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Evaluation of protein stability and *in vitro* permeation of lyophilized polysaccharides-based microparticles for intranasal protein delivery

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ABSTRACT

Biocompatible microparticles prepared by lyophilization were developed for intranasal protein delivery. To test for the feasibility of this formulation, stability of the incorporated protein and enhancement of *in vitro* permeation across the nasal epithelium were evaluated. Lyophilization was processed with hydroxypropylmethylcellulose (HPMC) or water soluble chitosan (WCS) as biocompatible polymers, hydroxypropyl-β-cyclodextrin (HP-β-CD) and d-alpha-tocopheryl poly(ethylene glycol 1000) succinate (TPGS 1000) as permeation enhancers, sugars as cryoprotectants and lysozyme as the model protein. As a result, microparticles ranging from 6 to 12 μm were developed where the maintenance of the protein conformation was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), circular dichroism and fluorescence intensity detection. Moreover, *in vitro* bioassay showed that the lysozyme activity was preserved during the preparation process while exhibiting less cytotoxicity in primary human nasal epithelial (HNE) cells. Results of the *in vitro* release study revealed slower release rate in these microparticles compared to that of the lysozyme itself. On the other hand, the *in vitro* permeation study exhibited a 9-fold increase in absorption of lysozyme when prepared in lyophilized microparticles with HPMC, HP-β-CD and TPGS 1000 (F4-2). These microparticles could serve as efficient intranasal delivery systems for therapeutic proteins.

1. Introduction

Intranasal drug delivery can serve as an attractive alternative route for protein and peptide drugs. These drugs are administered mainly by intravenous or subcutaneous injection because they tend to be degraded by enzymes at gastrointestinal tracts and show low bioavailability after oral administration (Illum, 2003). In order to facilitate the administration of these macromolecules and improve patient compliance, non-parenteral routes other than the oral route are in great need. Thus, intranasal drug delivery is one of the most convenient and reliable methods with advantages including relatively large absorptive surface area and high vascularity of the nasal mucosa (Ugwoke et al., 2001). Drugs can be absorbed into the network of blood vessels and be transported directly into the systemic circulation avoiding gastrointestinal and hepatic first-pass metabolism (Huh et al., 2010). In addition, the proteolytic activities in the mucosal membrane are lower in the nasal cavity than in gastrointestinal tracts. However, most proteins cannot be well absorbed from the nasal cavity when administered as aqueous solutions. Major factor limiting the absorption of nasally administered proteins is their poor permeability across nasal membranes due to their high molecular weight and the mucociliary clearance mechanism (12–15 min in normal human), which rapidly removes protein solutions from the absorption site (Illum, 2003). To overcome these disadvantages, two approaches have been mainly utilized: (1) use of permeation enhancers (surfactants, bile salts, cyclodextrins, phospholipids and fatty acids, etc.), which can promote the absorption of poorly absorbable drugs, (2) use of mucoadhesive systems (microparticles, bioadhesive liquid formulations and liquid gelling formulations) that reduces the mucociliary clearance of drug formulation and thereby increases the contact time between the drug and the absorption site (Costantino et al., 2007; Witschi and Mrsny, 1999).

Cyclodextrins are one of the well tolerated permeation enhancers in human nasal application (Merkus et al., 1999). Among the cyclodextrin derivatives, HP-β-CD showed superior tolerability, i.e., long-term nasal application of 20% HP-β-CD had no influence on the integrity of the mucosa in rats (Asai et al., 2002). Moreover, studies involving human cell cultures have shown that HP-β-CD has no significant cilio-inhibitory effect (Agu et al., 2000). On the other hand, TPGS 1000 has recently been described as an effective oral absorption enhancer for improving the bioavailability of poorly absorbed drugs (Collnot et al., 2007). TPGS 1000 was developed in the 1950s as a water-soluble form of vitamin E and is composed of a hydrophilic polar (water-soluble) head and a lipophilic (water-insoluble) alkyl tail group. Due to its surface active

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property, it can be used as a solubilizer, an emulsifier and a vehicle for lipid-based drug delivery formulations.

Among the various bioadhesive materials that have been considered for nasal delivery of protein, chitosan and HPMC were selected as biocompatible polymers in this investigation. Chitosan has been widely investigated as a biomaterial and as a pharmaceutical excipient for drug delivery (Illum et al., 2001; Lehr et al., 1992). Besides its ability to facilitate paracellular transport of peptides and proteins across mucosal barriers, it is also biodegradable and has very low toxicity (Illum et al., 2001; Van der Lubben et al., 2003). On the other hand, HPMC, a non-ionic polymer, exhibits bioadhesivity (Henriksen et al., 1996; Dyvik and Graffner, 1992) and is a generally regarded as safe (GRAS) excipient (Burdock, 2007), making it an attractive option for nasal drug delivery systems.

Recently, mucoadhesive microparticles have received much attention for protein delivery via the nasal route (Kraulanda et al., 2006; Tan et al., 2010). An intranasal protein delivery system that could guarantee a simple preparation process, high product yield, preservation of protein conformation and enhancement of absorption in the nasal cavity has been investigated where HP-β-CD/HPMC/TPGS 1000-based lyophilized microparticle was selected as the final formulation. Herein we report on the preparation process as well as the evaluation of the structural stability and bioactivity of the incorporated protein, permeation profiles across the HNE cell monolayers, and the cytotoxicity of the microparticles.

