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Evaluation and improvement of the properties of the novel cystine-knot microprotein McoEeTI for oral administration

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Abstract

Cystine-knot microproteins exhibit several properties that make them highly interesting as scaffolds for oral peptide drug delivery. It was therefore the aim of the study to evaluate the novel clinically relevant cystine-knot microprotein McoEeTI regarding its potential for oral delivery. Additionally, based on the gained results, important features of McoEeTI were improved. Enzymatic degradation was caused by chymotrypsin, trypsin and porcine small intestinal juice whereas McoEeTI was stable towards elastase, membrane bound proteases, pepsin and porcine gastric juice. Only minor McoEeTI degradation was observed during a 24 h incubation period in rat plasma. In the presence of various physiological ions about 50% of McoEeTI formed di- and/or trimers. P_{app} value of McoEeTI was determined to be $(7.4 \pm 0.4) \times 10^{-6}$ cm/s. Sodium caprate and polycarbophil-cysteine (PCP-Cys) had no beneficial effect on McoEeTI permeation, whereas the utilization of a chitosan-thiobutylamidine (Chito-TBA) system improved McoEeTI permeation 3-fold. Enzymatic stability could be strongly improved by the utilization of Bowman-Birk-Inhibitor (BBI) as well as PCP-Cys. In conclusion, this study indicates that McoEeTI represents a promising candidate as a novel scaffold for oral peptide drug delivery.

Keywords: Cystine-knot microprotein; Pharmacophoric scaffold; Oral peptide delivery; Thiomers

1. Introduction

Oral delivery of proteins and peptides has ever been a main challenge in pharmaceutical technology. Because of the obvious advantages of this route of administration including improved patient compliance and painless administration, the development of oral drug delivery systems has become of increasing interest in recent years. However, therapeutic peptides and proteins are encountered with several barriers during gastrointestinal (GI) passage leading consequently to low oral drug bioavailability. Limiting barriers are the diffusion barrier of the mucus layer covering GI epithelia (Bernkop-Schnürch and Fragner, 1996), the enzyme barrier caused by luminally secreted and membrane bound proteases (Woodley, 1994), comparatively poor drug permeation owing to the hydrophilic structure of proteins and peptides as well as protein aggregation (Swarbrick and Boylan, 2002). A novel approach to overcome these barriers is the use of cystine-knot microprotein technology. Cystine-knot microproteins (CKMs) are small peptides, typically consisting of about 30 amino acids. They are pharmacologically active substances with a defined structure based on intra-molecular disulfide bonds and a small triple stranded β-sheet (Craik et al., 2001). Their unique structure is responsible for their high thermal, chemical and enzymatic stability (Colgrave and Craik, 2004). Besides naturally occurring cystine-knot microproteins, a multitude of pharmacologically active cystine-knot microproteins of a desired phenotype can be generated by using recombinant technology or chemical synthesis. The pharmacophoric sequence is located in an exposed loop, whereas the knotted core is responsible for the stability (Craik et al., 2001). It is feasible to incorporate the pharmacophoric information into the amino acid sequence of a cystine-knot microprotein displaying desired features such as high enzymatic stability and good permeation behaviour. Therefore, the use of cystine-knot microproteins as

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pharmacophoric carriers in oral peptide delivery seems to be a promising novel approach. As demonstrated previously, certain cystine-knot microproteins permeate well through intestinal mucosa in comparison to other peptide drugs (Werle et al., 2006). So far evaluated cystine-knot microproteins have been demonstrated to be stable towards acidic conditions and pepsin, whereas degradation by luminally secreted proteases took place. Although it was possible to stabilize a cystine-knot microprotein towards chymotrypsin caused degradation by substituting the preferred theoretical cleavage site of this protease, stabilization towards trypsin by modifying the cystine-knot microprotein amino acid sequence is believed to be much more challenging, due to the broader cleavage site specificity of trypsin.

It was therefore the aim of this study to evaluate the properties of the novel structurally well characterized cystine-knot microprotein McoEeTI (Niemann et al., 2006) regarding its enzymatic stability towards GI proteolytic enzymes, permeation through intestinal mucosa, aggregation behaviour and isoelectric point. Additionally, based on this information, the properties of McoEeTI should be improved. Therefore, the effect of well-established enzyme inhibitors such as Bowman-Birk-Inhibitor (BBI) and polycarbophil-cysteine (PCP-Cys) as well as the effect of the permeation enhancers sodium caprate, chitosan-thiobutylamidine (Chito-TBA) and PCP-Cys were evaluated in vitro in order to gain essential information for the design of a potent delivery system for McoEeTI.

2. Materials and methods

2.1. Production and purification of McoEeTI

McoEeTI is a 30 amino acid engineered hybrid cystine-knot protein that consists of residues 6–21 of the naturally head-to-tail cyclized trypsin inhibitor MCoTI-II, of residues 16–28 of EETI-II, a naturally linear squash trypsin inhibitor and a C-terminal serine (Schmoldt et al., 2005). McoEeTI was expressed in *Escherichia coli* and purified essentially as described (Schmoldt et al., 2005).

