Available online at www.sciencedirect.com

SCIENCE DIRECT

European Journal of Pharmacology 502 (2004) 149–155

www.elsevier.com/locate/ejphar

# Segment-selective absorption of lysozyme in the intestine

Mikihisa Takano*, Yuka Koyama, Hiromi Nishikawa, Teruo Murakami, Ryoko Yumoto

*Department of Pharmaceutics and Therapeutics, Division of Clinical Pharmaceutical Science, Programs for Pharmaceutical Sciences, Graduate School of Biomedical Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan*

Received 25 March 2004; received in revised form 11 August 2004; accepted 19 August 2004
Available online 13 September 2004

**Abstract**

Absorption of fluorescein isothiocyanate-labeled lysozyme (FITC-lysozyme) was examined in rat small intestine. Messenger RNA of megalin, an endocytic receptor for lysozyme in the kidney, was expressed in the lower but not in the upper intestine. In in situ closed loop and recirculation methods, absorption of FITC-lysozyme from the upper intestine was much higher than from the lower intestine. The absorption rate of FITC-lysozyme in the upper intestine was significantly higher than FITC-dextran and was inhibited by unlabeled lysozyme in a concentration-dependent manner. The absorption of FITC-lysozyme was also inhibited by spermine and phenylarsine oxide. These results indicate that the intestinal absorption of lysozyme is segment-selective and occurs preferentially from the upper intestine. Megalin expressed in the lower intestine appears not to have a significant role in the absorption of lysozyme. In the upper intestine, lysozyme appears to be absorbed by an endocytic pathway, and cationic charge may be important for lysozyme absorption.


*Keywords:* Intestinal absorption; Protein drug; Lysozyme; Megalin; Endocytosis

## 1. Introduction

Recent developments in molecular biotechnology have made it possible to provide various protein and peptide drugs for clinical use. Generally, protein drugs are administered as injections and cannot be administered orally. However, oral medicines are self-manageable and much more convenient for patients. Thus, many attempts have been made to develop oral delivery systems for these macromolecular drugs (Fasano, 1998; Mahato et al., 2003; Swaan, 1998; Yamamoto, 2001). The main reason for their incompatibility with oral administration is poor intestinal absorption (Aungst, 1993). This poor absorption is mainly due to the large protein molecules, which cause poor permeability across the intestinal epithelium. Moreover, protein drugs are often unstable in the intestine, because the intestinal lumen and epithelial cells contain many enzymes that degrade proteins (Woodley, 1994). However, some protein drugs are clinically available in oral forms, such as lysozyme, cytochrome c, and bromelain. These drugs have been used for many years, but the characteristics and mechanisms underlying their absorption in the intestine remain unclear.

Megalin, a large glycoprotein (approximately 600 kDa) functioning as an endocytic receptor (Kerjaschki and Farquhar, 1982; Saito et al., 1994), is highly expressed in the renal proximal tubule epithelium (Zheng et al., 1994). The ligands for megalin include lysozyme, cytochrome c, and aminoglycosides (Christensen and Birn, 2001, 2002). In the kidney, megalin is reported to serve as a clearance receptor for low molecular weight proteins, such as lysozyme filtered through the glomerulus (Christensen et al., 1998; Leheste et al., 1999; Orlando et al., 1998). We also reported the high expression of megalin in the renal cortex and its important role in the uptake of aminoglycosides and lysozyme in renal proximal tubules (Nagai et al., 2001, 2002; Nagai and Takano, 2004). In addition to the kidney, megalin is expressed in intestinal epithelial cells and other

* Corresponding author. Tel.: +81 82 257 5315; fax: +81 82 257 5319.
*E-mail address:* takanom@hiroshima-u.ac.jp (M. Takano).

0014-2999/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2004.08.041

tissues (Christensen and Birn, 2001; Yammani et al., 2001). Therefore, it is possible that megalin is involved in the absorption of lysozyme in the intestine. However, this hypothesis has yet to be examined.

In this study, we compared upper and lower intestinal segments to examine the expression of megalin in the intestine and its relationship to lysozyme absorption. Certain characteristics of the intestinal absorption of lysozyme were also examined.

## 2. Materials and methods

### 2.1. Materials

Lysozyme chloride from egg white was obtained from Nacalai Tesque (Kyoto, Japan). Fluorescein isothiocyanate (FITC), FITC-dextran (FD-10, average molecular size, 9.4 kDa), spermine, and phenylarsine oxide were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals used were of the highest purity commercially available.

