring the release of protein and the activities of lactate dehydrogenase (LDH) from the jejunal epithelium. Finally, to elucidate the absorption enhancing mechanisms of the chitosan oligomers, the permeability of FD4 and transepithelial electrical resistance (TEER) with or without chitosan hexamer in the rat jejunal membranes were evaluated by an *in vitro* diffusion chamber method.

2. Materials and methods

2.1. Materials

Chitosan dimer, trimer, tetramer, pentamer and hexamer were obtained from Seikagaku Co. (Tokyo, Japan) and the chemical structure and physicochemical properties of these chitosan oligomers are listed in Table 1. FDs with different molecular weights (FD4, FD10 and FD70) were obtained from Sigma–Aldrich Chemical Co. (St. Louis, MO). Insulin was purchased from Nacalai Tesque Inc. (Kyoto, Japan). All other chemicals and solvents were of analytical grade.

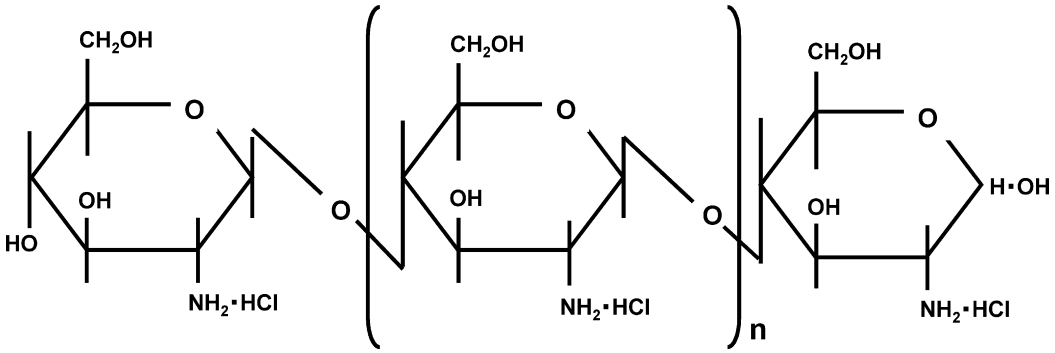
2.2. Preparation of drug solution

For an *in situ* closed loop absorption study, dosing solutions of FDs (FD4, FD10 and FD70) and insulin were prepared in the phosphate-buffered saline (PBS, pH 7.4) to yield a final concentration of 2 mg/ml and 20 IU/ml, respectively. For an *in vitro* transport study, 0.1 mM FD4 dissolved in PBS solution (pH 7.4) was prepared to examine the transport of FD4 across the jejunal membranes. Chitosan oligomers were added to the dosing solutions at a concentration of 0.1% (w/v), 0.5% (w/v), 1.0% (w/v), respectively. The solution containing chitosan hexamer (0.5%, w/v) was prepared for evaluating its intestinal membrane toxicity, permeability of FD4 and measuring the TEER values of rat jejunal membranes.

2.3. Absorption experiments

Absorption experiments were performed by an *in situ* closed loop method (Fetih et al., 2005; Gao et al., 2008). The studies were performed in accordance with the guidelines of animal ethics committee at Kyoto Pharmaceutical University. Animals were fasted for about 16 h before the experiment. Male Wistar rats, weighing 230–250 g, were anesthetized with sodium pentobarbital (50 mg/kg body weight) by intraperitoneal injection. The rats were kept in a supine position on a thermostatically controlled board at 37 °C. And then intestine was exposed through a midline abdominal incision and washed by phosphate-buffered saline (PBS, pH 7.4). The remaining buffer was expelled with air. Segments (jejunum and colon) were cannulated at both ends using polyethylene tubing, and the distal part was closed by clipping with a forceps. Drug solutions (1 ml), kept at 37 °C, were administered

Table 1
Chemical structure and physicochemical properties of various chitosan oligomers



Chitosan oligomer	*n*	MW (Da)	Solubility in water
Dimer	0	424.4	190 g/100 ml
Trimer	1	610.9	177 g/100 ml
Tetramer	2	808.5	158 g/100 ml
Pentamer	3	1006.1	86 g/100 ml
Hexamer	4	1203.7	68 g/100 ml

into the loop through a cannulated opening in the proximal part of the loop, which was then closed by clipping with another forceps. A closed loop was prepared of jejunum and colon. The jugular vein was exposed and blood samples were collected into heparinized syringes at predetermined time intervals up to 240 min. Samples were immediately centrifuged at 10,000 rpm for 5 min to obtain the plasma fraction, which was then kept in ice until determination. The intravenous administration of the drug was carried out separately via the femoral vein.

2.4. Analytical methods

Absorption of FDs was determined with a fluorescence spectrophotometer (Spectrafluor Plus, TECAN, Switzerland) at an excitation wavelength of 485 nm and emission wavelength of 535 nm, respectively.