2.2. HPLC analyses

HPLC analyses were performed as described previously (Werle et al., 2006). All HPLC separations were performed with Nucleosil 5 C_{18} columns (250 mm × 4.6 mm). A flow rate of 1 ml/min was maintained, using the solvents A (0.1% trifluoracetic acid in destilled H_2O) and B (0.1% trifluoracetic acid in acteonitril). The following gradient was used: 0–10.5 min (80–35% A), 10.5–12 min (35–80% A) and 12–17 min (80% A). FITC labelled McoEeTI and FITC labelled bacitracin was analyzed at λ_{ex} 495 nm, λ_{em} 525 nm using a fluorescence detector; unlabelled McoEeTI and nisin were analyzed at 220 nm using a diode array detector.

2.3. FITC labelling of McoEeTI and bacitracin

Bacitracin (Sigma) labelling was performed similar to a method described previously by our research group (Clausen and Bernkop-Schnürch, 2000; Werle et al., 2006). In brief, the peptide was dissolved in a Na_2CO_3 solution. Afterwards, fluorescein isothiocyanate (FITC) dissolved in dimethylsulfoxide (DMSO) was added dropwise under stirring. After incubation in the dark, NH_4Cl was added. FITC labelled peptides were isolated by gel filtration (Sephadex G25, mobile phase: demineralized H_2O) and lyophilized. Similarly, McoEeTI was labelled with equimolar amounts of FITC and fluorescently labelled microprotein was purified by RP-HPLC and lyophilized.

2.4. Synthesis and characterization of thiomers

Polycarbophil-cysteine (PCP-Cys) was synthesized as described previously (Bernkop-Schnürch and Thaler, 2000). In brief, the carboxylic acid moieties of polycarbophil were activated with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) and L-cysteine hydrochloride was added. Synthesis of chitosan-thiobutylamidine (Chito-TBA) was performed as described previously (Bernkop-Schnürch et al., 2003a,b). Chitosan was dissolved in acetic acid and 2-iminothiolane HCl was added. The thiomers were isolated by dialysis and thereafter lyophilized. The amount of immobilized free thiol groups of the resulting conjugates was determined via Ellman's test (Bernkop-Schnürch et al., 2003a,b).

2.5. Determination of the isoelectric point

The isoelectric point (p*I*) of McoEeTI was determined by gel-electrophoresis. First, 500 μg of McoEeTI was dissolved in 20 μl of lysis buffer (7 M urea, 2 M thiourea, 4% chaps, 40 mM Tris) and then 330 μl rehydration buffer (8 M urea, 2 M thiourea, 4% chaps, 18 mM dithiothreitol) was added. Gel strips for the IEF (Amersham, 18 cm) with a pH gradient between 6 and 11 were used. Electrophoresis was performed for 21 h (55,000 Vh, 8000 V max, 30 mA). Afterwards, McoEeTI was fixed with a solution of 40% ethanol, 10% acetic acid on the gel strip and stained with Coomassie blue.

2.6. Preparation of porcine small intestinal and porcine gastric juice

Gastric and small intestinal juice from a freshly slaughtered pig was collected. To 15 ml of collected small intestinal juice, 5 ml of distilled water was added in order to reduce viscosity. These liquids were filtered through sterile gaze. To remove undigested nutrient particles, samples were purified by centrifugation. The pH of porcine gastric juice was adjusted with 1 M HCl to pH 2 and the pH of porcine small intestinal juice was adjusted with 1 M NaOH to pH 8.0. The supernatants were stored at −20 °C until further use.

2.7. Enzymatic degradation

For enzyme degradation tests, chymotrypsin-TLCK treated (Worthington), elastase (Worthington), pepsin (Sigma) and trypsin-TPCK treated (Worthington) were chosen. Furthermore, tests with membrane bound proteases as well as tests in col-

Table 1
Predicted cleavage sites of McoEeTI caused by different luminally secreted and membrane bound peptidases

Trypsin: expected cleavage sites; substrate specifity for R, K

G-V-C-P-K↓I-L-K↓K↓C-R↓R↓D-S-D-C-L-A-G-C-V-C-G-P-N-G-F-C-G-S

Chymotrypsin: expected cleavage sites; substrate specifity for F, Y

G-V-C-P-K-I-L-K-K-C-R-R-D-S-D-C-L-A-G-C-V-C-G-P-N-G-F↓C-G-S

Elastase: expected cleavage sites; substrate specifity for A, G, I, L, V

G↓V↓C-P-K-I↓L↓K-K-C-R-R-D-S-D-C-L↓A↓G↓C-V↓C-G↓P-N-G↓F-C-G↓S

Carboxypeptidase A: expected cleavage sites; substrate specifity for Y, F, I, T, E, H, A

G-V-C-P-K-I-L-K-K-C-R-R-D-S-D-C·L-A-G-C-V-C-G-P-N-G-F-C-G-S

Carboxypeptidase B: expected cleavage sites; substrate specifity for R, K

G-V-C-P-K-I-L-K-K-C-R-R-D-S-D-C-L-A-G-C-V-C-G-P-N-G-F-C-G-S

Aminopeptidase N: expected cleavage sites; substrate specifity for many, especially A, L

G↓V-C-P-K-I-L-K-K-C-R-R-D-S-D-C-L-A-G-C-V-C-G-P-N-G-F-C-G-S

Pepsin: expected cleavage sites; hydrophobic, preferable aromatic residues in P1 and P1´position

G-V-C-P-K-I-L-K-K-C-R-R-D-S-D-C-L-A-G-C-V-C-G-P-N-G↓F-C-G-S

lected porcine gastric and small intestinal juice were performed. The preferred theoretical cleavage sites of various proteolytic enzymes are shown in Table 1.