### 2.2. Animal studies

Male Sprague–Dawley rats weighing 220 to 300 g (8–9 weeks old) were fasted overnight with free access to water, then anaesthetized with pentobarbital (30 mg/kg) by intraperitoneal injection before experiments. Rats used for the intestinal absorption studies were kept supine on a surface kept at 37 °C to maintain body temperature. All the animal experiments were performed in accordance with the Guide for Animal Experimentation from the Committee of Research Facilities for Laboratory Animal Sciences, Graduate School of Biomedical Sciences, Hiroshima University.

### 2.3. Analysis of mRNA expression of megalin in the intestine

The mucosa was scraped from the upper and lower half of the intestine of rats, and total RNA was isolated with Mag Extractor-RNA™ (Toyobo, Osaka, Japan). The total RNA (0.36 μg) was used for reverse transcription (RT) to generate cDNA using Rever Tra Dash™ (Toyobo), and the generated RT cDNA was used for the polymerase chain reaction (PCR) amplification using a Program Temp Control System PC-707 (ASTEC, Fukuoka, Japan). The primers for rat megalin were sense, 5′-ACACCGCTTCTGCCGTCT-3′; antisense, 5′-TCTGAGCACTCCCGAGGAAC-3′ (expected size of PCR product, 636 bp). The conditions for PCR were as follows: denaturation, 94 °C for 1 min; annealing, 68 °C for 1 min; and extension, 72 °C for 30 s (41 cycles). The PCR products were analyzed by electrophoresis on 2.0% agarose gel and visualized under ultraviolet light with ethidium bromide.

### 2.4. FITC labeling of lysozyme

Labeling of lysozyme with FITC was performed as described previously (Nagai et al., 2002). Briefly, 2 mg FITC and 200 mg lysozyme were dissolved in 0.1 M borate buffer (pH 9.0), and after incubation for 60 min at room temperature, pH was adjusted to 7.5 with 0.1 M boric acid. The solution was dialyzed by cellulose membrane for 48 h at 4 °C and concentrated by freeze-drying. When the lyophilized proteins were subjected to SDS-polyacrylamide gel electrophoresis (PAGE), a single band was observed by staining with Coomassie brilliant blue, and the band size was the same as an authentic lysozyme (14.3 kDa), indicating that there was no degradation of lysozyme during the FITC labeling.

### 2.5. Intestinal absorption of FITC-lysozyme from in situ closed loop

After flushing the small intestine with saline prewarmed to 37 °C, the upper (a 10-cm-long jejunal segment; 5–15 cm below the bile duct opening) or the lower (a 10-cm-long segment from the ileocecum) segment of the small intestine was ligated to form a closed loop. The femoral artery was cannulated with polyethylene tubing (PE50) for blood sampling. FITC-lysozyme was dissolved at a concentration of 0.7 mM in isotonic Dulbecco's phosphate-buffered saline (D-PBS; pH 7.4) containing 25 mM glucose. The solution (1 ml) was administered into the loop, and blood sample (0.25 ml) was collected from a femoral artery via the cannula at a designated time.

### 2.6. Intestinal absorption of FITC-lysozyme during in situ recirculating perfusion

The intestinal absorption of FITC-lysozyme was evaluated by a recirculating perfusion technique as described previously (Funakoshi et al., 2003; Yumoto et al., 2003). Briefly, FITC-lysozyme at a concentration of 0.35 μM was dissolved in D-PBS buffer containing 25 mM glucose. The upper segment (a 20-cm-long jejunal segment; 5–25 cm below the bile duct opening) or the lower segment (a 20-cm-long segment from the ileocecum) of the small intestine was perfused with the solution containing FITC-lysozyme (10 ml) at a rate of 1 ml/min in a recirculating perfusion manner and at a designated time, 100 μl of perfusate was taken for fluorescence measurement. The absorption rate of FITC-lysozyme from the intestine was estimated from the disappearance rate by measuring the FITC-lysozyme remaining in the perfusate. To exclude the effect of initial adsorption of FITC-lysozyme to the intestinal surface, the disappearance rate was calculated from the slope between 20 to 60 min, where a linear disappearance with time was observed. The absorption rate of FD-10 (0.35 μM) was estimated as described for FITC-lysozyme.

