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Improved paracellular uptake by the combination of different types of permeation enhancers

Davide Guggi[a], Andreas Bernkop-Schnürch[b,*]

[a] Institute of Pharmaceutical Technology, Center of Pharmacy, University of Vienna, Althanstr 14, 1090 Vienna, Austria, Europe
[b] Department of Pharmaceutical Technology and Biopharmaceutics, Institute of Pharmacy, Leopold-Franzens-University Innsbruck, Innrain 52, Josef Möller Haus, 6020 Innsbruck, Austria, Europe

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Abstract

This study had the purpose to improve the paracellular uptake of drugs by combining the thiomer/reduced glutathione (GSH) permeation-enhancing system with a proteolytic enzyme. Due to the covalent binding of 2-iminothiolane to chitosan the thiomer chitosan-TBA (chitosan-4-thiobutylamidine) was obtained. Permeation studies were performed with freshly excised intestinal mucosa of guinea pigs mounted in Ussing-type chambers using on the one hand the low-molecular size marker flurescein (Na-Flu) and on the other hand the high-molecular size marker FITC-dextran. Apparent permeability coefficient (P_{app}) as well as enhancement ratios (=P_{app} permeation-enhancing system/P_{app} control) were calculated.

Trypsin, papain and bromelain displayed a permeation-enhancing effect for Na-Flu on the small intestinal mucosa. Enhancement ratios of 1.84, 1.63 and 1.78 were identified for 2% trypsin, 0.5% papain and 2% bromelain solutions, respectively. However, only bromelain could guarantee a significant permeation enhancement of FITC-dextran with a P_{app} of $4.45 \pm 0.44 \times 10^{-6}$ cm/s representing an enhancement ratio of 1.57. A similar enhancement of FITC-dextran permeation was reached by the use of the chitosan-TBA (0.5%)/GSH (5%) system. Moreover, an additive permeation-enhancing effect of the chitosan-TBA/GSH system in combination with bromelain (2%) was observed, leading to a maximum P_{app} of $5.91 \pm 0.51 \times 10^{-6}$ cm/s, which corresponds to an enhancement ratio of 2.1.

According to these results, the combination of the thiomer/GSH system with bromelain might represent a new promising strategy in order to raise the in vivo efficacy of non-invasive administered hydrophilic macromolecular drugs.
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1. Introduction

Among the non-invasive routes of application the oral route is the most preferred, convenient and acceptable one for the majority of patients. However, the

* Corresponding author. Tel.: +43 512 507 5371; fax: +43 512 507 2933.
E-mail address: andreas.bernkop@uibk.ac.at (A. Bernkop-Schnürch).

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oral bioavailability of many hydrophilic drugs is often strongly limited by insufficient paracellular absorption from the GI tract. Attempts to reduce the absorption barrier are mainly based on the use of permeation enhancer as auxiliary agents in oral drug delivery systems (Lee, 1990; Aungst et al., 1996). Due to their low-molecular size, however, many permeation enhancers such as sodium salicylate and medium-chain glycerides are absorbed across the gut more rapidly than the drug itself (Aungst, 2000) causing systemic toxicity (Aungst et al., 1996).

In the last decade, proteolytic enzymes such as trypsin, chymotrypsin, papain and bromelain have gained interest as permeation enhancers, as their physiological function in the gut as well as a poor oral bioavailability of about 1% should cause neither local nor systemic toxicity (Kolac et al., 1996). These proteases have been reported to increase the transport of paracellular markers through Caco-2-cell monolayers (Bock et al., 1998; Kolac et al., 1996). The likely mechanism seems to be based on a loosening of the tightness of intercellular junctions, opening the paracellular route across the epithelium for normally non-absorbable compounds (Anderberg et al., 1992; Bock et al., 1998; Tomita et al., 1995).