Plasma glucose concentration was measured with the glucose oxidase method (Glucose B Test Wako Kit, Wako Pure Chemical Industries, Osaka, Japan) and the magnitude of the hypoglycemic response was calculated using the trapezoidal method as the area above the curve (AAC) for 0–4 h. The pharmacological availability (PA%) of insulin was calculated as follows:

$$\mathrm{PA\%} = \left\{ \frac{\mathrm{AAC(intestine)} \times \mathrm{dose(iv)}}{\mathrm{AAC(iv)} \times \mathrm{dose(intestine)}} \right\} \times 100$$

The plasma insulin concentration was estimated using an enzyme immunoassay method (Insulin-EIA TEST Kit, Wako Pure Chemical Industries, Osaka, Japan) and the total area under the insulin concentration curve (AUC) from 0 to 4 h was calculated with the sum of successive trapezoids between each data point.

2.5. Evaluation of intestinal membrane toxicity caused by chitosan hexamer

The release amount of tissue protein and the activity of LDH from the intestinal mucosa were studied to assess intestinal membrane damage caused by chitosan hexamer. PBS, chitosan hexamer (0.5%, w/v) and Triton X-100 (3%, v/v) were administered into the intestinal loop, respectively in a similar manner to that used for the absorption experiment. Rats were left for 4 h after administration and at the end of the experiment, the perfusate in the intestine was washed with PBS (5 ml) for the determination of the release amount of protein and the activity of LDH. The concentrations of protein were measured with bovine serum albumin as a standard using BCA™ Protein Assay Kit (Pierce Tech., USA). The activity of LDH was determined using the LDH CII assay kit (Wako Pure Chemical Industries, Ltd).

2.6. Measurement of TEER and the transport of FD4 in the presence of chitosan hexamer

The TEER values of the intestinal membranes were measured by an *in vitro* diffusion chamber method using stripped rat jejunal membranes. After surgical operation, the small intestine was isolated, the first 10 cm of the top of small intestine was cut away and the next 10 cm was used as the jejunum. The underlying muscularis of the jejunal membranes was removed and the jejunal segments were mounted in a diffusion chamber in which a surface area of 1.78 cm^2 was exposed. Two pairs of electrodes connected to a Short-Circuit Amplifier (CEZ-9100, NIHON KOHDEN) were inserted into each side of the diffusion chamber, respectively. PBS (7 ml) at pH 7.4 was added to the serosal side, and an equal volume of chitosan hexamer solution (0.5%, w/v) was added to the mucosal side. Each side of the chamber was bubbled with a mixture of 95% O_2 and 5% CO_2 in order to maintain the viability of the intestinal membranes. The temperature was maintained at 37 °C during the experiment by a circulating water bath. At different time intervals up to 2 h, the potential difference (PD) and the short-circuit current (I_{sc}) were measured, respectively, and then TEER values were calculated by Ohm's law. The initial value of TEER in the rat jejunal membranes was 46.4 ± 7.9 ohm cm^2.

The effect of chitosan hexamer on the permeability of FD4 was also examined by this *in vitro* diffusion chamber method. This method was already reported by our previous papers (Yamamoto et al., 1992, 2001; Yodoya et al., 1994) and the operation was the same as the TEER experiments as indicated above. Drug solution (7 ml) containing 0.1 mM of FD4 was applied to the mucosal side, whereas an equal volume of buffer solution was added to the serosal solution. In certain experiments, chitosan hexamer (0.5%, w/v) was applied to the drug solution in the mucosal side. At predetermined times up until 150 min, solution was sampled from the serosal side and the concentrations of drugs were determined spectrofluorometrically. The apparent permeability coefficients (P_{app}) were calculated by the following equation.

$$P_{app} = \frac{dX_R}{dT} \cdot \frac{1}{A} \cdot \frac{1}{C_0}$$

where P_{app} is the apparent permeability coefficient in centimeters per second, X_R is the amount of the drugs in moles in the receptor side, A is the diffusion area (i.e., in square centimeters), C_0 is the initial concentration of drugs in the donor side in moles per milliliter.

The viability of intestinal membrane during the test period was monitored by measuring the transport of trypan blue dye and electrophysiological parameters, including potential difference, short-circuit current and membrane resistance. There was no transport of dye during the incubation and no remarkable change of the electrical parameters, confirming that the viability of the intestinal membrane was maintained during the transport experiment.

2.7. Statistical analyses

Each value is expressed as the mean ± S.E. and statistical analysis was performed by the analysis of variance (ANOVA) with a one-way layout for comparisons with $P < 0.05$ as the minimum levels of significance.