2.7.1. Enzymatic degradation of McoEeTI by various proteases

To 120 μl of a 1 mg/ml solution of McoEeTI in Tris buffer (50 mM, pH 6.5), 120 μl of chymotrypsin-TLCK treated (6.6 U) and trypsin-TPCK treated (3.1 U) dissolved in Tris buffer (50 mM, pH 6.5) were added in each case, respectively. The elastase solution was prepared by dissolving elastase in 1% KCl and then adding Tris buffer (50 mM, pH 6.5) to obtain a concentration of 0.0356 U elastase/ml. The concentration of the three utilized proteases was in the physiological range present in the intestinal fluid (Bernkop-Schnürch, 1998). For pepsin degradation tests, pepsin (1.6 U) was dissolved in 120 μl of 0.08 M HCl which was adjusted with 1 M NaOH to pH 2 and then added to the McoEeTI solution (1 mg McoEeTI in 1 ml 0.08 M HCl; pH 2). Furthermore, McoEeTI stability in collected porcine small intestinal as well as porcine gastric juice was evaluated in the same way. The solution was shaken (300 rpm) and incubated at 37 °C during the sampling period. At predetermined time points (0, 5, 15, 30, 60, 120 and 180 min) aliquots (30 μl) were withdrawn and the reaction was stopped by the addition of 30 μl of 0.1% trifluoroacetic acid solution or 30 μl of 1 M NaOH, respectively. Samples were immediately cooled to 4 °C and afterwards analyzed by HPLC as described above.

2.7.2. Enzymatic degradation caused by membrane bound proteases

To investigate whether any degradation caused by membrane bound proteases occurs, Ussing-type chambers with a surface area of 0.64 cm^2 were used. The method has been described previously (Marschütz et al., 2002). Rat small intestine (jejunum) was excised immediately after sacrificing the animal. Onto the acceptor chamber, an impermeable membrane (Parafilm M, Sigma) was mounted to prevent permeation. Rat jejunum was mounted in the Ussing-type chamber, the basolateral side facing the para-film, and the donor and acceptor compartments of the chamber were filled with 1.0 ml of the freshly prepared incubation containing 250 mM NaCl, 2.6 mM $MgSO_4$, 10 mM KCl, 40 mM glucose and 50 mM $NaHCO_3$. This incubation medium was buffered with 40 mM *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethane-sulfonic acid) (HEPES, Sigma) and the pH was adjusted to 6.5. Membrane bound protease degradation studies were performed in an atmosphere of 95% O_2 and 5% CO_2 at 37 °C and were started 15 min after the mounting of the tissue. The solution in the donor chamber was replaced with incubation medium containing 0.4 mg/ml of FITC labelled McoEeTI. To demonstrate the activity of the membrane bound proteases, experiments were also performed with an aqueous 0.1% solution of the aminopeptidase N substrate leu-*p*-nitroanilide. Aliquots (30 μl) were withdrawn from the donor chamber after 0, 5, 15, 30, 60, 120 and 180 min.

2.7.3. Plasma stability studies

To 150 μl of rat plasma, 20 μl of an aqueous McoEeTI-FITC solution (1 mg/ml) was added. The McoEeTI-FITC-plasma solution was incubated at 37 °C while shaking (300 rpm) during the sampling period. Samples (10 μl) were withdrawn at 0, 4, 8, 16 and 24 h and added to 40 μl of a 0.05% trifluoroacetic acid solution to stop enzyme activity. Samples were analyzed via HPLC as described above.

2.8. Aggregation studies

Aggregation studies were performed as described previously (Werle et al., 2006). For comparison, nisin was chosen as a model drug. Nisin exhibits a molecular mass (MW = 3354.1 g/mol) and a p*I* (8.0) in the same range as McoEeTI (MW = 3087.7 g/mol). To gain information concerning aggregate size, ultrafiltration devices (vivaspin 500, vivascience) with two different filters (molecular mass cut off of 5 and 10 kDa) were used for aggregation studies. First, 200 μg of lyophilized McoEeTI were dissolved in 1 ml of freshly prepared buffer containing 250 mM NaCl, 2.6 mM $MgSO_4$, 10 mM KCl, 40 mM glucose and 50 mM $NaHCO_3$. This solution was buffered with 40 mM *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethane-sulfonic acid) (HEPES, Sigma). The pH was adjusted with 5 M NaOH to 7.0. In parallel, 40 mg of commercially available milk solids containing 2.5% nisin (Sigma) were dissolved in 2 ml of the same buffer. The insoluble solids were removed by centrifugation (1500 rcf, 10 min, Megafuge 1.0, Heraeus Sepatech). Then the concentrator of the ultrafiltration device was filled with 300 μl of the vortexed McoEeTI and nisin solution, respectively. The samples were centrifuged for 45 min at 10,000 rpm (Eppendorf, minispin) and afterwards the supernatant was diluted with buffer to obtain the same volume as the filtrate. Aliquots were withdrawn from the diluted supernatant and the filtrate and analyzed via HPLC as described above.