On the other hand, an alternative class of permeation enhancer that has received a lot of attention are high-molecular mass polymers such as polyacrylates or chitosans (Borchard et al., 1996). These polymers combine permeation-enhancing properties with mucoadhesive features and are not absorbed from the GI-tract due to their high-molecular mass. In addition, further improvements of the permeation-enhancing features of established polymers could be achieved by the covalent fixation of sulhydryl groups (Clausen and Bernkop-Schnürch, 2000), thus leading to thiolated polymers, or so-called thiomers. Thiolated polymers were shown to display a high potential as drug carrier matrices for oral administration of hydrophilic drugs (Caliceti et al., 2003; Guggi et al., 2003a; Marschütz et al., 2000). Moreover, several ex vivo studies demonstrated that glutathione (GSH) mediates and improves the permeation-enhancing effect of thiolated polymers (Bernkop-Schnürch et al., 2004; Clausen et al., 2002), thus leading to an even higher in vivo efficacy of oral delivery systems based on thiomers (Guggi et al., 2003a,b; Kast et al., 2003).

It was the aim of this study to evaluate the possibility of improving the paracellular uptake by the combination of a proteolytic enzyme with the thiomer/GSH system, representing two types of permeation enhancers, which are believed to act in different ways. Permeation studies were performed with Ussing-type chambers using freshly excised small intestinal mucosa of guinea pigs. On the one hand, the effect of trypsin, papain and bromelain, possessing the strongest permeation-enhancing features among proteolytic enzymes (Bock et al., 1998; Kolac et al., 1996), should be evaluated using the low-molecular size (MM 376 Da) marker sodium fluorescein (Na-Flu) as well as the high-molecular size marker FITC-dextran (MM 4300 Da). On the other hand, the permeation-enhancing effect of the thiolated polymer chitosan-TBA (chitosan-4-**t**hio**b**utyl**a**midine) (Bernkop-Schnürch et al., 2003a, 2004; Guggi et al., 2003a,b; Roldo et al., 2003) in combination with GSH should be investigated using the larger marker FITC-dextran. Thereafter, the combined effect of the chitosan-TBA/GSH system with the most promising protease should be evaluated.

2. Materials and methods

2.1. Materials

Trypsin (EC 3.4.21.4) (9.82 units/mg protein, Sigma, St. Louis, MO), papain (EC 3.4.22.2) (14 units/mg protein), bromelain (EC 3.4.22.32) (5.1 units/mg protein), Ellman's reagent (5,5′-dithiobis(2-nitrobenzoic acid)), L-cysteine hydrochloride anhydrous, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES), sodium fluorescein (Na-Flu), fluorescein isothiocyanate-dextran (FITC-dextran) and glutathione reduced form (GSH) were all purchased from Sigma, St. Louis, Missouri. Chitosan (medium molecular mass: 400 kDa) was obtained from Fluka Chemie, Buchs, Switzerland. 2-Iminothiolane HCl (Traut's reagent) was purchased from Pierce, Oud Beijerland, Netherlands.

2.2. Synthesis and purification of the chitosan-TBA

The chitosan-TBA conjugate was synthesized according to a method described previously (Bernkop-

Schnürch et al., 2003a). In brief, 500 mg of chitosan were dissolved in 50 ml of 1% acetic acid. The pH was adjusted to 6 with 5 M NaOH and 200 mg of 2-iminothiolane HCl was added. After 24 h of incubation at room temperature under continuous stirring, the resulting chitosan-4-thio-butylamidine conjugate (chitosan-TBA) was dialyzed against 5 mM HCl, two times against 5 mM HCl containing 1% NaCl, against 5 mM HCl and finally against 0.4 mM HCl. A sample prepared in the same way but omitting the addition of 2-iminothiolane HCl served as control. Polymers were adjusted to pH 7, then freeze-dried at −30 °C and 0.01 mbar (Christ Beta 1–8 K, Germany) and stored at 4 °C until further use.

2.3. Determination of the degree of modification of the chitosan-TBA

The total amount of sulfhydryl groups fixed on the polymer is represented by the summation of reduced thiol groups and of oxidised thiol moieties available in form of disulfide bonds.

Ellman's reagent was used to quantify photometrically the amount of free sulfhydryl groups on modified chitosan as described previously (Hornof et al., 2003).