3. Results

3.1. Effect of chitosan oligomers on the absorption of FDs from the jejunum and the colon in rats

Fig. 1 shows the effects of various chitosan oligomers on the absorption of FD4 from the jejunum (A) and the colon (B). As shown in Fig. 1(A), chitosan hexamer and chitosan dimer significantly increased the absorption of FD4 from the jejunum, but its absorption was not enhanced in the presence of other chitosan oligomers. On the other hand, in the colonic absorption of FD4 with or without various chitosan oligomers (Fig. 1(B)), only chitosan pentamer and chitosan dimer showed moderate increase in the absorption of FD4 following colonic administration, while we found no significant increase in the absorption of FD4 from the colon in the presence of other chitosan oligomers including chitosan hexamer. These findings indicated that chitosan hexamer appeared to be a most effective absorption enhancer in the jejunum among all the chitosan oligomers and the absorption enhancing effect of chitosan hexamer on the absorption of FD4 was much greater in the jejunum than that in the colon.

Fig. 2 shows the effect of different concentrations of chitosan hexamer on the intestinal absorption of FD4 from the jejunum (A)

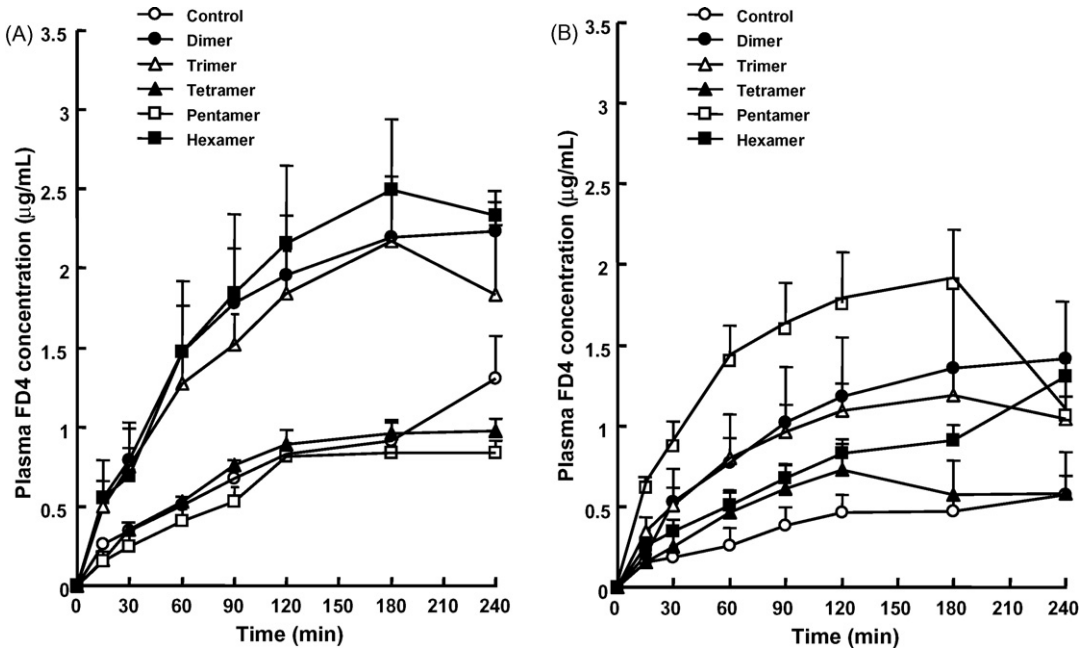


Fig. 1. Effects of various chitosan oligomers (0.5%, w/v) on the absorption of FD4 from the jejunum (A) and colon (B) in rats. Results are expressed as the mean ± S.E. of 3–5 rats. Keys: (○) control, (●) chitosan dimer, (△) chitosan trimer, (▲) chitosan tetramer, (□) chitosan pentamer, (■) chitosan hexamer.

and the colon (B). As shown in Fig. 2(A), the absorption enhancing effect of chitosan hexamer in the jejunum was influenced by its concentration and a greatest absorption enhancement effect was obtained at the concentration of 0.5% (w/v) chitosan hexamer following jejunal administration. In contrast, no significant increase in the absorption of FD4 from the colon was observed even though 0.5% (w/v) of chitosan hexamer was co-administered with FD4 to the colon (Fig. 2(B)).

Table 2 summarizes the AUC values of FD4 with or without various chitosan oligomers and their absorption enhancing ratio after jejunal and colonic administration. The absorption of FD4 from the jejunum without any absorption enhancers was limited. However, the AUC value of FD4 was greatly improved by the addition of chitosan hexamers (0.5%, 1.0%, w/v) and dimer (0.5%, w/v) from jejunum and the absorption enhancement ratio in the presence of 0.5% chitosan hexamer, 1.0% chitosan hexamer and 0.5% dimer