2.9. Permeation studies

Permeation studies with Ussing-type chambers were performed as described previously (Clausen and Bernkop-Schnürch, 2000) with FITC labelled McoEeTI and FITC labelled bacitracin. The prearrangement procedure was performed as described above without mounting an impermeable membrane on the basolateral side so that permeation could take place. Three different formulations were used to improve permeation: sodium caprate in combination with Bowman-Birk-Inhibitor, PCP-Cys and Chito-TBA. The solution in the donor chamber was replaced with incubation medium containing the formulations provided in Table 2. During a 3 h incubation period, 100 μl samples were withdrawn from the acceptor chamber every 30 min and the volume was replaced by the same medium equilibrated at 37 °C. The samples were analyzed via HPLC. Cumulative corrections were made for the previously removed samples in determining the total amount permeated.

2.10. Protection of McoEeTI against intestinal degradation

2.10.1. Protection of McoEeTI against intestinal degradation by Bowman-Birk-Inhibitor

Porcine small intestinal juice prepared as described above was thawed at 4 °C, centrifuged (15 min at 10,000 × *g*/rpm). The pH was determined to be 8.0. Bowman-Birk-Inhibitor (BBI) was added in different concentrations (0.1, 0.5, 1 and 2%) to a solution of 0.1% McoEeTI in 50 mM Tris buffer pH 7.6 and incubated for 20 min at 37 °C and shaking (300 rpm). To 120 μl of these McoEeTI/inhibitor solution 120 μl of porcine small intestinal juice (37 °C) were added and samples were withdrawn after 0, 5, 15, 30, 60, 120 and 180 min. The enzymatic reaction was stopped by adding 0.25 M NaOH and samples were analyzed via HPLC.

2.10.2. Protection of McoEeTI against intestinal degradation by polycarbophil-cysteine

PCP-Cys was added in a final concentration of 0.5% as well as 1% to a solution of 0.1% McoEeTI in 50 mM Tris buffer pH 7.6 and incubated for 20 min at 37 °C and shaking (300 rpm). To 250 μl of this McoEeTI/polymer mixture 250 μl of thawed and centrifuged (15 min at 10,000 × *g*/rpm) porcine small intestinal juice as described above was added and incubated at 37 °C. Aliquots (50 μl) were withdrawn after 0, 5, 15, 30, 60, 120 and 180 min. The enzymatic reaction was stopped immediately by adding 50 μl 0.25 M NaOH and samples were centrifuged (20 min at 10,000 × *g*/rpm) and analyzed via HPLC.

2.11. Data analyses

Apparent permeability coefficient (P_{app}) for McoEeTI-FITC and FITC labelled bacitracin (Bac-FITC) was calculated with data gained after 3 h in permeation studies using the following equation:

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

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 chamber (cm^2), c the initial concentration of the test compound in the donor compartment ($\mu g/cm^3$) and t is the total time of the experiment.

Table 2
P_{app} values and enhancement ratios by using different permeation enhancers

McoEeTI-FITC (mg/ml)	PCP-Cys (%)	Chito-TBA (%)	GSH (%)	Sodium caprate (%)	BBI (%)	P_{app} value (cm/s) ($\times 10^{-6}$)	Transport enhancement ratio (P_{app} sample/P_{app} control)
0.4	–	–	–	–	–	6.3 ± 1.1	1.0
0.2	–	–	–	0.5	0.1	3.7 ± 0.1	0.6
0.2	0.5	–	5	–	–	8.1 ± 1.0	1.3
0.4	0.5	–	2.5	–	0.1	8.2 ± 3.6	1.3
0.4	–	0.5	2.5	–	–	18.3 ± 4.9	2.9
0.4	–	0.5	2.5	–	0.1	18.6 ± 7.9	3.0

2.12. Statistical data analysis

Statistical data analysis was performed using the Student's *t*-test, with $p < 0.05$ as the minimal level of significance unless indicated otherwise.

3. Results

3.1. Polymer synthesis

The amount of immobilized free thiol groups of PCP-Cys was determined to be 216 μmol/g polymer. Chito-TBA displayed 227 μmol/g polymer.

3.2. Determination of the isoelectric point

If ionic delivery systems such as polyacrylates, chitosan or derivatives thereof are used as delivery systems, it is important to determine the p*I* of the drug. The p*I* of McoEeTI was calculated using two different calculation programs (http://scansite.mit.edu/cgi-bin/calcpi and http://www.embl-heidelberg.de/cgi/pi-wrapper.pl) and was determined to be 8.3 and 8.7, respectively. The isoelectric point determined by isoelectric focusing was 8.5, which is in good correlation with the theoretical p*I*s.