2. Materials and methods

2.1. Materials

Chicken egg white lysozyme, HPMC (viscosity 80–120 cP, 2% in H_2O at 20 °C), sucrose, maltose, trehalose and TPGS 1000 were purchased from Sigma Co. (St. Louis, MO). HP-β-CD was purchased from TCI Co. (Tokyo, Japan). Water soluble chitosan (WCS) was obtained from JAKWANG Co. Ltd. (Ansung, Korea). Transwell® (0.4 μm pore size, 12 mm diameter, polyester) was obtained from Costar Co. (Cambridge, MA). BEGM bullet kit was purchased from Cambrex Bio Science Inc. (Walkersville, MD) and other cell culture reagents were obtained from Invitrogen Co. (Grand Island, NY).

2.2. Preparation of lyophilized microparticle containing lysozyme

The biocompatible microparticles were prepared by the freeze-drying method. Briefly, 1% (w/w) of HP-β-CD solution was prepared in deionized distilled water (DDW) containing lysozyme (about 14.4 kDa), WCS, HPMC, TPGS 1000 and sugars (Table 1). Then the solution was stirred for 24 h and filtered through a 0.2 μm membrane filter. The filtrate was lyophilized to obtain the microparticles. The lyophilization was performed as follows; the vials containing the filtrate were frozen at −20 °C for 2 h and stored at −80 °C for 24 h. The samples were then lyophilized in a freeze dryer at 40 mbar vacuum for 48 h (OPERON FDU-8612, Korea). The lyophilized samples were then desiccated for 48 h to remove the residual water content.

2.3. Particle characterization and SEM images

The particle size and size distribution were recorded using a NICOMP 370 Submicron Particle Sizer (Particle Sizing Systems, Santa Barbara, CA, USA) for microparticles above 0.7 μm. The mean particle size in this study represents the volume mean diameter of the microparticles, and was obtained from measurements of at least three batches of microparticles unless stated otherwise.

Product yield was calculated by the following equation:

$$\text{Product yield (\%)} = \frac{\text{weight of microparticles after lyophilization}}{\text{total weight of ingredients before lyophilization}} \times 100 \quad (1)$$

The morphological shapes of microparticles were examined by SEM (Hitachi S-4100, Tokyo, Japan). Samples were prepared using sticky tape covered with microparticles onto aluminum stubs and coated with gold particle at 100 mTorr for 5 min. Under 15 kV of accelerating voltage, the images of micrographs were taken.

2.4. Stability tests of lysozyme in microparticles

2.4.1. SDS-PAGE

SDS-PAGE was carried out to evaluate the effect of the microparticle preparation process on protein conformation. It was performed using 15% of running gel and 5% of stacking gel followed by Laemmli's discontinuous method (Laemmli, 1970). Mixed formulation solution of 10 μl in DDW with Laemmli sample buffer and protein marker (Precision Plus Protein™ Standards, Bio-Rad Laboratories, Inc., CA) were loaded into the well. The loaded amount of protein was equal to 10 μg of lysozyme per sample. The gels were run for 1 h at 130 V. After electrophoresis, it was stained with Coomassie R250 staining solution (Bio-Rad Laboratories, Inc., CA) for 30 min and destained overnight.

2.4.2. Circular dichroism analysis

The secondary structures of lysozyme at 0.1 mg/ml in 5 mM phosphate buffer (pH 7.2) was determined by using a spectropolarimeter (JASCO model J-715, Jasco, Tokyo, Japan) with a quartz cell (0.1 cm of path length). This study was performed for both pure lysozyme and the microparticle formulations. Data were acquired at bandwidth of 1.0 nm, response of 4 s and scanning speed of 100 nm/min. Each measurement was repeated at least three times and the average value was plotted. Molar ellipticity ($[\theta]$, deg cm^2 d mol^{-1}) was calculated by the following equation:

$$[\theta] = \frac{\theta \cdot \text{Mp}}{10,000 \cdot n \cdot C \cdot l} \quad (2)$$

where Mp is the molecular weight of lysozyme (14,400 Da), n is the number of amino acid residues of lysozyme (129), C is the concentration of lysozyme solution (0.0001 g/ml) and l is the path length of the cell (0.1 cm). Spectra were presented as the plot of molar ellipticity to wavelength.

Table 1
Compositions of lyophilized microparticles containing lysozyme.

Formulation	Lysozyme (w/w, %)	HP-β-CD (w/w, %)	Biocompatible polymer (w/w, %)	TPGS 1000 (w/w, %)	Sugars (w/w, %)
F1	1	1	WCS 0.2%	–	–
F2	1	1	WCS 0.2%	–	Sucrose 2%
F3	1	1	WCS 0.2%	–	Maltose 2%
F4	1	1	WCS 0.2%	–	Trehalose 2%
F4-1	1	1	HPMC 0.2%	–	Trehalose 2%
F4-2	1	1	HPMC 0.2%	0.5	Trehalose 2%

The content of α-helix (%) was calculated from $[\theta]_{208}$ by the following equation (Greenfield and Fasman, 1969):

$$\alpha\text{-helix}\ \ (\%) = \frac{-[\theta]_{208} - 4000}{33{,}000 - 4000} \times 100 \quad (3)$$

2.4.3. Fluorescence intensity measurement

Fluorescence spectra of protein residues were observed to detect conformational changes in the tertiary structure. Lysozyme solution (0.1 mg/ml) prepared from pure lysozyme or lysozyme-loaded microparticle was used to obtain emission spectra (300–500 nm) at fixed excitation wavelength of 280 nm using the fluorescence spectrometer FP-6500 (JASCO Co., Tokyo, Japan).