### 2.7. Evaluation of intact FITC-lysozyme in the plasma and intestinal perfusate

The intactness of FITC-lysozyme in the plasma and intestinal perfusate was evaluated after separation by SDS-PAGE. The plasma and perfusate samples were solubilized in a loading buffer consisting of 2% SDS, 62.5 mM Tris–HCl, 7% glycerol, and 5% 2-mercaptoethanol. Then, the sample was subjected to SDS-PAGE with 12.5% polyacrylamide gel. After SDS-PAGE, the fluorescence intensity of the gel was analyzed by fluoroimage analyzer FLA-2000 (Fuji Photo Film, Tokyo, Japan); the image was obtained with the aid of a Macintosh personal computer and a software provided with the analyzer.

### 2.8. Analytical methods

Fluorescence of FITC-lysozyme and FD-10 in biological samples was measured using a Hitachi fluorescence spectrophotometer F-3000 (Tokyo, Japan) at an excitation wavelength of 500 nm and an emission wavelength of 520 nm. All the experiments were repeated three times, and the data were expressed as the mean±S.E.M. The statistical analysis was performed by Student's *t*-test or by one-way analysis of variance followed by the Tukey test for multiple comparisons. The level of significance was set at $*P<0.05$ and $**P<0.01$.

## 3. Results

### 3.1. Expression of megalin mRNA in the intestine

The expression of megalin mRNA was compared between the upper and lower segments of the small intestine using RT-PCR (Fig. 1). The PCR product of the expected size was clearly observed in the sample of renal cortex after reverse transcription (lane 5, positive control). A band with the same size was also observed in the lower (lane 3) but not in the upper (lane 1) intestine, although the level in the lower intestine seemed to be much lower than that in the renal cortex. Thus, it was found that megalin was expressed only in the lower segment of the intestine.

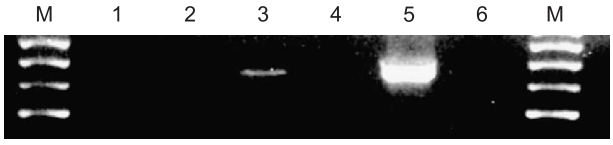


Fig. 1. RT-PCR analysis of the expression of megalin mRNA in the intestine and renal cortex. The PCR products were analyzed by electrophoresis on 2.0% agarose gel and visualized under ultraviolet light with ethidium bromide. M: size marker; lane 1: upper intestinal sample with reverse transcription [RT(+)]; lane 2: upper intestinal sample without reverse transcription [RT(−)]; lane 3: lower intestinal RT(+) sample; lane 4: lower intestinal RT(−) sample; lane 5: renal cortical RT(+) sample; and lane 6: renal cortical RT(−) sample.

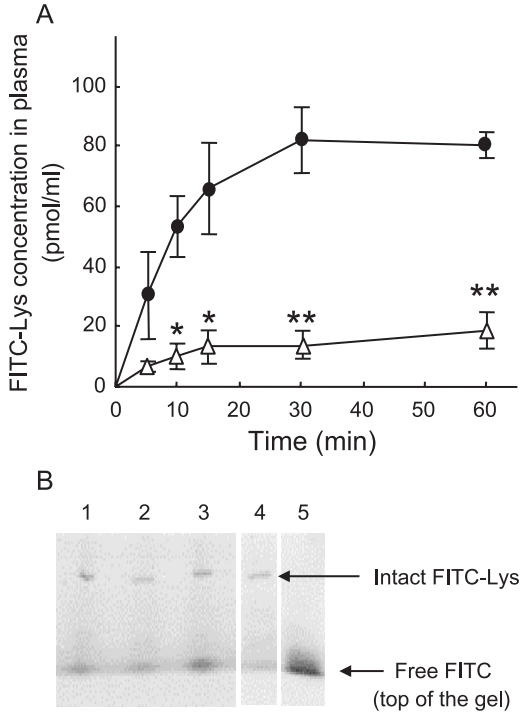


Fig. 2. Absorption of FITC-lysozyme from the upper and lower intestine evaluated by in situ closed loop method (A) and evaluation of intact FITC-lysozyme in the plasma samples (B). (A) FITC-lysozyme (FITC-Lys, 0.7 mM) was administered into the loop, and blood samples (0.25 ml) were collected at 5, 10, 15, 30, and 60 min. Total fluorescence in the plasma samples was measured at an excitation wavelength of 500 nm and an emission wavelength of 520 nm, and the data were expressed as FITC-Lys concentrations in the plasma. $*P< 0.05$, $**P< 0.01$, significantly different from the upper intestine. (B) The intactness of FITC-Lys in the plasma samples obtained at 120 min was evaluated by a fluoroimage analyzer after separation by SDS-PAGE. Lanes 1–3: three separate plasma samples; lane 4: intact FITC-Lys (standard); and lane 5: free FITC.