To determine the total amount of bound thiol functions 0.5 mg of the conjugate were swelled in 1 ml of 0.05 M tris(hydroxymethyl)–aminomethane buffer pH 6.8. After 30 min, 1 ml of a freshly prepared 4% sodium-borohydride solution was added to the polymer suspension. The mixture was then incubated for 1 h in an oscillating waterbath at 37 ± 0.5 °C. Thereafter 200 μl of 5 M HCl were added and the mixture was agitated for 10 min in order to destroy the remaining sodium-borohydride. The mixture was neutralized by the addition of 1 ml of 1 M phosphate buffer pH 8.5 and then immediately 100 μl of 0.4% (m/v) Ellman's reagent dissolved in 0.5 M phosphate buffer pH 8.0 was added. After incubation for 15 min at room temperature aliquots of 200 μl were transferred to a 96-well microtitration plate and the absorbance was measured at 450 nm with a microtitration-plate reader (Anthos Reader 2001, Salzburg, Austria). The quantity of bound iminothiolane was calculated using a standard curve obtained by the sulfhydryl group determination of a series of solutions containing increasing concentrations of cysteine hydrochloride (Sigma–Aldrich, Steinheim, Germany). The amount of disulfide bonds was calculated by subtracting the quantity of free thiol groups from the totality of thiol moieties present on the polymer.

2.4. Permeation studies

According to previous studies Ussing-type chambers with a volume of 1 ml (=1 cm^3) in the donor and acceptor chamber and a permeation area of 0.64 cm^2 were used to carry out permeation studies with Na-Flu and FITC-dextran (Clausen and Bernkop-Schnürch, 2001). The pH of the freshly prepared incubation medium containing 250 mM NaCl, 2.6 mM $MgSO_4$, 10 mM KCl, 40 mM glucose and 50 mM $NaHCO_3$ buffered with 40 mM HEPES was adjusted to 6.8. Immediately after sacrificing the animal the first 15 cm of the small intestine (duodenum) of the guinea pig was excised and mounted in the Ussing-type chamber, without stripping off the underlying muscle layer. All experiments were performed four times in an atmosphere of 95% O_2 and 5% CO_2 at 37 °C. After 15–20 min of preincubation the solution in the donor chamber was substituted by the same medium but containing the proteolytic enzymes trypsin, papain or bromelain in different concentrations. Sodium fluorescein 0.001% (w/v) was additionally added as paracellular marker. Further studies were carried out by substituting with the proteolytic enzymes trypsin, papain or bromelain in incubation medium containing 0.1% FITC-dextran. The effect of 0.5% chitosan-TBA conjugate in combination with 5% GSH as well as the effect of 0.5% chitosan-TBA conjugate in combination with 5% GSH and with 2% bromelain in a 0.1% FITC-dextran incubation medium was also tested. Control studies were carried out with Na-Flu or FITC-dextran in incubation medium. Over a 3 h incubation period 100 μl samples were taken from the acceptor chamber every 30 min and the volume was replaced by incubation medium preequilibrated at 37 °C. The permeation of Na-Flu or FITC-dextran was evaluated by measuring the amount of permeated test compound in the acceptor chamber using a fluorimeter (SLT, Spectra Fluor, Tecan, Austria). Cumulative corrections were made for the previously removed samples in determining the total amount permeated.

2.5. Measurement of the transepithelial electrical resistance (TEER)

A Millicell® ERS meter (Millipore Corp., Bedford, MA) connected to a pair of side-by-side electrodes was used to monitor the effect on the TEER of the intestinal mucosa. Measurements were performed before applying the proteolytic enzymes and then every 30 min within 3 h. Thereafter, the solutions in the acceptor- and donor-chamber were substituted by incubation medium preequilibrated at 37 °C in order to investigate the reversibility of the effect of the proteolytic enzymes on the TEER. Measurements were then performed after 40 min as well as after 70 min.

2.6. Data analyses

Apparent permeability coefficients (P_{app}) for Na-Flu and FITC-dextran were calculated according to the following equation:

$$P_{app} = \frac{Q}{Act} \tag{1}$$

where P_{app} is the apparent permeability coefficient (cm/s), Q the total amount permeated throughout the incubation time (μg), A the diffusion area of the Ussing-type chamber (cm^2), c the initial concentration of the marker in the donor compartment (μg/cm^3), and t the total time of the experiments.

Transport enhancement ratios (R) were calculated from P_{app} values by:

$$R = \frac{P_{app}(\text{sample})}{P_{app}(\text{control})} \tag{2}$$

Statistical data analyses were performed using the students t-test with $P < 0.05$ as the minimal level of significance. Calculations were done using the software Xlstat version 5.0 (b8.3).