3.3. Enzymatic degradation

An exact knowledge about the stability of a drug towards GI enzymes is an important prerequisite to render possible a drug for oral administration or not. Furthermore, the development of oral delivery systems should be based on these data. Neither elastase nor pepsin caused degradation to McoEeTI, whereas chymotrypsin as well as trypsin caused degradation to McoEeTI. Nevertheless, after an incubation period of 3 h still 30–40% of undegraded McoEeTI was present (Fig. 1). McoEeTI was also degraded by native porcine small intestinal juice. Membrane bound proteases caused no damage to McoEeTI within a 3 h incubation period (data not shown). Additionally, McoEeTI was stable towards collected porcine gastric juice (data not shown), so that degradation during stomach passage can definitely be excluded. Furthermore, McoEeTI exhibits high stability towards enzymes of rat plasma. After 24 h, still about 60% of undegraded McoEeTI could be detected in the plasma (Fig. 2).

3.4. Aggregation studies

Due to their large size, aggregates commonly cannot permeate through membranes as good as the corresponding monomers. According to the results shown in Fig. 3, McoEeTI displays certain aggregation effects in a solution containing various ions and glucose, whereas the model drug nisin exhibits no significant aggregation properties. Aggregates are in a range between 5 and 10 kDa which indicates that, besides McoEeTI monomers (approximately 50%), also di- and/or trimers (approximately 50%) occur.

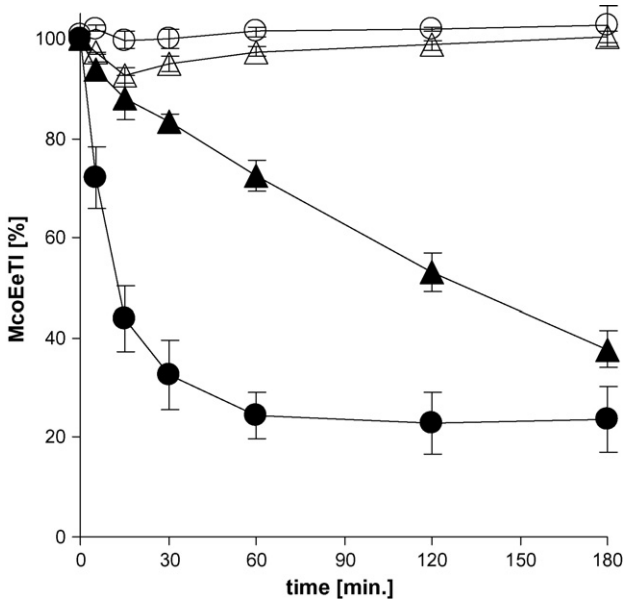

Fig. 1. Enzymatic degradation of McoEeTI caused by pepsin (◯), chymotrypsin (●), elastase (△), and trypsin (▲); each point represents the mean ± S.D. of at least $n = 3$ experiments.

3.5. Permeation studies

Data gained by in vitro permeation studies often correlate with in vivo results of oral bioavailability studies. Results of McoEeTI permeation studies are shown in Fig. 4. Compared to the FITC labelled model drug bacitracin (Bac-FITC), McoEeTI-FITC permeates well through rat small intestinal mucosa. P_{app} values were determined to be $(7.4 \pm 0.4) \times 10^{-6}$ for McoEeTI and $(4.6 \pm 0.5) \times 10^{-6}$ for Bac-FITC ($p < 0.0014$).

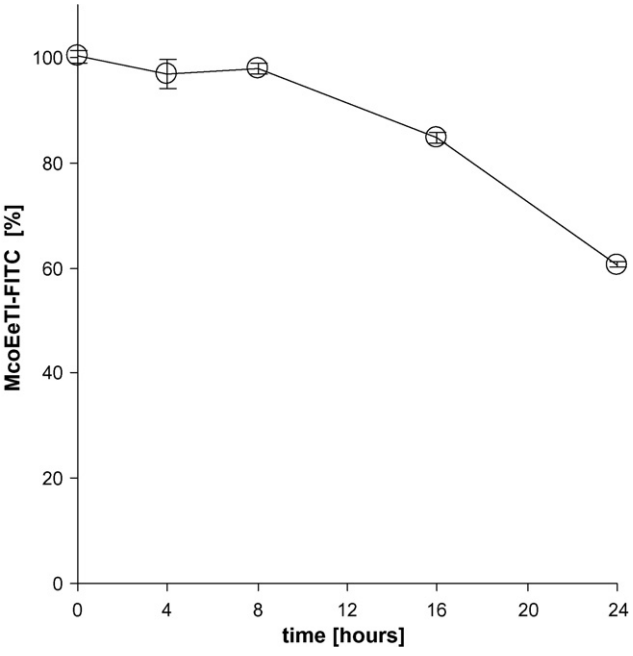

Fig. 2. Enzymatic degradation of McoEeTI in rat plasma; each point represents the mean ± S.D. of at least $n = 3$ experiments.