### 3.2. Intestinal absorption of FITC-lysozyme evaluated by in situ loop method

The intestinal absorption of FITC-lysozyme was examined by the in situ closed loop method. A solution containing FITC-lysozyme (0.7 mM) was administered into either the upper or lower intestinal loop, and fluorescence appearing in the plasma was measured over time. The data were expressed as FITC-lysozyme concentrations, although the fluorescence in the plasma does not necessarily reflect intact FITC-lysozyme, as described below. As shown in Fig. 2A, FITC-lysozyme concentration in the plasma increased gradually with time and reached an almost constant level at about 30 min. In some experiments, plasma concentration of FITC-lysozyme was measured until 120 min, but the concentration was almost the same after 30 min (data not shown). The plasma concentration of FITC-lysozyme and the area under the concentration–time curve ($AUC_{0–60}$) after administration into the upper intestine were much higher than after administration into the lower intestine ($AUC_{0–60}$: upper, 4.1 ± 0.6 nmol min/ml; lower, 0.8 ± 0.3 nmol min/ml, $**P< 0.01$).

The plasma samples were analyzed to evaluate the intactness of FITC-lysozyme by a fluoroimage analyzer after SDS-PAGE. In the plasma samples obtained even 120 min after administration into the upper intestine, intact FITC-lysozyme was detected (Fig. 2B). In addition, fluorescence signals from the smaller molecule were observed near the top of the gel, the position where free FITC was detected.

### 3.3. Intestinal absorption of FITC-lysozyme evaluated by in situ recirculation method

The intestinal absorption of FITC-lysozyme was also examined by the in situ recirculation method. The initial concentration of FITC-lysozyme in intestinal perfusate was adjusted to 0.35 μM, a similar concentration to the $K_d$ value of lysozyme for megalin (0.32 μM; Leheste et al., 1999). In these experiments, the absorption rate from the intestine was estimated from the disappearance rate of FITC-lysozyme from the perfusate. The absorption rate of FITC-lysozyme was approximately fivefold higher in the upper intestine (0.98 ± 0.08 nmol/h) than in the lower intestine (0.19 ± 0.19 nmol/h, *$P$< 0.05; Fig. 3A), in good agreement with the results from the loop method. During recirculating perfusion, FITC-lysozyme was found to be stable in the upper (Fig. 3B) and lower (data not shown) intestinal perfusate.

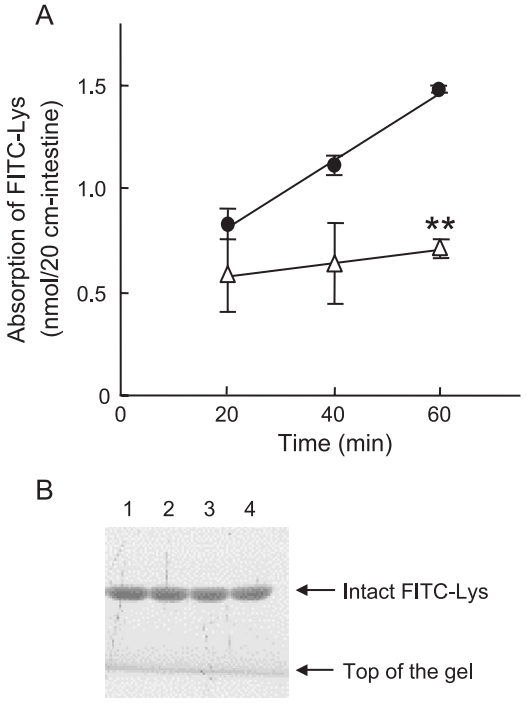


Fig. 3. Absorption of FITC-lysozyme from the upper and lower intestine evaluated by in situ recirculation method (A) and evaluation of intact FITC-lysozyme in the intestinal perfusate (B). (A) The upper or the lower segment of the intestine was perfused with the solution containing FITC-lysozyme (FITC-Lys, 0.35 μM), and the fluorescence in the intestinal perfusate was measured. **$P$< 0.01, significantly different from the upper intestine. (B) The intactness of FITC-Lys in the intestinal perfusate was evaluated by a fluoroimage analyzer after separation by SDS-PAGE. The perfusate after 60 min of intestinal reperfusion was used. Lane 1: intact FITC-Lys (standard); lanes 2–4: three separate perfusate samples.