3. Results

3.1. Characterisation of the chitosan-TBA

The immobilization of thiol groups on chitosan was achieved by the use of Traut's reagent. The obtained polymer was slightly yellowish, odourless and showed a fibrous structure. A full characterisation of the properties of the chitosan-TBA conjugate, including an evaluation of the disintegration time, the swelling behaviour, the mucoadhesiveness and the permeation-enhancing effect has already been performed (Bernkop-Schnürch et al., 2004; Roldo et al., 2003). The features of the polymer derivative described here were in good accordance with them. The amount of reduced thiol groups, determined to be 185 ± 12 μmol per gram polymer ($n = 4$, mean ± S.D.), as well as the amount of thiol groups available in form of disulfide bonds, determined to be 43 μmol per gram polymer ($n = 4$), were quantified via Ellman's reagent.

3.2. Permeation studies

3.2.1. Effect of trypsin, papain and bromelain on the permeation of Na-Flu

In this study permeation-enhancing effect of the mammalian serine protease trypsin and of the plant cysteine proteases papain (from *Carica papaya*) and bromelain (from *Ananas comosus*) was evaluated in vitro using small intestinal tissue isolated from guinea pigs. A time- and concentration-dependent increase of Na-Flu transport across the mucosa was observed for all three tested enzymes. The calculated apparent permeability coefficients and the according enhancement ratios are shown in Table 1. The highest transport of marker across the mucosa was achieved after 3 h incubation with a 2% trypsin solution (Fig. 1). However, this permeation-enhancing effect was not significantly higher than the effect obtained by using a 0.5% papain solution (see Fig. 2) or a 2% bromelain solution (see Fig. 3). Accordingly, a comparable increase in the transport of Na-Flu from the donor chamber to the acceptor chamber was observed after incubation with 2% trypsin, 0.5% papain and 2% bromelain solutions. Papain displayed the strongest concentration-dependent effect, but its low solubility in the incubation medium prevented the evaluation of higher concentrations.

3.2.2. Influence of trypsin, papain and bromelain on the permeation of FITC-dextran

From the investigations with the low-molecular size marker Na-Flu emerged that 2% trypsin, 0.5% papain and 2% bromelain were the more appropriate enzyme concentrations, respectively, in order to guarantee an increased paracellular permation (see Table 1).

Table 1
Comparison of the influence of trypsin, papain, bromelain, chitosan-TBA/GSH and chitosan-TBA/GSH/bromelain on the apparent permeability coefficient (P_{app}) for sodium flurescein and FITC-dextran across the intestinal mucosa of guinea pigs

Paracellular marker	Test compound	Apparent permeability coefficient ($P_{app} \times 10^{-6}$ (cm/s)), means ± S.D., $n = 4$	Enhancement ratio (P_{app} enhancer system/P_{app} control)
Sodium fluorescein	control (buffer only)	6.85 ± 0.67	1
	0.5% trypsin	8.87 ± 1.06	1.29
	2% trypsin	12.64 ± 0.64	1.84
	0.2% papain	8.53 ± 0.94	1.24
	0.5% papain	11.23 ± 1.11	1.63
	0.5% bromelain	9.90 ± 0.56	1.44
	2% bromelain	12.22 ± 0.45	1.78
FITC-dextran	control (buffer only)	2.82 ± 0.51	1
	2% trypsin	3.48 ± 0.65	1.23
	0.5% papain	2.96 ± 0.20	1.05
	2% bromelain	4.45 ± 0.44	1.57
	0.5% chitosan-TBA/5% GSH	4.23 ± 0.66	1.5
	0.5% chitosan-TBA/5% GSH/2% bromelain	5.91 ± 0.51	2.1

Accordingly, the influence of these same concentrations of the proteases on the transport across the intestinal mucosa of the high-molecular mass marker FITC-dextran was evaluated as well. Because of its molecular size and mass (MM 4300 Da) FITC-dextran mimicks better hydrophilic macromolecular drugs and should therefore allow to make more reliable previsions about the effect of permeation enhancers on the paracellular transport of such drugs. Results of these experiments are displayed in Fig. 4 and Table 1. In contrast to the results obtained with Na-Flu, a significantly increased permeation of FITC-dextran was only provided by bromelain (2% solution). A P_{app}