2.5. Lysozyme bioassay

To evaluate the biological activity of the lysozyme in the freeze-dried microparticle, the method reported earlier was applied with slight modification (Elkordy et al., 2004). Briefly, *Micrococcus lysodeikticus* (ATCC 4698) was suspended in phosphate buffer (66 mM, pH 6.2) at 0.2 mg/ml concentration. Enzyme solutions (0.2 mg/ml) of unprocessed pure lysozyme and lysozyme-loaded lyophilized microparticles were prepared in the same buffer. Aliquots (20 μl) of each lysozyme solution were mixed with 1.3 ml of bacterial suspension. The decrease in the absorption rate at 450 nm was monitored using a UV–vis spectrophotometer (Lambda 25, PerkinElmer, MA). The decreasing slope was measured in the linear region.

2.6. Cytotoxicity in primary HNE cells

Earlier reported methods for isolation and culture of HNE cells were used (Cho et al., 2011; Lee et al., 2005). Briefly, bronchial epithelial cell growth medium (BEGM) supplemented with insulin (5 μg/ml), triiodothyronine (6.5 μg/ml), epidermal growth factor (0.5 ng/ml human recombinant), epinephrine (0.5 μg/ml), amphotericin-B (50 μg/ml), gentamycin (50 μg/ml), hydrocortisone (0.5 μg/ml), transferrin (10 μg/ml) and retinoic acid (0.1 ng/ml) (all supplied by Cambrex Bio Science Inc., Walkersville, MD) were used for the HNE cell culture. After HNE cells of passage 1 and 2 obtained 70–80% confluency, the cells were detached from cell culture plate and seeded on a 48 well plate at 1.0×10^5 cells density per well. The cell culture media was aspirated after 24 h and 500 μl of fresh culture media containing 0.5 or 1.0 mg of formulations was added into each well and incubated for 1 h at 37 °C in 5% CO_2 atmosphere and 95% relative humidity condition. The HNE cells were then treated with 50 μl of thiazolyl blue tetrazolium bromide solution and the cell viability was measured by typical MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay procedure (Mosmann, 1983). The absorbance was detected at 560 nm wavelength by EMax precision microplate reader (Molecular Devices, CA).

2.7. In vitro release study

The *in vitro* release profile of lysozyme from the microparticles was determined at 37 °C using a shaking bath at 50 rpm. Phosphate buffered saline (PBS, pH 6.4 adjusted with phosphoric acid) was used as release media and Transwell® (0.4 μm pore size, 12 mm diameter, polyester membrane) insert was used for release study. The microparticle formulation equivalent to 2 mg of lysozyme was added to the apical side and 1 ml of release medium was added to the basolateral side. At determined times (15, 30, 45, 60, 90 and 120 min), 1 ml of release medium was collected and equivalent volume of fresh release medium was supplemented. The protein content in the samples was analyzed by BCA™ protein assay kit (Pierce, IL).

2.8. In vitro permeation study in HNE cell monolayers cultured by air–liquid interface (ALI) method

The HNE cells were isolated and cultured for transport study by ALI culture method as reported in our previous studies (Cho et al., 2010, 2011; Lee et al., 2005; Lin et al., 2007). When HNE cells acquired about 70–80% confluency, the cells were detached and seeded on Transwell® at a density of 1.5×10^5 cells/cm^2. The apical (0.5 ml) and basolateral side (1.5 ml) of Transwell® were filled with BEGM:DME/F12 (50:50) supplemented with BEGM bullet kit (Cambrex Bio Science Inc., Walkersville, MD). Cell culture media were replaced with fresh media after 1 day and the cell culture media in the basolateral side were changed every other day. The incubator was maintained at 37 °C in 5% CO_2 atmosphere and 95% relative humidity. The *in vitro* permeation study was performed on the cell monolayers cultured at least 10 days by ALI method. Transwell® inserts with transepithelial electrical resistance (TEER) value higher than 500 Ω cm^2 were selected for the transport study. TEER value was measured by EVOM voltohmmeter device (WPI, Sarasota, FL). The amount of microparticles (lysozyme, F4, F4-1 and F4-2) equivalent to 2 mg of lysozyme was loaded in the apical side to evaluate the permeation of lysozyme from apical to basolateral direction. After 2 h of incubation in the shaking bath (37 °C, 50 rpm), samples (1 ml) were withdrawn from the basolateral compartment and analyzed by high performance liquid chromatography (HPLC).

2.9. Quantitative analysis of lysozyme and statistical analysis of data

The quantitative analysis of lysozyme was performed by HPLC using gradient method. The Waters HPLC system was equipped with a pump (Waters 515 HPLC pump, Milford, MA), an automatic injector (Waters 717plus autosampler, milford, MA) and UV detector (Waters 2487, Milford, MA). The separation was performed in a C-18 column (Jupiter, 250 mm × 4.6 mm, 5 μm, 300 Å, Phenomenex, CA). The mobile phases were (A) 0.1% (v/v) trifluoroacetic acid and (B) acetonitrile. The gradient condition was as follows: 0 min, 40% A, 60% B; 10 min, 80% A, 20% B. The eluent was monitored at 280 nm at a flow rate of 1.0 ml/min and the injection volume was 20 μl. Precision and accuracy values were within acceptable range (<10%).