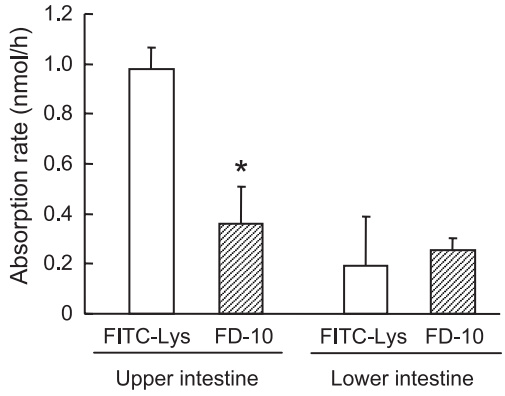


Fig. 4. Absorption rates of FITC-lysozyme and FD-10 in the upper and lower intestine evaluated by in situ recirculation method. The absorption rates of FITC-lysozyme (FITC-Lys, 0.35 μM) and FD-10 (035 μM) were estimated in the same manner as described in Fig. 3. *$P$< 0.05, significantly different from the absorption rate of FITC-Lys in the upper intestine.

### 3.4. Characteristics of intestinal absorption of FITC-lysozyme evaluated by in situ recirculation method

The absorption rate of FITC-lysozyme was compared with that of FD-10, a marker for nonspecific absorption. In the upper intestine, the absorption rate of FITC-lysozyme was about 2.5-fold higher than that of FD-10 (Fig. 4). In the lower intestine however, no significant difference was observed in the absorption rates between these two compounds.

To further characterize the absorption of FITC-lysozyme from the upper intestine, the effects of various concentrations of unlabeled lysozyme were examined. As shown in Fig. 5, the absorption rate of FITC-lysozyme from the upper intestine was inhibited by unlabeled lysozyme in a concentration-dependent manner. The effects of spermine, a

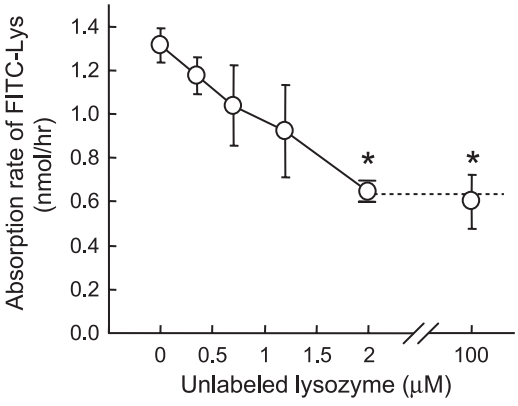


Fig. 5. Effect of unlabeled lysozyme on the absorption rate of FITC-lysozyme from the upper intestine. The absorption rate of FITC-lysozyme (FITC-Lys, 0.35 μM) was evaluated by in situ recirculation method in the absence (control, 0 μM) or presence of various concentrations of unlabeled lysozyme. *$P$<0.05, significantly different from control.

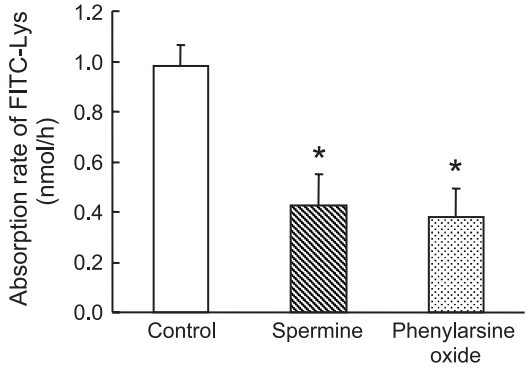


Fig. 6. Effect of spermine and phenylarsine oxide on the absorption rate of FITC-lysozyme in the upper intestine. The absorption rate of FITC-lysozyme (FITC-Lys, 0.35 μM) was evaluated by in situ recirculation method in the absence or presence of spermine (100 μM). To examine the effect of phenylarsine oxide, the upper intestinal lumen was preperfused with D-PBS buffer containing 25 mM glucose and 10 μM phenylarsine oxide for 10 min, and after washing the intestinal lumen with D-PBS buffer containing 25 mM glucose, the absorption rate of FITC-Lys was evaluated in the absence of phenylarsine oxide. *$P$<0.05, significantly different from control.

polycation, and phenylarsine oxide, an inhibitor of clathrin-mediated endocytosis, were also examined. The absorption rate of FITC-lysozyme was significantly inhibited by the presence of spermine in the perfusate and by pretreatment of the intestinal lumen with phenylarsine oxide (Fig. 6).