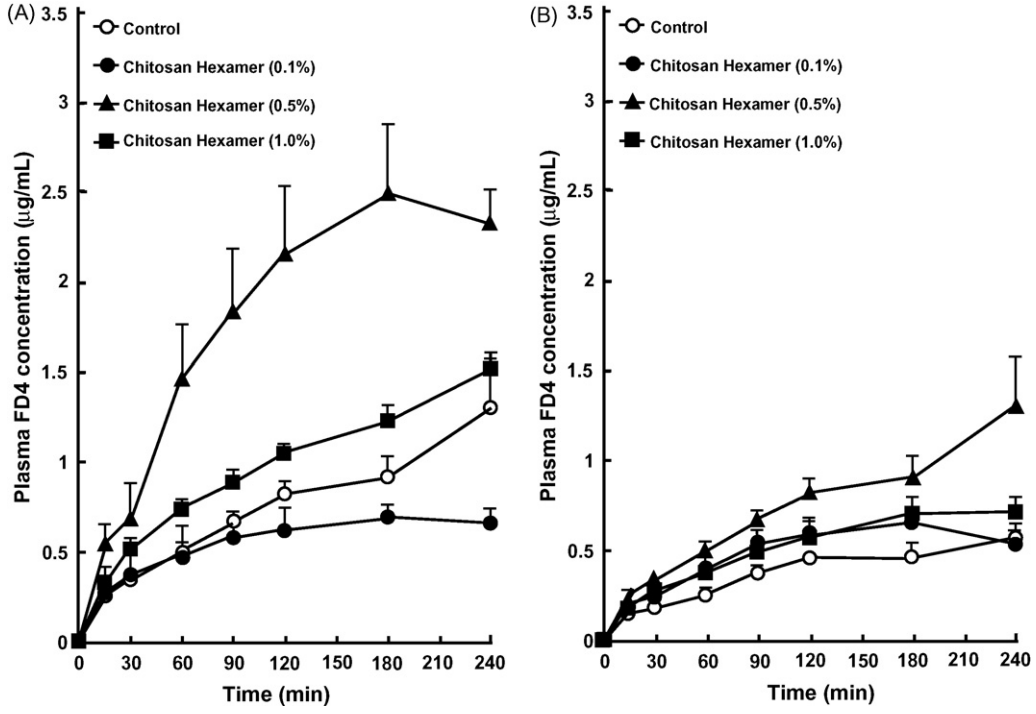


Fig. 2. Effects of various concentrations of chitosan hexamer on the absorption of FD4 from the jejunum (A) and colon (B) in rats. Results are expressed as the mean ± S.E. of 3–5 rats. Keys: (○) control, (●) 0.1% (w/v) chitosan hexamer, (▲) 0.5% (w/v) chitosan hexamer, (■) 1.0% (w/v) chitosan hexamer.

Table 2
AUC values of FD4 after intestinal administration with or without chitosan oligomers

	Jejunum		Colon	
	AUC (μg min/ml)	Ratio	AUC (μg min/ml)	Ratio
Control	180.1 ± 15.1	–	145.1 ± 12.7	–
Chitosan dimer (0.5%)	366.4 ± 50.4*	2.0	226.4 ± 27.5*	1.6
Chitosan trimer (0.5%)	269.7 ± 50.7	1.5	221.6 ± 33.5	1.5
Chitosan tetramer (0.5%)	175.8 ± 10.4	1.0	124.6 ± 33.3	0.9
Chitosan pentamer (0.5%)	147.5 ± 7.6	0.8	246.9 ± 58.0*	1.7
Chitosan hexamer (0.1%)	134.1 ± 25.3	0.7	91.7 ± 10.6	0.6
Chitosan hexamer (0.5%)	440.2 ± 66.6**	2.4	156.3 ± 36.7	1.1
Chitosan hexamer (1.0%)	232.4 ± 8.3*	1.3	118.7 ± 27.7	0.8

Results are expressed as the mean ± S.E. of 3–5 rats. (*) $P < 0.05$, (**) $P < 0.01$, compared with the control.

were 2.4, 1.3 and 1.5 times, respectively, compared with the control studies. On the other hand, a moderate increase in the colonic absorption of FD4 was observed in the presence of chitosan pentamer (0.5%, w/v) and dimer (0.5%, w/v), although other chitosan oligomers had almost no absorption enhancing effect in the colon.

To confirm whether the absorption enhancing effect of chitosan hexamer is dependent on the molecular weights of drugs, we next examined the effect of chitosan hexamer (0.5%, w/v) on the jejunal absorption of FDs with different molecular weights. As shown in Fig. 3, chitosan hexamer (0.5%, w/v) significantly increased the AUC values of FD4 and FD10 in the jejunum, but we found no significant increase in the AUC value of FD70 even in the presence of chitosan hexamer (0.5%, w/v). Overall, the absorption enhancing effect of chitosan hexamer in the jejunum decreased as the molecular weight of FDs increased.