Statistical analysis was performed using analysis of variance (ANOVA). All experiments were performed at least three times and the data were represented as mean ± standard deviation (SD).

3. Results and discussion

3.1. Development and characterization of the microparticles

The microparticles were prepared by lyophilization because of its easiness in scale up and high product yield over the spray drying method. However, as proteins are very labile molecules against stress factors, the stability of the loaded proteins during the lyophilization process should be carefully monitored. In this study, 2% (w/w) of sugars were used as cryoprotectants for the protein molecules. WCS or HPMC (0.2%, w/w) was used as biocompatible polymers, and HP-β-CD and TPGS 1000 were used as permeation enhancer. Lysozyme, as a model protein used in this study, is a 14.4 kDa protein composed of 129 amino acids and abundant in tear, saliva and mucus. As each protein has different physicochemical properties, such as molecular weight, the number of amino acid, solubility, isoelectric point (pI) and stereostructure,

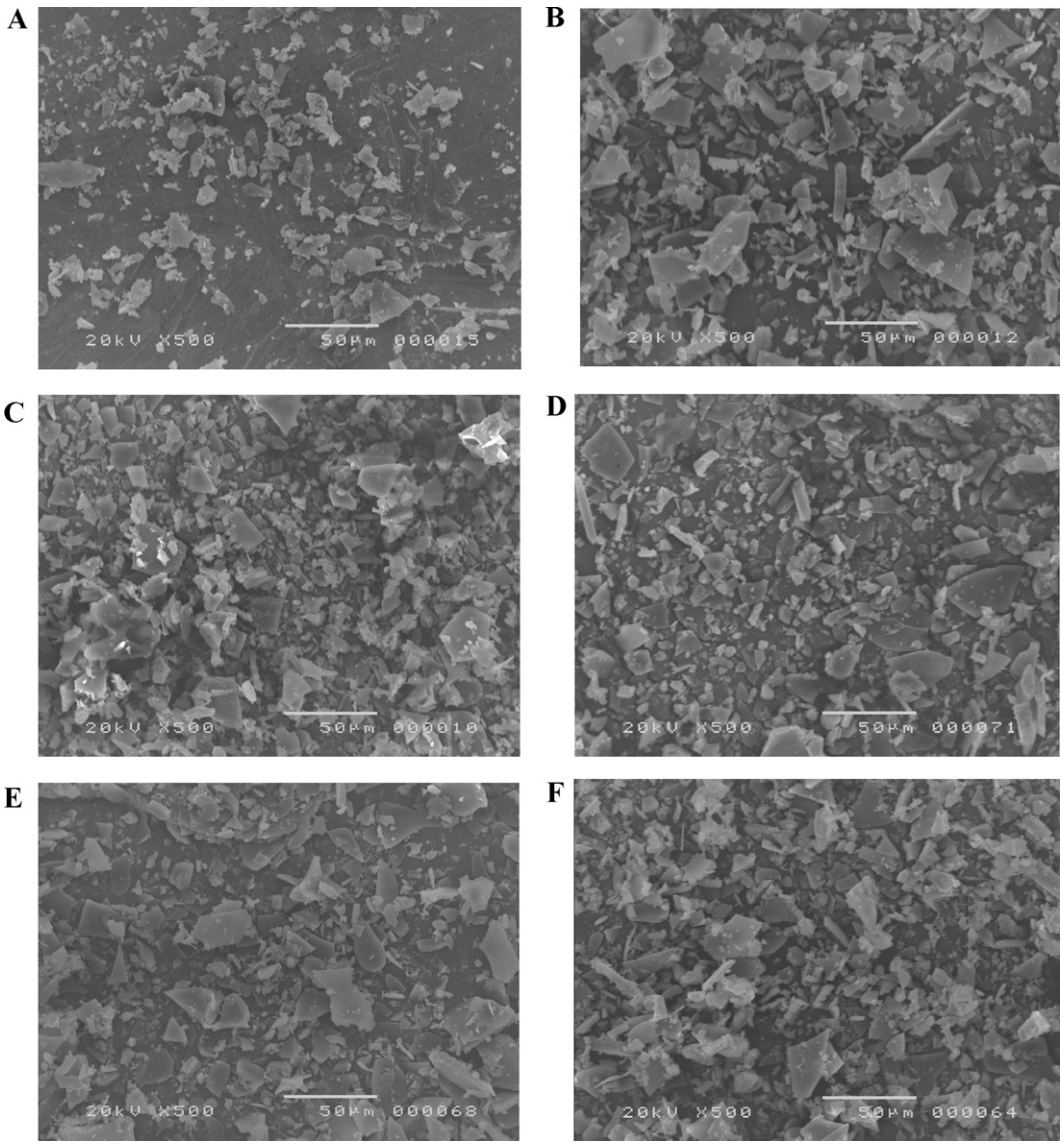

Fig. 1. SEM images of lyophilized microparticles (A) F1, (B) F2, (C) F3, (D) F4, (E) F4-1 and (F) F4-2.

the results about structural and biological stability of protein might be dependent on the kind of protein therapeutics. The formulation was optimized upon trials of various compositions of ingredients in the formulations (Table 1). Since the stability of the protein during lyophilization was a major concern (Arakawa et al., 1993), it was considered as a crucial formulation parameter during the development of microparticles. Table 2 shows the characteristics of the lysozyme-loaded lyophilized microparticles where the product yields were high (80–90%) for all formulations and the particle size slightly increased as the number of ingredients increased. SEM images (Fig. 1) revealed that the developed microparticles were in irregular shape.