## 4. Discussion

Lysozyme is a cationic protein (p$I$=11) with a molecular size of approximately 14 kDa. It is administered orally for the treatment of chronic sinusitis and to promote expectoration in patients with respiratory disease. Some studies indicate that lysozyme is absorbed from the intestine in rats as well as in humans, although the extent of absorption is not as high (Hashida et al., 2002; Nishikawa et al., 2002; Yuzuriha et al., 1975). However, little information is available concerning the underlying mechanisms of that intestinal absorption.

To clarify the role of megalin in the intestinal absorption of lysozyme, we examined the expression of megalin mRNA in the upper and lower intestine, and the relationship with lysozyme absorption. The expression of megalin mRNA was observed only in the lower intestine, and the expression level was much lower than that in the renal cortex (Fig. 1). Our results are comparable to those reported by Yammani et al. (2001), in which megalin expression in rat intestine was shown by immunoblotting to be limited to the distal (lower) region. On the other hand, the absorption of FITC-lysozyme from the upper intestine was much higher than from the lower intestine when examined by the in situ closed loop method (Fig. 2A). Using our present and previous data (Nagai et al., 2002), in which FITC-lysozyme was intravenously injected in rats, the bioavailability of FITC-lysozyme (0–60 min) administered into the intestinal loop was estimated, assuming that the fluorescence in the plasma reflected the absorbed FITC-lysozyme. The bioavailability was 1.47±0.20% in the upper intestine and 0.29±0.10% in the lower intestine (*$P$<0.01). Thus, although the extent of absorption from the intestinal loop was fairly low as reported elsewhere (Nishikawa et al., 2002; Yuzuriha et al., 1975), there was a marked segmental difference in the absorption of FITC-lysozyme along the intestine. In addition, using a fluoroimage analyzer after SDS-PAGE, intact FITC-lysozyme, as well as fluorescence signals derived from degradation products, was detected in the plasma samples (Fig. 2B). This result would strongly indicate that FITC-lysozyme is absorbed from the upper intestine as its intact form at least partly. Some previous reports using immunological or biological methods also indicated the intestinal absorption of intact proteins including lysozyme (Castell et al., 1997; Hashida et al., 2002; Nishikawa et al., 2002; Yuzuriha et al., 1975).

The concentration of FITC-lysozyme used in the in situ loop method was 0.7 mM, which was necessary to detect FITC-lysozyme in the plasma. However, the concentration is much higher than the $K_d$ value for lysozyme binding to megalin described above (0.32 μM; Leheste et al., 1999). Under these experimental conditions, megalin-mediated endocytosis would be completely saturated, and the involvement of megalin in the absorption of lysozyme, if any, may not be detectable. Therefore, the intestinal absorption of FITC-lysozyme was also examined at a much lower concentration (0.35 μM), comparable to its $K_d$ value for megalin, using the in situ intestinal recirculation method. However, even at this lower concentration of FITC-lysozyme, the absorption rate was much higher (about fivefold) in the upper intestine than in the lower intestine (Fig. 3A). During the recirculating perfusion, FITC-lysozyme remained intact in the intestinal perfusate (Fig. 3B), indicating that the difference in the absorption rates of FITC-lysozyme between the upper and lower intestine is not due to the degradation of lysozyme. Taken together, these findings indicate that megalin does not play a crucial role in the intestinal absorption of lysozyme, unlike the kidney (Christensen and Birn, 2001; Leheste et al., 1999). Although the reason for this apparent discrepancy between the kidney and intestine in the role of megalin for lysozyme uptake is not clear, the level of megalin in the intestine may be too low to take up substantial amounts of lysozyme from the intestinal lumen.