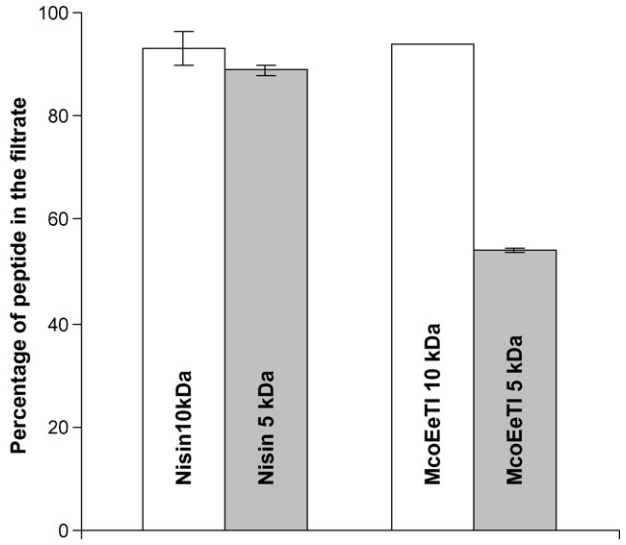

Fig. 3. Percentage of McoEeTI and nisin in the filtrate (white bars: 10 kDa molecular mass cut off; grey bars: 5 kDa molecular mass cut off); each point represents the mean ± S.D. of at least $n = 3$ experiments.

Sodium caprate led to a decrease in McoEeTI permeation of factor 0.6, whereas PCP-Cys/GSH non-significantly ($p < 0.15$) improved permeation 1.3-fold and finally Chito-TBA/GSH significantly improved McoEeTI permeation 3-fold ($p < 0.018$). Co-administration of BBI to the thiomer/GSH systems had no effect on McoEeTI permeation. Results are shown in Fig. 5. A summary of P_{app} values is provided in Table 2.

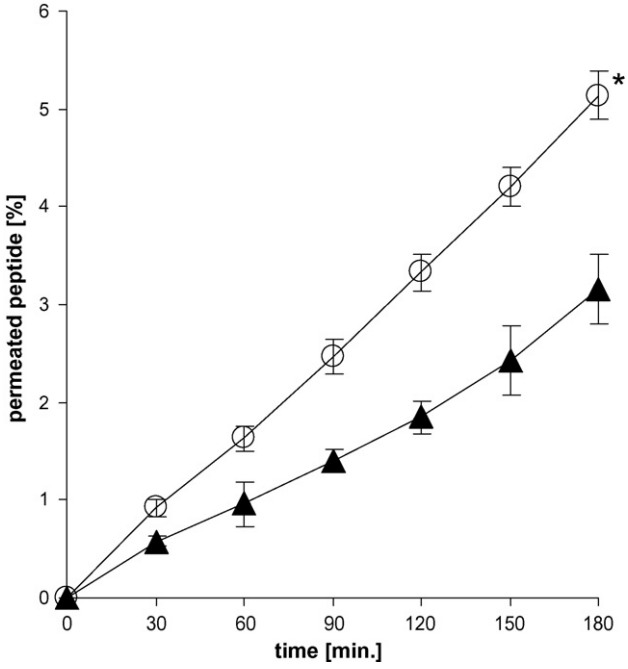

Fig. 4. Permeation of McoEeTI-FITC (◯) and Bac-FITC (▲) across rat small intestine; (◯, *) differs from control $p < 0.0014$; each point represents the mean ± S.D. of at least $n = 3$ experiments.

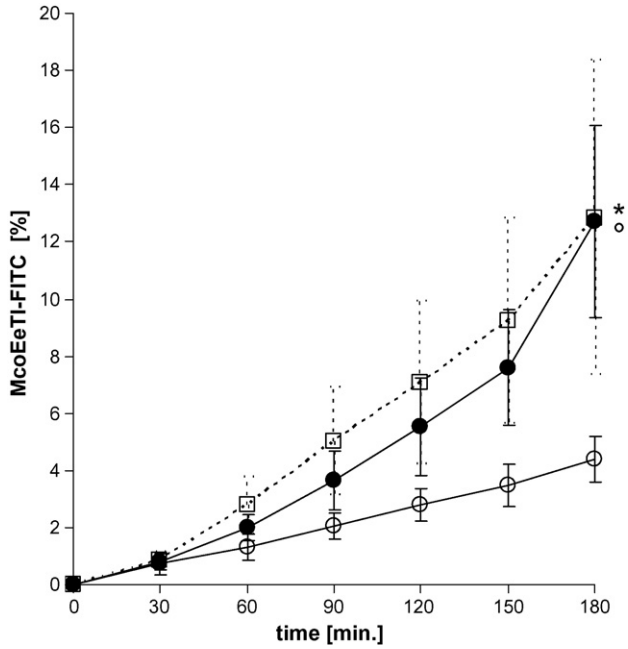

Fig. 5. Permeation enhancing effect of Chito-TBA ((●) McoEeTI-FITC + 0.5% Chito-TBA + 2.5% GSH, (□) McoEeTI-FITC + 0.5% Chito-TBA + 2.5% GSH + 0.1% BBI, (◯) McoEeTI-FITC in buffer, (×) Bac-FITC in buffer); (□, *) differs from control $p < 0.058$, (●, °) differs from control $p < 0.018$; each point represents the mean ± S.D. of at least $n = 3$ experiments.