Table 2
Characterization of lyophilized microparticles.

Formulations	Product yield (%)[a]	Mean diameter (mean ± SD, μm)
F1	78.32	7.47 ± 0.77
F2	89.71	9.48 ± 0.61
F3	93.74	10.44 ± 0.54
F4	86.86	11.40 ± 1.32
F4-1	89.19	12.53 ± 1.83
F4-2	89.68	12.78 ± 1.37

[a] Product yield (%) was calculated by Eq. (1).

3.2. Stability tests of lysozyme in lyophilized microparticles

The conformational stability of lysozyme in the microparticles is of great importance from the pharmaceutical point of view. In this study, primary, secondary and tertiary structures of lysozyme included in the lyophilized microparticles were investigated by SDS-PAGE, circular dichroism and fluorescence intensity measurement, respectively. SDS-PAGE analysis was used to verify the change of amino acid sequence of the lysozyme loaded in the microparticle (Fig. 2). Lysozyme in formulations was compared with the protein standard and untreated lysozyme. No significant difference was observed in the lanes with various lyophilized microparticle solutions (lanes D–I) when compared with that of the pure lysozyme (lane C). The formation of dimer or multimer as well as degradation of the lysozyme were not observed in lanes loaded with lyophilized microparticle solutions, indicating that the stability of the primary structure of lysozyme was maintained during the lyophilization process.

The secondary structure of lysozyme was analyzed by circular dichroism. It was observed that the secondary structure of lysozyme entrapped in lyophilized microparticles was not significantly different from that of the pure lysozyme as illustrated in

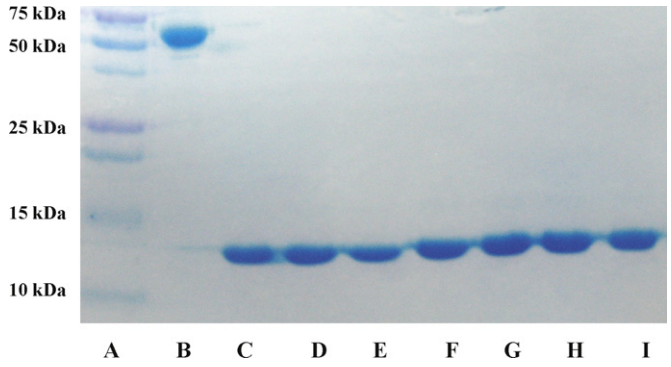

Fig. 2. SDS-PAGE images of (A) protein standard marker, (B) bovine serum albumin (M.W.: 66.4 kDa), (C) Lysozyme (MW: 14.4 kDa), (D) F1, (E) F2, (F) F3, (G) F4, (H) F4-1 and (I) F4-2.

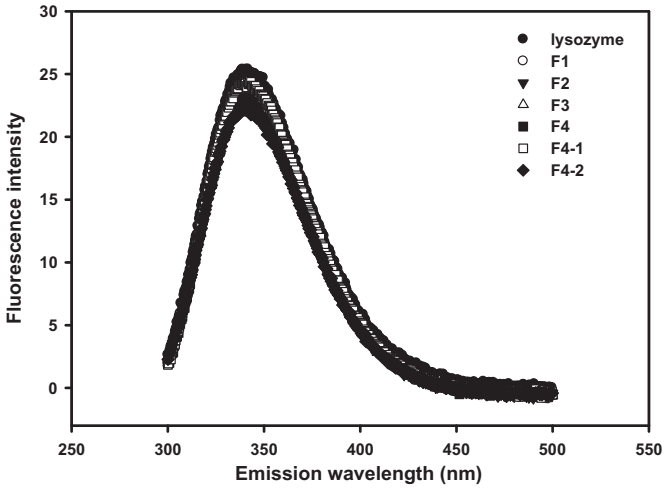

Fig. 4. Fluorescence intensity measurement of pure lysozyme was compared with that in microparticles. The emission spectrum (300–500 nm) at fixed excitation wavelength of 280 nm is shown. Each point is the mean value of three repeated experimental data.

the circular dichroism spectra (200–260 nm) in Fig. 3. In the far UV spectra (Fig. 3), minima were shown at 208 nm and the two minimum peak at 208 and 222 nm indicated the contribution of $n \rightarrow \pi^*$ transfer for the peptide bond of α-helix (Price, 2000). The slightly negative peak around 217 nm indicated the property of β-sheet structure of lysozyme (Lewis et al., 2010). Though several equations and programs have been reported to calculate the contents of helix (Deléage and Geourjon, 1993; Greenfield and Fasman, 1969; Moffitt and Yang, 1956; Perczel et al., 1992; Sreerama and Woody, 2000), Eq. (3) was used in this study as it is one of the representative methods (Greenfield and Fasman, 1969). The calculated contents of α-helix were 28.88, 31.90, 32.25, 24.34, 25.32, 27.94 and 26.37% for the pure lysozyme, F1, F2, F3, F4, F4-1 and F4-2, respectively. These results confirmed that the secondary structure of lysozyme in microparticle was conserved during the lyophilization process. Especially in F4-1 and F4-2, the α-helix contents of these formulations were as close as possible to the pure lysozyme (Chen et al., 1972; Witoonsaridslip et al., 2010).