FD-10 is a similar but somewhat smaller molecule compared with lysozyme and was used here as a nonspecific absorption marker, which may be absorbed by fluid-phase endocytosis and/or via a paracellular pathway. The absorption rate of FITC-lysozyme was significantly higher than that of FD-10 in the upper but not in the lower intestine (Fig. 4). We have previously examined gentamicin uptake by cultured renal epithelial cells LLC-PK1 in comparison with fluid-phase endocytosis markers, horseradish peroxidase,

and lucifer yellow (Takano et al., 1994). We found that the cellular uptake of gentamicin is saturable and higher than those of fluid-phase endocytosis markers, and concluded that gentamicin is taken up by adsorptive endocytosis in LLC-PK1 cells. Similarly, specific mechanism(s), such as adsorptive or receptor-mediated endocytosis (transcytosis), may be involved in the absorption of FITC-lysozyme from the upper intestine. The involvement of saturable and nonsaturable processes in the absorption of FITC-lysozyme from the upper intestine was evident from the inhibition study with unlabeled lysozyme (Fig. 5). Assuming that the saturable transport was completely inhibited by 2 μM of unlabeled lysozyme, the apparent $IC_{50}$ value was found to be about 0.9 μM.

Nishikawa et al. (2002) examined the role of electric charges of protein molecules in their intestinal absorption. They compared the jejunal absorption rates of native, anionized, and cationized lysozyme in rats, and found that the absorption rate was in the order of cationized>native>anionized lysozyme. In this study, the absorption of FITC-lysozyme from the upper intestine was inhibited by spermine, a polycation (Fig. 6). Thus, the cationic charge of lysozyme may be an important determinant for its absorption from the upper intestine. The absorption of FITC-lysozyme from the upper intestine was also inhibited by phenylarsine oxide (Fig. 6). Phenylarsine oxide, a trivalent arsenical that specifically reacts with vicinal sulfhydryls to form stable ring structures, is known to inhibit clathrin-mediated endocytic pathways (Rückert et al., 2003; Visser et al., 2004). Thus, clathrin-mediated endocytosis may be involved in the absorption of FITC-lysozyme from the upper intestine in rats.

In conclusion, this study showed that the endocytic receptor megalin probably does not have a crucial role in the intestinal absorption of lysozyme. In addition, intestinal absorption of lysozyme was segment-selective, and lysozyme was preferentially absorbed from the upper region of the intestine, probably by clathrin-mediated endocytosis. The cationic charge of lysozyme may be important for its absorption from the upper intestine. At present, systemic oral delivery of protein drugs is one of the most challenging goals in the field of intestinal drug delivery, and many investigations have sought to find new and efficient oral drug delivery systems for protein drugs and other macromolecules. Further studies to clarify the molecular mechanisms underlying the intestinal absorption of lysozyme and other protein drugs may provide useful information in developing a new oral drug delivery system for these macromolecules.

## Acknowledgement

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture in Japan.

## References

Aungst, B.J., 1993. Novel formulation strategies for improving oral bioavailability of drugs with poor membrane permeation or presystemic metabolism. J. Pharm. Sci. 82, 979–987.

Castell, J.V., Friedrich, G., Kuhn, C.-S., Poppe, G.E., 1997. Intestinal absorption of undegraded proteins in men: presence of bromelain in plasma after oral intake. Am. J. Physiol. 273, G139–G146.

Christensen, E.I., Birn, H., 2001. Megalin and cubilin: synergistic endocytic receptors in renal proximal tubule. Am. J. Physiol., Renal Physiol. 280, F562–F573.

Christensen, E.I., Birn, H., 2002. Megalin and cubilin: multifunctional endocytic receptors. Nat. Rev., Mol. Cell Biol. 3, 256–266.

Christensen, E.I., Birn, H., Verroust, P., Moestrup, S.K., 1998. Membrane receptors for endocytosis in the renal proximal tubule. Int. Rev. Cytol. 180, 237–284.

Fasano, A., 1998. Novel approaches for oral delivery of macromolecules. J. Pharm. Sci. 87, 1351–1356.

Funakoshi, S., Murakami, T., Yumoto, R., Kiribayashi, Y., Takano, M., 2003. Role of *p*-glycoprotein in pharmacokinetics and drug interactions of digoxin and beta-methyldigoxin in rats. J. Pharm. Sci. 92, 1455–1463.

Hashida, S., Ishikawa, E., Nakamichi, N., Sekino, H., 2002. Concentration of egg white lysozyme in the serum of healthy subjects after oral administration. Clin. Exp. Pharmacol. Physiol. 29, 79–83.

Kerjaschki, D., Farquhar, M.G., 1982. The pathogenic antigen of Heymann nephritis is a membrane glycoprotein of the renal proximal tubule brush border. Proc. Natl. Acad. Sci. U. S. A. 79, 5557–5561.