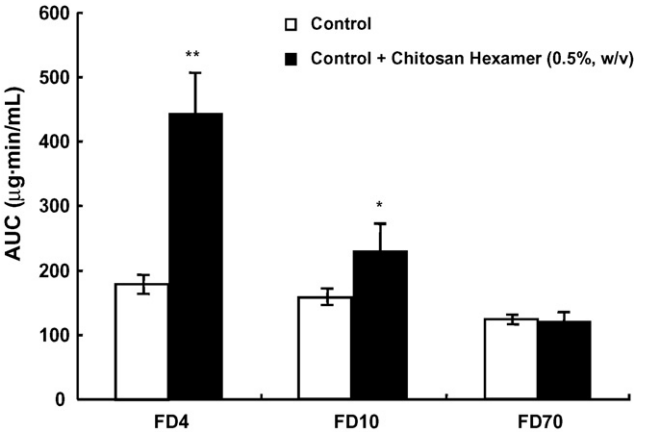


Fig. 3. Effect of chitosan hexamer (0.5%, w/v) on the absorption of FDs with various molecular weights from the jejunum in rats. Results are expressed as the mean ± S.E. of 3–5 rats. (*) $P < 0.05$, (**) $P < 0.01$, compared with the control.

3.2. Effect of chitosan hexamer on the absorption of insulin from the rat jejunum

We next examined the effect of chitosan hexamer on the absorption of insulin from the jejunum in rats. In this study, chitosan hexamer was chosen as a model of absorption enhancer, because it had a greatest absorption enhancing effect for improving the absorption of FD4 from the jejunum as described above. Fig. 4 shows concentration–time profiles of insulin (A) and glucose (B) in plasma after jejunal administration of insulin with various concentrations of chitosan hexamer. As shown in Fig. 4(A), we found a significant increase in plasma insulin levels after jejunal administration

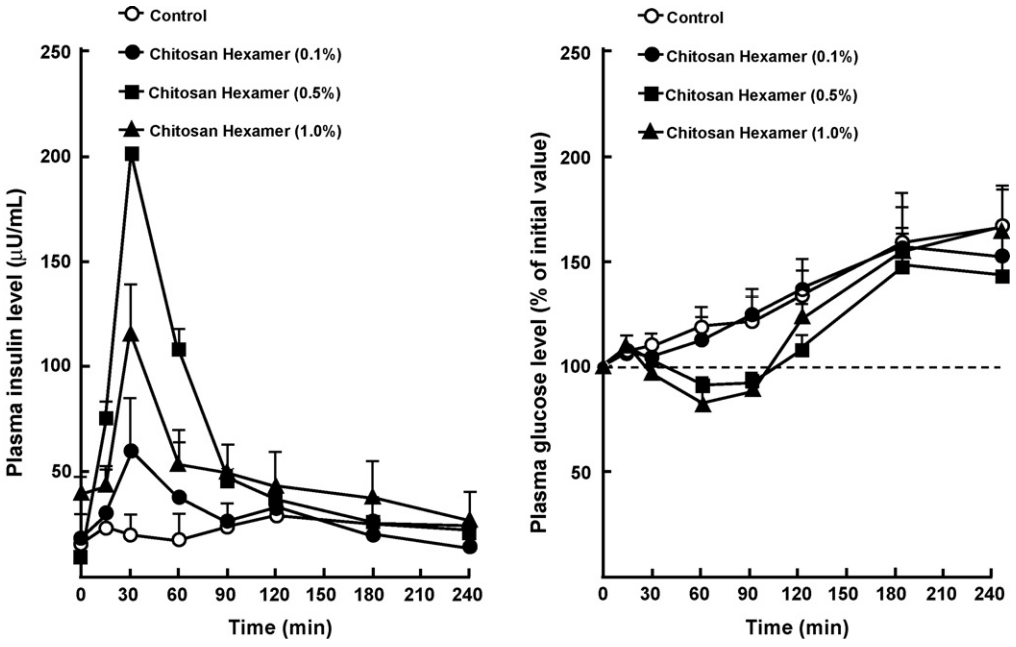


Fig. 4. Concentration–time profiles of insulin (A) and glucose (B) in plasma after jejunal administration of insulin with or without different concentrations of chitosan hexamer. Results are expressed as the mean ± S.E. of 3–5 rats. Keys: (○) control, (●) 0.1% (w/v) chitosan hexamer, (▲) 0.5% (w/v) chitosan hexamer, (■) 1.0% (w/v) chitosan hexamer.

Table 3
Pharmacokinetic parameters of insulin following jejunal administration with or without different concentrations of chitosan hexamer (CH)

	C_{max} (μU/ml)	T_{max} (min)	AUC (μU min/ml)	BA (%)	AAC (% glu.reduc.min)	PA (%)
Control	23.3 ± 19.7	15.1 ± 0.0	5742.9 ± 1334.7	0.28	0.0 ± 0.0	0.0
0.1% CH	60.0 ± 25.4	28.3 ± 4.6	6958.0 ± 2030.1	0.34	0.0 ± 0.0	0.0
0.5% CH	202.1 ± 7.6	30.3 ± 6.1	14391.1 ± 1125.2**	0.70**	1229.2 ± 157.3	0.38
1.0% CH	116.0 ± 23.6	31.9 ± 4.7	11637.5 ± 1679.3*	0.57*	503.9 ± 78.2	0.16

Results are expressed as the mean ± S.E. of 3–5 rats. (*) $P < 0.05$, (**) $P < 0.01$, compared with the control.