The integrity of the tertiary structure of the lysozyme in microparticles was analyzed by fluorescence intensity detection (Fig. 4). The fluorescence emission spectrum of lysozyme at excitation wavelength 295 nm is dominated by tryptophan fluorescence and shows maximal emission at 339 nm which is in good accordance with earlier studies (Teichberg and Sharon, 1970; Imoto et al., 1972). Moreover, it was reported that the emission spectrum in the range of 330–345 nm represents tryptophan residue (Zemser et al., 1994) and approximately 80% of fluorescence intensity from

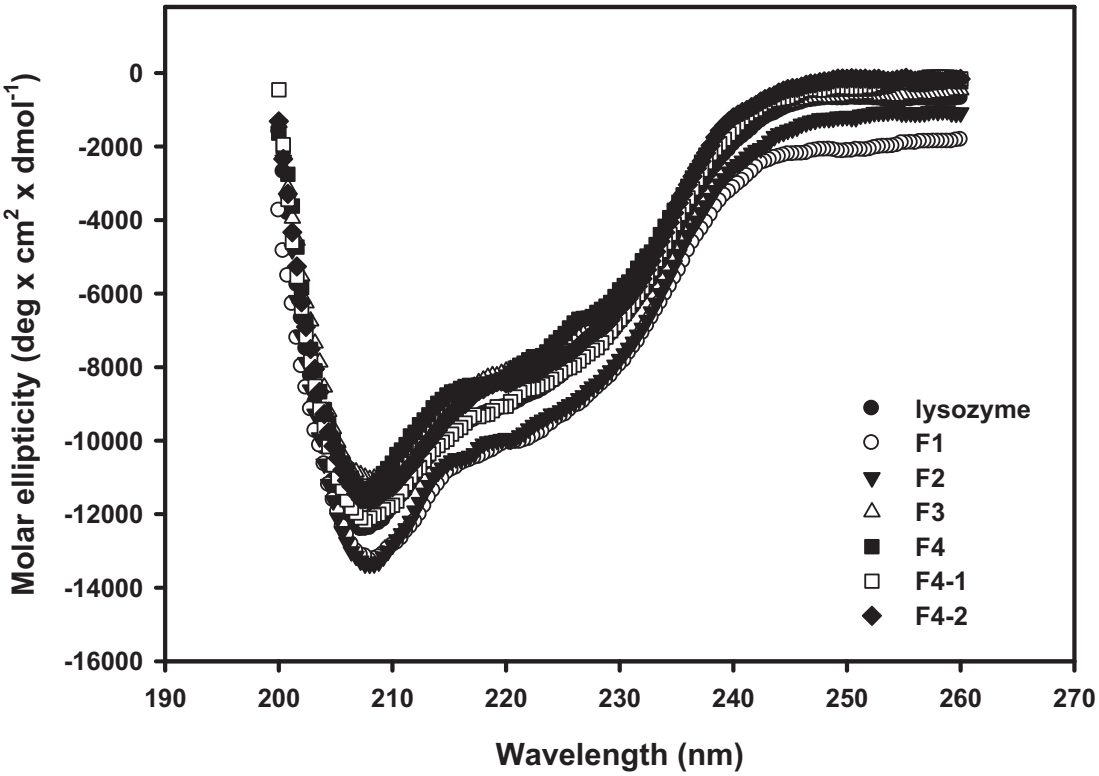

Fig. 3. The conformational change of lysozyme included in microparticles measured by circular dichroism. Molar ellipticity values of lysozyme are ploted and each point represents the mean value of three repeated experiments.

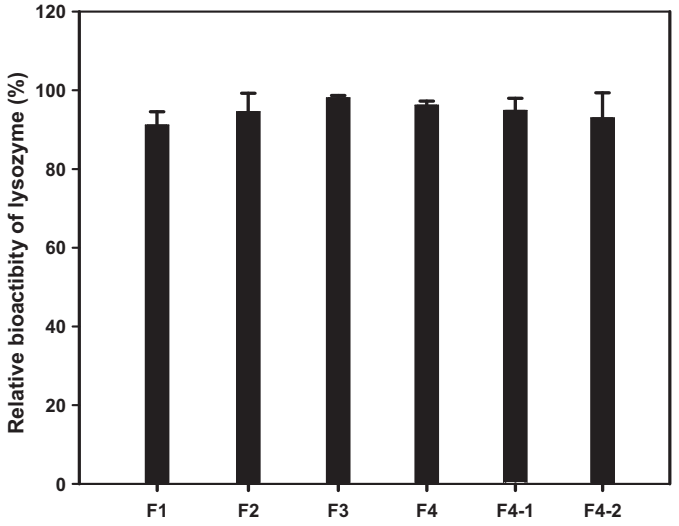

Fig. 5. Relative bioactivity of lysozyme after lyophilization process determined with *M. lysodeikticus* (ATCC 4698) cell suspension. Relative bioactivity presents the ratio of each value to that of pure lysozyme ($n = 3$).