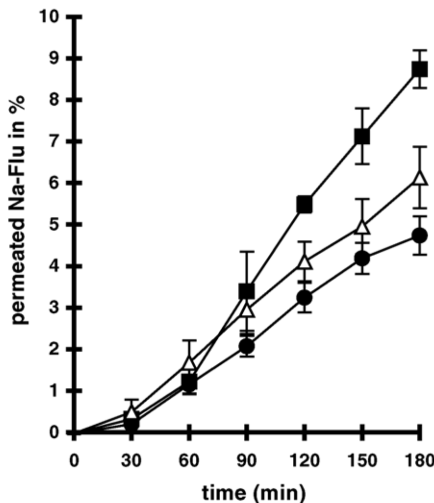

Fig. 1. Transport of Na-Flu across freshly excised small intestinal mucosa of guinea pigs. Transport data are expressed as percentage of the total dose of Na-Flu applied to the luminal side of the mucosa. A 2% (w/v) trypsin (■), 0.5% (w/v) trypsin (△), buffer solution only (●). Indicated values are means of at least four experiments ±S.D.

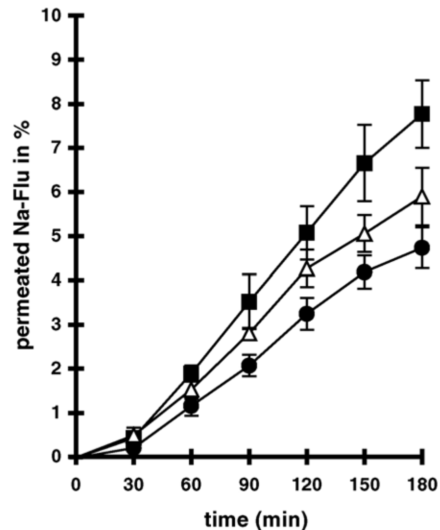

Fig. 2. Transport of Na-Flu across freshly excised small intestinal mucosa of guinea pigs. Transport data are expressed as percentage of the total dose of Na-Flu applied to the luminal side of the mucosa. A 0.5% (w/v) papain (■), 0.2% (w/v) papain (△), buffer solution only (●). Indicated values are means of at least four experiments ±S.D.

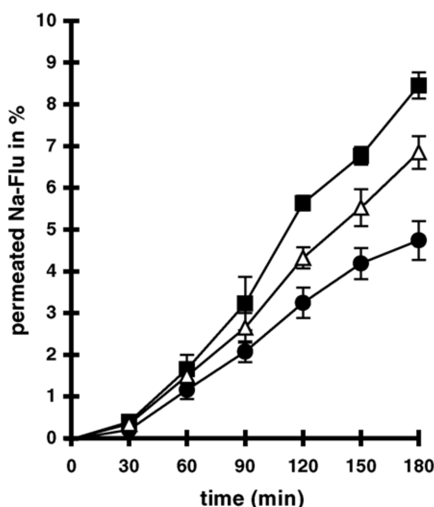

Fig. 3. Transport of Na-Flu across freshly excised small intestinal mucosa of guinea pigs. Transport data are expressed as percentage of the total dose of Na-Flu applied to the luminal side of the mucosa. A 2% (w/v) bromelain (■), 0.5% (w/v) bromelain (△), buffer solution only (●). Indicated values are means of at least four experiments ±S.D.