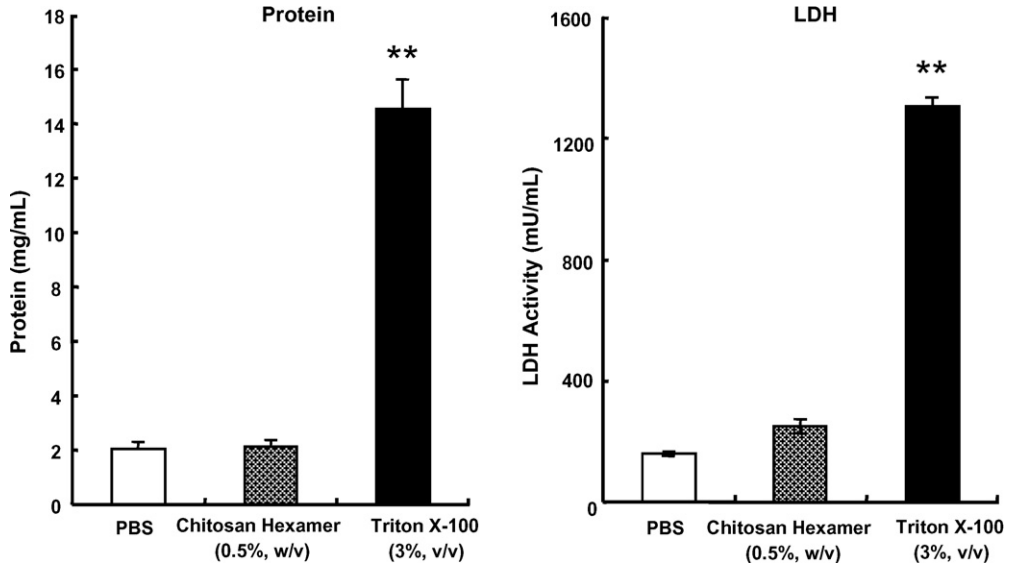


Fig. 5. Amount of total protein and the activity of lactate dehydrogenase (LDH) at 4 h after jejunal administration of chitosan hexamer (0.5%, w/v). Results are expressed as the mean ± S.E. of 3–5 rats.

of insulin with 0.5–1.0% (w/v) of chitosan hexamer. In addition, a corresponding significant hypoglycemic effect was also obtained after jejunal administration of insulin with 0.5–1.0% (w/v) of chitosan hexamer, as indicated in Fig. 4(B), Of these concentrations of chitosan hexamer, 0.5% (w/v) of chitosan hexamer appeared to be a greatest absorption enhancing effect for improving the jejunal absorption of insulin in rats.

Table 3 summarizes the pharmacokinetic and pharmacodynamic parameters of insulin in the presence or absence of various concentrations of chitosan hexamer. As indicated in Table 3, chitosan hexamer, especially at the concentration of 0.5% (w/v) significantly increased the peak plasma concentration (C_{max}), AUC, bioavailability (BA) %, AAC and PA% of insulin as compared with the control, suggesting that the chitosan hexamer could improve the absorption of insulin from the rat jejunum as well as other hydrophilic macromolecular drugs including FDs.

3.3. Evaluation of intestinal membrane toxicity of chitosan hexamer by measuring the amount of protein and lactate dehydrogenase released from the intestinal epithelium

We also examined the intestinal membrane toxicity of chitosan hexamer by measuring the amount of protein and the activity of LDH released from the intestinal epithelium. The effect of chitosan hexamer on the amount of protein and the activity of LDH released from the jejunal epithelium are shown in Fig. 5. As shown in this figure, chitosan hexamer (0.5%, w/v) did not increase the amount of protein and the activity of LDH released from the jejunal epithelium as compared with the control (no enhancer), although we found a significant increase in these biochemical parameters in the presence of Triton X-100 (3%, v/v) as a positive control. These findings suggested that chitosan hexamer might not cause any membrane damage to the intestinal epithelium and this compound was quite safe after intestinal administration.

3.4. Effect of chitosan hexamer on TEER and the transport of FD4 in rat jejunal membranes

To elucidate the absorption enhancing mechanism of chitosan hexamer, the TEER and the permeability of FD4 in rat jejunal membranes with or without chitosan hexamer (0.5%, w/v) were