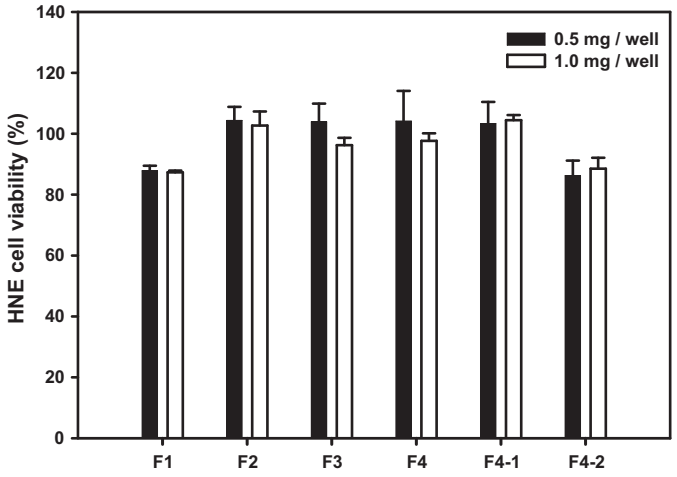

Fig. 6. Microparticle amount-dependent (0.5 and 1.0 mg per well) cell viability in HNE cells measured by MTT assay ($n = 3$).

lysozyme was produced by tryptophan residues 62 and 108 (Imoto et al., 1972). It was noted that the fluorescence spectra of lysozyme in the microparticles showed no significant difference from that of the pure lysozyme (Fig. 4).

Over all, the lysozyme in microparticles seemed to be well preserved during the lyophilization process. This might be due to the presence of cryoprotectants (HP-β-CD and sugars) in the microparticles. HP-β-CD has also been reported for stabilizing protein molecules in the pharmaceutical formulations (Brewster et al., 1991). The stabilizing effect of HP-β-CD is due to the fact that the aromatic phenylalanine, tyrosine and tryptophan residues of proteins could fit in the hydrophobic internal cavity of CD (Haeberlin et al., 1996). Moreover, it was also reported that sugars with HP-β-CD provide better stability in protein formulations than HP-β-CD alone (Iwai et al., 2007). In this study, the disaccharides (sucrose, maltose and trehalose) were used, which have been reported to be effective and common stabilizers in protein formulation (Arakawa et al., 1993).

3.3. Bioassay of lysozyme

To make sure that the preparation process of microparticle did not affect the bioactivity of protein, an *in vitro* bioassay of lysozyme was performed. Fig. 5 presents the relative bioactivity of lysozyme from the lyophilized microparticles. The lysozyme in lyophilized microparticles showed similar bioactivity with that of the pure lysozyme, which indicated that the biological property of lysozyme was preserved well in the microparticles during the lyophilization process. Results were coincided with the results of structural stability tests as shown in Section 3.2.

3.4. In vitro cytotoxicity in HNE cells

The cytotoxicity of the lysozyme-loaded microparticles on primary HNE cells was evaluated by assessing cell viability using the MTT assay (Fig. 6). The cell viability of HNE cells for the pure lysozyme and microparticle formulations (0.5 or 1 mg per well) showed no obvious difference (Fig. 6), indicating the possibility of low cytotoxicity. Further *in vivo* study is under way to elucidate the effect on the nasal epithelial barrier to establish safety of the formulation for clinical application.

3.5. In vitro release study

The release profiles of the microparticle formulations (Table 1) are shown in Fig. 7. It was reported that the pH value of nasal secretion ranged form 5.5 to 6.5 in adults, which might slightly vary according to the region of nasal cavity, the age of subjects and the physiological conditions (Behl et al., 1998). In this investigation, pH 6.4, a representative pH value, was selected for the *in vitro* release study as we previously reported (Cho et al., 2010, 2011; Huh et al., 2010). Since the spherical mean diameter of lysozyme is 3.2 nm based on its partial specific volume (Kisler et al., 2001), Transwell® insert with 0.4 μm pore size (12 mm diameter, polyester membrane) is considered to be enough for the penetration of lysozyme through pores in the polymer membrane. As shown in Fig. 7, untreated lysozyme was released much faster than the prepared microparticles, and the release profile was in the order of lysozyme > F4-1 > F4-2 > F1, F2, F3 and F4. It has been reported that the presence of other excipients, including polymers, influence the interaction between drug substance and HP-β-CD. The polymers such as water-soluble cellulose derivatives and chitosan can interact with drug-HP-β-CD complex and different drug release pattern can be observed compared to that of the HP-β-CD

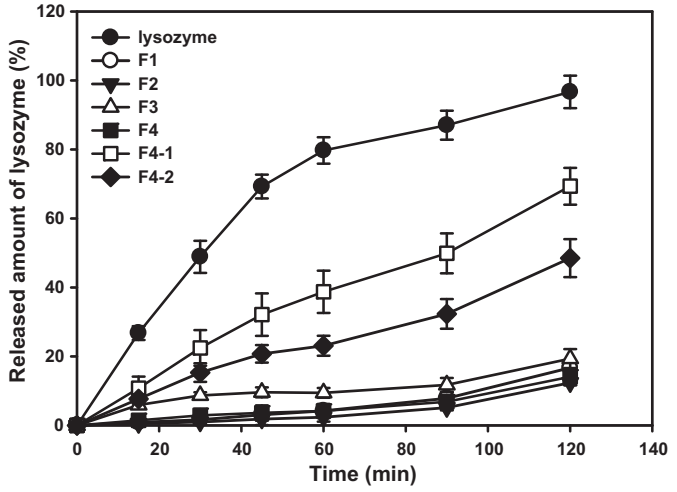

Fig. 7. *In vitro* release profiles of lysozyme from lyophilized microparticles. Corresponding weight of each microparticle to 2 mg of lysozyme was loaded into the apical side. Each point represents mean ± SD ($n = 3$).