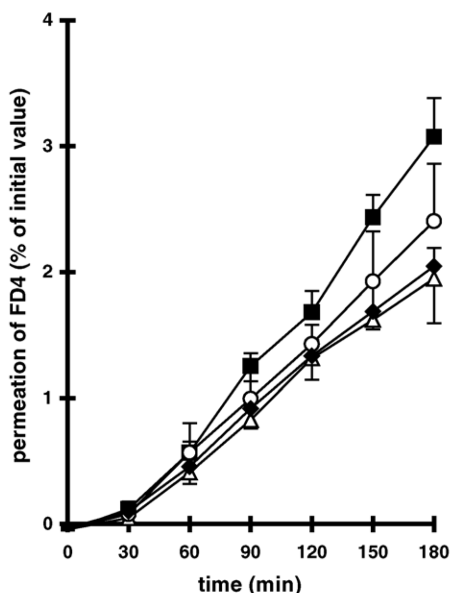

Fig. 4. Transport of FITC-dextran across freshly excised small intestinal mucosa of guinea pigs. Transport data are expressed as percentage of the total dose of FITC-dextran applied to the luminal side of the mucosa. Bromelain (2%) (■), trypsin (2%) (○), papain (0.5%) (♦), buffer solution only (△). Indicated values are means of at least four experiments ±S.D.

of $4.45 \pm 0.44 \times 10^{-6}$ cm/s was reached with this enzyme, which represents an enhancement ratio of 1.57 (Table 1). Trypsin (2%) increased only slightly the permeation of FITC-dextran, whereas papain (0.5%) showed a negligible effect.

3.2.3. Influence of trypsin, papain and bromelain on the TEER

TEER measurements, being performed during permeation studies with Na-Flu, served on the one hand as an indicator for the opening of the tight junctions. On the other hand, investigations regarding the reversibility of the effect of the proteolytic enzymes on the TEER should provide information about permanent damages of the intestinal mucosa. Results of these experiments, shown in Fig. 5, evidenced a very different concentration- and time-dependent effect of trypsin, papain and bromelain on the TEER. Incubating the mucosa for 60 min with a 0.5% papain solution caused the TEER to decrease significantly by $22.2 \pm 3.3\%$ (means ± S.D., $n = 3$) and no recovery was observed by removing the enzyme solution after 180 min incubation. A similar decrease of the TEER-values without recovering was induced by incubating the mucosa with a 2% trypsin solution. In contrast, bromelain was the only tested enzyme leading to a reversible TEER reduction in a concentration (2%), which caused a significant increase in the mucosal permeability (see Fig. 3). Although lower concentration of trypsin (0.5%) and papain (0.2%) provoked an invertible TEER reduction, these solutions could not guarantee a significantly increased transport of Na-Flu across the intestinal mucosa (Figs. 1 and 2).

3.2.4. Additive effect of the chitosan-TBA/GSH system and of bromelain on the permeation of FITC-dextran

The chitosan-TBA conjugate/GSH permeation-enhancing system has been proven successful in increasing the permeation of the low-molecular mass paracellular marker rhodamine 123, leading to an enhancement ratio of 3.6 (Bernkop-Schnürch et al., 2004). In the present study it has been firstly shown that the chitosan-TBA/GSH system can guarantee a significantly enhanced permeation of a high-molecular size marker like FITC-dextran as well (see Fig. 6). The P_{app} of FITC-dextran in the presence of 0.5% chitosan-TBA