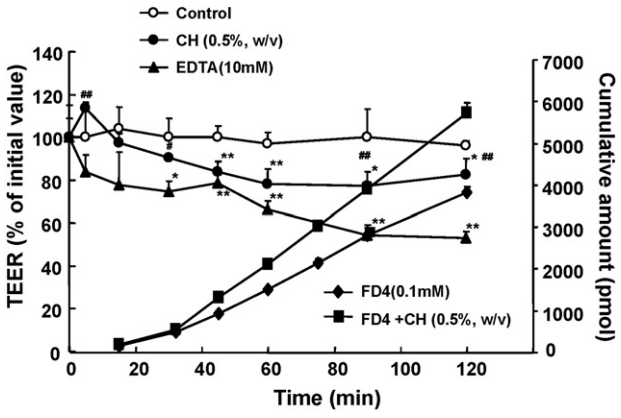


Fig. 6. Transport of FD4 and transepithelial electrical resistance (TEER) with or without chitosan hexamer (0.5%, w/v) in the rat jejunum. Results are expressed as the mean ± S.E. of at least 4 rats. (*) $P < 0.05$, (**) $P < 0.01$, compared with the control group, and (#) $P < 0.05$, (##) $P < 0.01$, compared with the EDTA group. Keys: (○) TEER values without enhancer (control), (●) TEER values with 0.5% (w/v) chitosan hexamer, (▲) TEER values with 10 mM EDTA, (♦) permeability of FD4 (Control), (■) permeability of FD4 with 0.5% (w/v) chitosan hexamer.

examined by an *in vitro* diffusion chamber method (Fig. 6). As shown in Fig. 6, chitosan hexamer (0.5%, w/v) moderately decreased the TEER values of rat jejunal membrane, while this value remarkably decreased in the presence of EDTA (10 mM) as a positive control. Moreover, we found a corresponding increase in the permeability of FD4, a paracellular marker compound, in the presence of chitosan hexamer (0.5%, w/v). The P_{app} value of FD4 with chitosan hexamer in the jejunum was $5.65 \pm 0.31\ cm/s \times 10^{-6}$, which was significantly greater than that without chitosan hexamer ($3.87 \pm 0.29\ cm/s \times 10^{-6}$) used as the control. These findings suggested that chitosan hexamer might loosen the tight junction of the intestinal epithelium, thereby improving the intestinal permeability of hydrophilic macromolecular compounds via a paracellular pathway.

4. Discussion

Although many researchers already reported that chitosan derivatives as absorption enhancers could effectively improve the absorption of hydrophilic drugs after nasal and intestinal administration (Schipper et al., 1996; Kotze et al., 1997a, 1997b; Thanou et al., 2000, 1999; Hamman et al., 2002), few studies have been examined on the effects of chitosan derivatives with water-soluble and low molecular weight compounds on the intestinal absorption of poorly absorbable drugs. Therefore, in the present study, we studied the effect of chitosan oligomers, water-soluble and relatively low molecular weight compounds on the intestinal absorption of hydrophilic drug including FDs with different molecular weights and insulin in rats. In this study, the membrane toxicity caused by chitosan hexamer was also examined to assess the safety of chitosan hexamer. We also measured the TEER in order to elucidate the absorption enhancing mechanism of chitosan hexamer.

The present study demonstrated that chitosan hexamer had a greatest absorption enhancing effect for improving the intestinal absorption of FD4 and insulin among various chitosan oligomers (Figs. 1, 2 and 4). These findings were well correlated with the results of Yamada et al. (2005) who showed that chitosan hexamer had the greatest absorption enhancing effect for improving the pulmonary absorption of interferon α in rats. The present study also found that the number of sugar ring of these chitosan oligomers was not related to their absorption enhancing effects. Although the absorption enhancing mechanism of these chitosan oligomers is still unknown, we can conclude that the number of sugar rings of these chitosan oligomers is not a main factor regulating their potencies of absorption enhancing effects. We also demonstrated that there exist regional differences in the absorption enhancing effect of these chitosan oligomers for improving the intestinal absorption of FD4 in rats. That is, the absorption of FD4 from the jejunum was improved in the presence of chitosan hexamer, while this compound did not improve the absorption of FD4 from the colon. This result was remarkably different from the previous findings that the absorption enhancing effects of many absorption enhancers in the large intestine were generally much greater than those in the small intestine (Yamamoto et al., 1997). However, we recently found that the absorption enhancing effects of NO donors and polyamines in the small intestine was almost same as those in the large intestine for improving the intestinal absorption of insulin in rats (Fetih et al., 2005; Gao et al., 2008). Therefore, this finding suggests that there exist some absorption enhancers, which show greater or similar absorption enhancing effects in the small intestine compared with those in the large intestine.

We found that the absorption enhancing effect of chitosan hexamer for improving the intestinal absorption of FD4 was affected by its concentration and a maximal absorption enhancing effect was observed at a concentration of 0.5% (w/v). Yamada et al. (2005) reported that the effects of chitosan hexamer on the pulmonary absorption of interferon α were also influenced by its concentration and chitosan hexamer at a concentration of 0.5% (w/v) had a greatest absorption enhancing effect for improving the pulmonary absorption of interferon α. Artursson et al. (1994) also reported that the absorption enhancing effect of chitosan for mannitol transport across Caco-2 cells was dependent on its concentration, but its absorption enhancing effect was almost saturable up to 0.5% (w/v), although the type of chitosan and administration route of chitosan were different. Therefore, our present result is consistent with the previous reports and chitosan oligomers as well as other chitosan derivatives have some optimal concentration to show the greatest absorption enhancing effects for improving the intestinal and pulmonary absorption of poorly absorbable drugs in rats.