3.6. Protection against enzymatic degradation

Both, Bowman-Birk-Inhibitor and PCP-Cys effectively protected McoEeTI towards native porcine chymotrypsin as well as trypsin (Figs. 6 and 7). The utilized different concentrations (0.1, 0.5, 1 and 2% in the case of Bowman-Birk-Inhibitor and 0.5 and 1% in the case of PCP-Cys) had no significant effect on

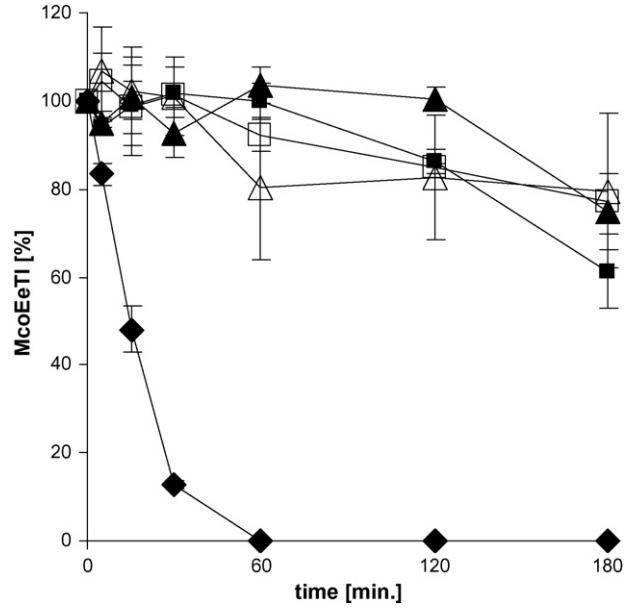

Fig. 6. McoEeTI degradation in porcine small intestinal juice ((♦) control without inhibitor; (□) 2% BBI; (△) 1% BBI; (▲) 0.5% BBI; (■) 0.1% BBI); each point represents the mean ± S.D. of at least $n = 3$ experiments.

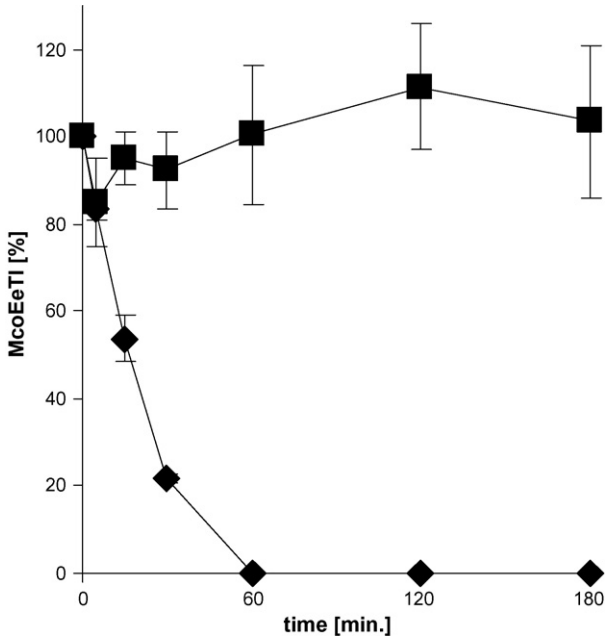

Fig. 7. McoEeTI degradation in porcine small intestinal juice ((♦) control without inhibitor; (■) 0.5% PCP-Cys); each point represents the mean ± S.D. of at least $n = 3$ experiments.

the inhibition rate, which suggests that even small doses of these systems inhibit the responsible proteases completely.

4. Discussion

McoEeTI is a hybrid cystine-knot microprotein that is derived from two microproteins of the squash inhibitor family (Schmoldt et al., 2005). This microprotein has been engineered for ease of recombinant production in *E. coli* (Schmoldt et al., 2005) and structure determination (Niemann et al., 2006) and to serve as a scaffold for the introduction of novel functionalities via replacement of functional loops. McoEeTI has been shown to be an inhibitor of trypsin (Schmoldt et al., 2005) and of human mast cell tryptase (unpublished results).

Based on the known specificity of the utilized proteolytic enzymes (Table 1) a prediction of the expected cleavage of McoEeTI was made. Interestingly, neither elastase nor pepsin caused degradation to McoEeTI, although various predicted elastase cleavage sites occur in the cystine-knot microprotein. This might be explained by the unique folding of cystine-knot microproteins which form an extraordinary stable and rigid structure that is believed to complicate the fit into the active site of the protease. Furthermore, these results are in good correlation with results of studies performed previously (Werle et al., 2006). Chymotrypsin as well as trypsin caused degradation to McoEeTI. Nevertheless, after an incubation period of 3 h still 30–40% of undegraded McoEeTI was present (Fig. 1). McoEeTI was not affected by membrane bound proteases. Furthermore, McoEeTI was stable over 3 h in a solution containing pepsin (Fig. 1) as well as in porcine gastric juice so that the possibility of gastric degradation can be definitely excluded. The high stability of McoEeTI towards the acidic milieu and proteolytic enzymes of the stomach correlates well with data of similar cystine-knot microproteins evaluated previously (Werle et al., 2006). Also only minor McoEeTI degradation over an incubation period of 24 h in rat plasma was observed (Fig. 2). Drug stability in plasma is of particular interest for pharmacokinetic parameters such as plasma half-life time. Beside of fast renal clearance, plasma stability is the most important factor affecting half-life time of peptides and proteins. Referring to McoEeTI plasma stability, sufficient plasma half-life time can be anticipated, although the effect of glomerular filtration has to be evaluated in vivo to approve this hypothesis.