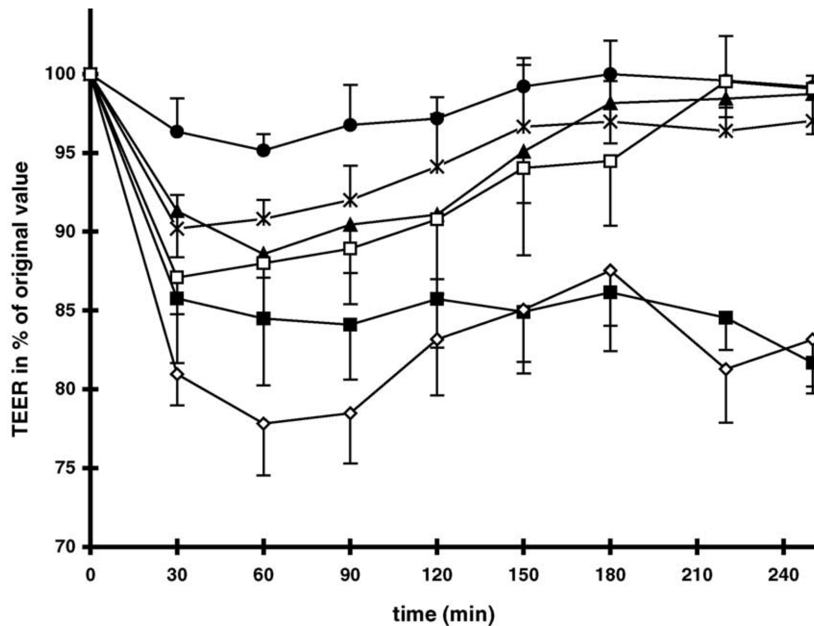

Fig. 5. Effect of different concentrations of proteolytic enzymes on the TEER of small intestinal mucosa of guinea pigs at pH 7.0. Each point represents the mean ± S.D. of at least three experiments. Keys: 2% (w/v) trypsin (■), 0.5% (w/v) trypsin (▲), 0.5% (w/v) papain (◇), 0.2% (w/v) papain (□), 2% (w/v) bromelain (*), buffer solution only (●), reversibility experiment started after 180 min of incubation (means ± S.D., $n = 3$).

conjugate and 5% GSH was $4.23 \pm 0.66 \times 10^{-6}$ cm/s, representing an enhancement ratio of 1.5 (Table 1). Moreover, the efficacy of this permeation-enhancing system could be clearly raised due to the addition of 2% bromelain (Fig. 6). Thereby a maximum P_{app} of $5.91 \pm 0.51 \times 10^{-6}$ cm/s was achieved, which corresponds to an enhancement ratio of 2.1. An additive permeation-enhancing effect of the chitosan-TBA/GSH system in combination with the proteolytic enzyme bromelain can therefore be postulated.

4. Discussion

Thiolated polymers were shown to be a valuable tool for the non-invasive application of hydrophilic macromolecules (Bernkop-Schnürch et al., 2003b), due to their mucoadhesive (Bernkop-Schnürch et al., 1999), cohesive and permeation-enhancing properties (Clausen et al., 2002). In particular, the chitosan-TBA conjugate, possessing the strongest so far known mucoadhesive features (Roldo et al., 2003), was shown to enhance the oral bioavailability of macromolecular drugs (Guggi et al., 2003a), especially when combined with the permeation mediator GSH (Guggi et al., 2003a,b). The underlying mechanism of permeation enhancement by thiomers seems to be based on the inhibition of protein tyrosine phosphatase (PTP). This results in a higher extent of phosphorylated tyrosine groups on the extracellular loops of the membrane protein occludin, leading to the opening of the tight junctions. The inhibition of PTP can be achieved by a disulfide bond formation of the active site cysteine of the protein. GSH released by intestinal cells oxidises the cysteine groups on PTP (Barrett et al., 1999). It is believed that reduced thiol functions on the thiolated polymer reduce the oxidised glutathione, thereby raising the amount of GSH at the absorption area for PTP inhibition. Accordingly, a significantly improved permeability of the tight junctions was observed (Clausen et al., 2002; Bernkop-Schnürch et al., 2004). Moreover, former TEER studies showed that the effect of the thiolated polymer/GSH system on the intercellular junctions is reversible and does not lead to permanent cell damages (Clausen and Bernkop-Schnürch, 2000).

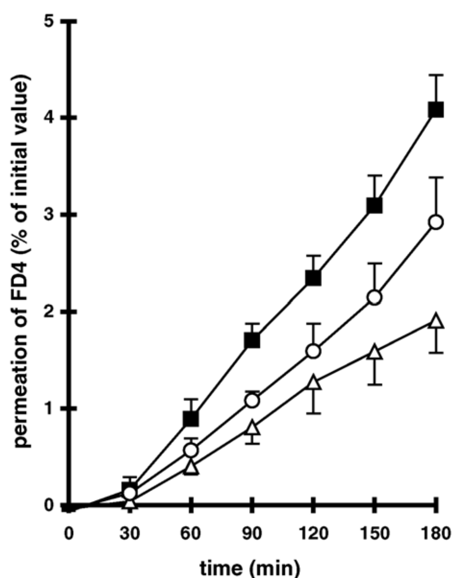

Fig. 6. Transport of FITC-dextran across freshly excised small intestinal mucosa of guinea pigs. Transport data are expressed as percentage of the total dose of FITC-dextran applied to the luminal side of the mucosa. Combination of chitosan-TBA (0.5%) with GSH (5%) and bromelain (2%) (■), combination of chitosan-TBA (0.5%) with GSH (5%) (◯), buffer solution only (Δ). Indicated values are means of at least four experiments ± S.D.