The present study indicated that chitosan oligomers not only enhanced the intestinal absorption of FD4 but also improved the intestinal absorption of insulin in rats. One possible mechanism for improving the intestinal absorption of insulin by the chitosan hexamer is that this compound may inhibit the degradation of insulin in the intestine, thereby increasing the intestinal absorption of insulin from the intestine. Indeed, it was previously reported that chitosan–EDTA conjugate strongly inhibit the enzymatic activity (Bernkop-Schnurch and Krajicek, 1998). However, the characteristics of macromolecular chitosans were totally different from those of chitosan oligomers, and chitosan hexamer could improve the intestinal absorption of FD4, which was a quite stable compound in the intestine. Therefore, it may not be plausible that this chitosan hexamer might increase the stability of insulin in the intestine and enhance its intestinal absorption in rats, although we do not have enough evidence to support this hypothesis at present.

When absorption enhancers are applied in clinical use, their potential local toxicity should be considered. The membrane damage of these absorption enhancers is usually estimated by measuring the biological markers including protein and LDH released from the intestinal epithelial cells. Generally, protein is an index of membrane damage, because protein is one of the components of biological membrane and is released if the biomembrane was damaged (Lee et al., 1991). LDH is a cytosolic enzyme and its presence in the luminal fluid is generally regarded as evidence of cell membrane damage. As shown in Fig. 5, chitosan hexamer did not increase the amount of protein and the activity of LDH, although we observed a significant increase in these biological markers in the presence of 3% Triton X-100, as a positive control. Yamada et al. (2005) reported that the total amount of protein and the activity of LDH in the presence of 0.5% (w/v) chitosan hexamer at 5 and 24 h after intratracheal administration were the same levels as compared with PBS. It was also reported that chitosan polymer with the molecular weight of larger than 100 kDa were toxic towards B16F10 cells (Carreno-Gomez and Duncan, 1997). These findings suggested that chitosan hexamer (0.5%, w/v) with low molecular weight might not cause any significant membrane damage to the intestinal epithelium.

The TEER of rat jejunal membranes in the presence of chitosan hexamer (0.5%, w/v) was measured in order to elucidate absorption enhancing mechanisms of chitosan hexamer. As shown in Fig. 6, TEER value moderately decreased in the presence of 0.5% (w/v) chitosan hexamer compared with the control, indicating that chitosan hexamer might loosen the tight junction of the intestinal epithelium, thereby increasing the permeability of drugs via paracellular pathway. Many researchers previously examined the absorption enhancing mechanisms of chitosan derivatives for improving the transport and absorption of drugs. Schipper et al. (1997) reported that the binding and absorption enhancing effects of chitosans on epithelial cells were mediated through

their positive charges and the interaction of chitosans with the cell membrane resulted in a structural reorganization of tight junction-associated proteins which is followed by enhanced transport through the paracellular pathway. Artursson et al. (1994) reported that the mechanism of the enhancement of the transport of mannitol across the nasal mucosal membrane was proposed to be a combination of mucoadhesion and an effect on tight junctions in the epithelium. In order to determine which protein kinase C (PKC) isozymes were responsible for the chitosan-mediated tight junction disruption, the activation of the PKC isozymes alpha, beta and delta was investigated by Smith et al. (2005) and a chitosan-mediated translocation of PKC alpha but not PKC beta or delta from the cytosol to the membrane fraction was observed. Thus, treatment of Caco-2 cells with chitosan may result in the activation of PKC-dependent signal transduction pathways which may affect tight junction integrity (Smith et al., 2005). Smith et al. (2004) also concluded that chitosan-mediated tight junction disruption was caused by a translocation of tight junction proteins from the membrane to the cytoskeleton. Probably, these mechanisms might be related to the opening the tight junction of the intestinal epithelium and the increased permeability of drugs across the intestinal membranes caused by chitosan hexamer. Nevertheless, further investigation on the mechanism of opening the tight junction by these chitosan oligomers should be examined for developing the pharmaceutical dosage forms of poorly absorbable drugs.

5. Conclusions

In conclusion, the present study demonstrated that the absorption of insulin and FD4 from the jejunum could be improved in the presence of chitosan hexamer. In addition, chitosan hexamer did not cause any serious membrane damage to the intestinal epithelium. These findings indicated that chitosan hexamer is an efficient and safe absorption enhancer for oral delivery of poorly absorbable macromolecular drugs including peptide and protein drugs.

Acknowledgements

This work was supported by 21 Century Center of Excellent Program “Development of Drug Discovery Frontier Integrated from Traditional to Proteome” from Ministry of Education, Science, Sports and Culture in Japan.

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