Referring to the results gained in enzymatic degradation studies, protection towards trypsin and chymotrypsin caused degradation seems to be crucial to achieve sufficient and less mutable oral bioavailability of McoEeTI. To overcome enzymatic degradation, the effect of the well-known chymotrypsin/trypsin inhibitor Bowman-Birk-Inhibitor was tested in four different concentrations (0.1, 0.5, 1 and 2%). The efficacy of the Bowman-Birk inhibitor to inhibit trypsin, chymotrypsin and elastase has been demonstrated in various studies (Reseland et al., 1996; Ushirogawa, 1992). Another promising excipient which was evaluated in concentrations of 0.5 and 1% was the thiolated polymer polycarbophil-cysteine (PCP-Cys) which is known to inhibit proteases like trypsin, aminopeptidase N as well as carboxypeptidase A and B (Bernkop-Schnürch and Thaler, 2000; Bernkop-Schnürch and Walker, 2001) and moreover exhibits permeation enhancing effects (Clausen and Bernkop-Schnürch, 2000). The efficacy of these inhibitors was investigated under physiological conditions in porcine small intestinal juice at 37 °C and pH 8, which is the pH optimum of porcine chymotrypsin and trypsin. Although it was shown within this study that only chymotrypsin and trypsin degrade McoEeTI, it was important to further confirm these results by incubating McoEeTI in collected small intestinal juice comprising of a mixture of various different proteases. Results are shown in Figs. 6 and 7 and demonstrate the efficacy of Bowman-Birk-Inhibitor as well as polycarbophil-cysteine to protect McoEeTI from luminal secreted intestinal enzymes. These results also indicate that degradation caused by other intestinal proteases can be excluded. The different utilized concentrations had no effect on the inhibition rate of both inhibitors, leading to the conclusion that even little concentrations of both evaluated compounds protects McoEeTI efficiently towards GI metabolism.

Results of aggregation studies indicate the formation of di- and/or trimers (Fig. 3). Formation of aggregates can lead to a decrease in oral bioavailability of drugs. The P_{app} value of McoEeTI-FITC was determined to be $(7.4 \pm 0.4) \times 10^{-6}$ cm/s which is in good correlation with the P_{app} values of similar cystine-knot microproteins gained in previous studies (Werle et al., 2006). McoEeTI exhibited better permeation properties than bacitracin, which is known to permeate well through intestinal mucosa in comparison to various other peptide drugs (Fig. 4). To further improve McoEeTI mucosal uptake, permeation studies were performed with the well-known permeation enhancers sodium caprate (Tomita et al., 1988; Tomita et al., 1995) as well as with the thiomers Chito-TBA (Bernkop-Schnürch et

al., 2003a,b) and PCP-Cys (Clausen and Bernkop-Schnürch, 2000). Also a possible influence of the enzyme inhibitors on McoEeTI permeation was evaluated. All evaluated formulations are listed in Table 2. Sodium caprate had no beneficial effect on McoEeTI permeation; P_{app} value was even decreased. Also PCP-Cys did not significantly improve McoEeTI permeation, which is believed to be due to the anionic character of PCP-Cys. The possibility of ionic interactions between PCP-Cys and the slightly cationic McoEeTI – exhibiting a p*I* of 8.5 as determined within this study – seems likely. However, such a complexation may allow a sustained McoEeTI uptake. Nevertheless, according to the potent enzyme inhibitory properties of PCP-Cys (Fig. 7), an improved mucosal uptake of McoEeTI in vivo can be anticipated. Utilization of Chito-TBA led to strongly enhanced McoEeTI permeation. This cationic polymer, which is well known to improve permeation of various peptide drugs in vitro (Bernkop-Schnürch et al., 2004) as well as in vivo (Bernkop-Schnürch et al., 2005) increased McoEeTI permeation 3-fold (Fig. 5). Co-administration of BBI had no influence on McoEeTI permeation in vitro and is therefore a promising auxiliary agent for in vivo studies. All P_{app} values are summarized in Table 2.

5. Conclusion

Within the current study, important parameters of the novel CKM McoEeTI regarding its potential for the use as a scaffold for oral peptide drug delivery were evaluated. Moreover, various auxiliary agents including BBI, PCP-Cys and Chito-TBA which are able to improve enzymatic stability or permeation behaviour of McoEeTI were identified. The results of the current study provide substantial information for the design of oral CKM delivery systems and point out the potential of McoEeTI as a novel pharmacophoric carrier for oral peptide drug delivery.

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