In this study the permeation-enhancing effect of the chitosan-TBA/GSH system could be further improved by the addition of a protease, being the first time that two types of permeation enhancers acting in different ways were combined in order to reach a higher paracellular uptake. Proteases such as trypsin, papain and bromelain have often been used in the last decades for the therapy of inflammatory diseases such as arteriosclerosis, phlebitis, rheumatism and many others (Emele et al., 1966; Izaka et al., 1972), in particular because of their safety and the lack of drug interactions and of undesired side-effects.

Kolac et al. (1996) found that plant proteolytic enzymes were more effective in increasing the Na-Flu transport across a Caco-2-cell monolayer than mammalian proteases and identified the following ranking: papain > bromelain > trypsin. Results of ex vivo investigations with freshly excised intestinal mucosa, being a model much closer to in vivo conditions than cell culture models, confirmed the strongest efficacy of papain (Fig. 2, Table 1). However, the difference of capacity of papain as permeation enhancer in comparison to the other two enzymes was significantly reduced. Furthermore, bromelain and trypsin displayed almost the same permeation-enhancing effect on the mucosa. The diverse efficacy of the proteases observed in this study can be mainly explained by the presence of the mucus layer, constituting the so-called diffusion barrier, on the intestinal tissue used for the trials. Indeed, it has been shown that especially papain is strongly inhibited by mucin, whereas the inhibition of bromelain is lower and trypsin is not at all affected by this physiological component (Kolac et al., 1996). Moreover, both trypsin and bromelain were shown to be highly active mucolytically, due to the cleavage of protein substructures in the glycoprotein that builds up the mucus gel layer (Bernkop-Schnürch et al., 2000).

Also the determination of the TEER showed a lower difference in efficacy in opening the intercellular junctions between papain and trypsin than described previously using cell monolayers. Both enzymes led to a similar decrease of the TEER in concentrations providing similarly increased permeations of Na-Flu. In contrast, bromelain was the only enzyme causing a clearly slighter and reversible decrease of the TEER in a concentration, which caused a significant increase in the mucosal permeability (see Figs. 3 and 5). These observations suggest, as already other investigations did (Bock et al., 1998), that there are differences in the mechanisms of action between the proteolytic enzymes, which remain to be elucidated in more detail. Furthermore, such differences in the mechanism of action of the proteases as well as the already mentioned different interactions of the proteases with the mucus layer represent the only logic explanation for the outcomings of the permeation studies with FITC-dextran (see Fig. 4). Although the 2% trypsin solution, the 0.5% papain solution and the 2% bromelain solution caused comparable permeation enhancement for Na-Flu, only the 2% bromelain solution could guarantee a significantly increased permeation of the larger molecular size model compound FITC-dextran. Bromelain was therefore the protease chosen to be combined with the chitosan-TBA/GSH system. Thereby, an additive permeation-enhancing effect could be reached (Fig. 6), providing more evidence for the supposed different mechanism of action of these two permeation-enhancing systems.

Results of this study evidenced that the combination of permeation enhancers acting in different ways rep-

resents a new promising strategy for peroral delivery of hydrophilic macromolecular drugs as additive effects are feasible. In particular, the combination of the thiomer/GSH system with the plant protease bromelain should raise the in vivo efficacy of thiomer-based oral delivery systems for drugs such as plasmid DNA or oligonucleotides, which are known to be stable towards proteolytic digestion. In the future also the absorption of enzymatically undergradable chemically modified or synthetic peptides might be improved successfully using the approach suggested in this study.

5. Conclusion

The proteolytic enzymes trypsin, papain and bromelain were found to have dramatic effects on the permeability of the small intestine for a low-molecular size compound like Na-Flu. However, only bromelain caused this effect for the high-molecular model compound FITC-dextran. Moreover, bromelain provided an additive permeation enhancement for FITC-dextran in combination with the chitosan-TBA/GSH system. Therefore, bromelain could be adequate to be used in combination with the thiomer/GSH permeation-enhancing system in order to enhance the mucosal absorption of hydrophilic macromolecular compounds in vivo as well.

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