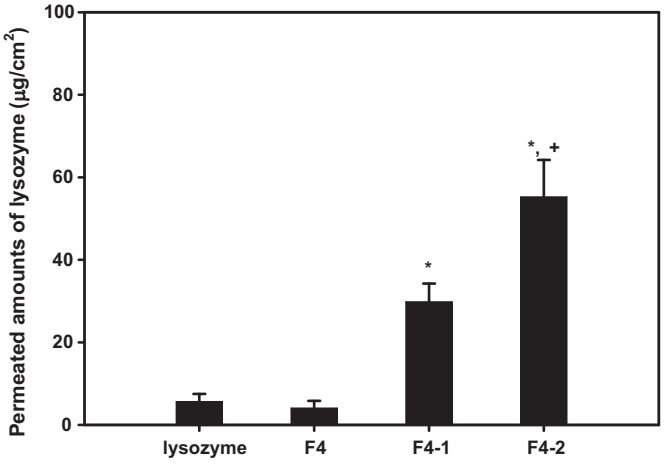

Fig. 8. *In vitro* permeation study of lyophilized microparticles on the HNE cell monolayers cultured by ALI method. Corresponding weight of each microparticle to 2 mg of lysozyme was added in the apical side of the Transwell® insert. Each point represents mean ± SD ($n = 3$). $^{*}p < 0.05$ compared to pure lysozyme, $^{+}p < 0.05$ compared to F4-1.

alone (Carrier et al., 2007; Mura et al., 2010). The type of sugars used in the formulation did not play any role on the release profile of lysozyme. Microparticles containing HPMC showed higher release profile compared to chitosan-contained formulations. The slow release profile observed with F4-2 compared to F4-1 might be due to the presence of TPGS 1000 although its mechanism was not clarified. It was observed that the release profile of lysozyme could be controlled according to the composition of the formulation. In particular, the initial burst release presented by the pure lysozyme release profile may result in the complete elimination of released protein by mucociliary clearance in the nasal cavity. It is expected that the controlled release of lysozyme could influence on the absorption profile across the nasal mucosa and further on the *in vivo* pharmacokinetics.

3.6. *In vitro* transport study

In order to evaluate whether the prepared lyophilized microparticles could provide satisfactory absorption of the lysozyme across the epithelial cellular membranes, their *in vitro* permeation in HNE cell monolayers was evaluated. This is important because the limitation of intranasal protein delivery is normally the inadequate absorption of proteins. The HNE cell monolayers were cultured by ALI method for 10–14 days and the TEER values of used Transwell® inserts covered with HNE cell monolayers were $693.08 \pm 174.23\ \Omega\ cm^2$. In our previous studies, HNE cell monolayers cultured by ALI method have been successfully used for the permeation study of drug from microspheres and microparticles (Cho et al., 2010; Huh et al., 2010). F4-1 and F4-2 were selected for further study as they exhibited better release profile than other formulations, and lysozyme and F4 were selected as control groups. Apart from the improvement of dissolution, these formulations were selected as they contained trehalose which was known as a preferable lyoprotectant for biomacromolecules among disaccharides due to its high glass transition temperature (Crowe et al., 1996). The permeation study was conducted for 2 h and the amount of permeated lysozyme across HNE cell monolayer is presented in Fig. 8. It was noted that F4-2 showed significantly higher permeability than those of lysozyme itself ($p < 0.05$) and F4-1 ($p < 0.05$). As shown in Fig. 8, the permeated amounts from F4-1 and F4-2 were 5.27 and 9.76 times higher than that of lysozyme itself ($p < 0.05$). The low permeation observed with F4 might be due to its low and incomplete drug release profile. The higher permeation rates of F4-1 and F4-2 could be explained by the hydrophilic property of HPMC which is known to synergistically enhance permeation with cyclodextrin (Marttin et al., 1998). Although the absorption enhancing mechanism of cyclodextrin on the macromolecules has not been clearly proven, its absorption enhancing potential across the mucosal membrane was reported previously (Soares et al., 2007). Permeation enhancing effect of TPGS 1000, which is contained in F4-2, has already been reported in the delivery of small chemicals (Sethia and Squillante, 2004). It has been reported that over 1.65 mM of TPGS 1000 in nasal formulation would lead to a progressive fluidization of the cell membrane (Collnot et al., 2007). It implies that amphophilic TPGS 1000 can be used as a permeation enhancer in macromolecule delivery across mucosal membrane. Based on the reported individual absorption enhancing effect of cyclodextrin and TPGS 1000, the permeation enhancing effect in this investigation may be related to paracellular transport. It was reported that TPGS 1000 induced the reduction of TEER value in MDCK cell monolayer (Xie et al., 2010) and HP-β-CD also reduced the TEER value in Caco-2 cell monolayers (Shah et al., 2004; Takahashi et al., 2002). The reduction of the TEER value implies tight junction opening which is followed by paracellular transport. Although the identification of the exact permeation enhancing mechanism in the nasal epithelial cell monolayers should be further investigated, the present data indicates that formulation F4-2 is the strongest potential carrier for the nasal delivery of protein therapeutics.

4. Conclusion

Lyophilization technique was used successfully to prepare polysaccharides-based microparticles containing the model protein lysozyme. Superior physicochemical and high drug loading capacity were observed as a promising nasal delivery system. The prepared microparticles were able to preserve the structural stability and biological activity of the loaded protein during the lyophilization process. Moreover, the *in vitro* permeation study in HNE cell monolayers of the optimized microparticle formulations showed that the microparticles significantly enhanced the permeation of the loaded protein compared to that of pure lysozyme. Overall, results of this study indicate that the prepared microparticulate system could serve as a useful and potential protein carrier for intranasal delivery.

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