Leheste, J.-R., Rolinski, B., Vorum, H., Hilpert, J., Nykjaer, A., Jacobsen, C., Aucouturier, P., Moskaug, J.Ø., Otto, A., Christensen, E.I., Willnow, T.E., 1999. Megalin knockout mice as an animal model of low molecular weight proteinuria. Am. J. Pathol. 155, 1361–1370.

Mahato, R.I., Narang, A.S., Thoma, L., Miller, D.D., 2003. Emerging trends in oral delivery of peptide and protein drugs. Crit. Rev. Ther. Drug Carr. Syst. 20, 153–214.

Nagai, J., Takano, M., 2004. Molecular aspects of renal handling of aminoglycosides and strategies for preventing the nephrotoxicity. Drug Metab. Pharmacokineti. 19, 159–170.

Nagai, J., Tanaka, H., Nakanishi, N., Murakami, T., Takano, M., 2001. Role of megalin in the renal handling of aminoglycosides. Am. J. Physiol., Renal Physiol. 281, F337–F344.

Nagai, J., Katsube, T., Murakami, T., Takano, M., 2002. Effect of gentamicin on pharmacokinetics of lysozyme in rat: interaction between megalin substrates in the kidney. J. Pharm. Pharmacol. 54, 1491–1496.

Nishikawa, M., Hasegawa, S., Yamashita, F., Takakura, Y., Hashida, M., 2002. Electrical charge on protein regulates its absorption from the rat small intestine. Am. J. Physiol., Liver Physiol. 282, G711–G719.

Orlando, R.A., Rader, K., Authier, F., Yamazaki, H., Posner, B.I., Bergeron, J.J., Farquhar, M.G., 1998. Megalin is an endocytic receptor for insulin. J. Am. Soc. Nephrol. 9, 1759–1766.

Rückert, P., Bates, S.R., Fisher, A.B., 2003. Role of clathrin- and actin-dependent endocytotic pathways in lung phospholipid uptake. Am. J. Physiol., Lung Cell. Mol. Physiol. 284, L981–L989.

Saito, A., Pietromonaco, S., Loo, A.K., Farquhar, M.G., 1994. Complete cloning and sequencing of rat gp330/megalin, a distinctive member of the low density lipoprotein receptor gene family. Proc. Natl. Acad. Sci. U. S. A. 91, 9725–9729.

Swaan, P.W., 1998. Recent advances in intestinal macromolecular drug delivery via receptor-mediated transport pathways. Pharm. Res. 15, 826–834.

Takano, M., Ohishi, Y., Okuda, M., Yasuhara, M., Hori, R., 1994. Transport of gentamicin and fluid-phase endocytosis markers in the LLC-PK1 kidney epithelial cell line. J. Pharmacol. Exp. Ther. 268, 669–674.

Visser, C.C., Stevanovic, S., Heleen, V.L., Gaillard, P.J., Crommelin, D.J., Danhof, M., De Boer, A.G., 2004. Validation of the transferrin receptor for drug targeting to brain capillary endothelial cells in vitro. J. Drug Target. 12, 145–150.

Woodley, J.F., 1994. Enzymatic barriers for GI peptide and protein delivery. Crit. Rev. Ther. Drug Carr. Syst. 11, 61–95.

Yamamoto, A., 2001. Improvement of transmucosal absorption of biologically active peptide drugs. Yakugaku Zasshi 121, 929–948.

Yammani, R.R., Seetharam, S., Seetharam, B., 2001. Cubilin and megalin expression and their interaction in the rat intestine: effect of thyroidectomy. Am. J. Physiol.: Endocrinol. Metab. 281, E900–E907.

Yumoto, R., Murakami, T., Takano, M., 2003. Differential effect of acute hepatic failure on in vivo and in vitro *p*-glycoprotein functions in the intestine. Pharm. Res. 20, 765–771.

Yuzuriha, T., Katayama, K., Fujita, T., 1975. Studies on biotransformation of lysozyme: I. Preparation of labeled lysozyme and its intestinal absorption. Chem. Pharm. Bull. 23, 1309–1314.

Zheng, G., Bachinsky, D.R., Stamenkovic, I., Strickland, D.K., Brown, D., Andres, G., McCluskey, R.T., 1994. Organ distribution in rats of two members of the low-density lipoprotein receptor gene family, Gp330 and LRP/α2MR, and the receptor-associated protein (RAP). J. Histochem. Cytochem. 42, 531–542.