Amino Acids (2008) 35: 195–200
DOI 10.1007/s00726-007-0569-1
Printed in The Netherlands

Amino Acids

Characterisation of the barrier caused by luminally secreted gastro-intestinal proteolytic enzymes for two novel cystine-knot microproteins

M. Werle[1], **H. Kolmar**[2], **R. Albrecht**[1], and **A. Bernkop-Schnürch**[3]

[1] ThioMatrix GmbH, Research Center Innsbruck, Innsbruck, Austria
[2] TU Darmstadt, Clemens Schöpf Institut, Fachbereich Biochemie, Darmstadt, Germany
[3] Department of Pharmaceutical Technology, Leopold Franzens Universität Innsbruck, Innsbruck, Austria

Received March 30, 2007
Accepted May 21, 2007
Published online July 6, 2007;

Summary. It was the aim of this study to evaluate the stability of two novel cystine-knot microproteins (CKM) SE-ET-TP-020 and SE-MC-TR-020 with potential clinical relevance towards luminally secreted proteases of the gastrointestinal tract in order to gain information about their potential for oral administration. Therefore, the stability of the two CKM and the model-drug insulin towards collected porcine gastric and small intestinal juice as well as towards isolated proteolytic enzymes was evaluated under physiological conditions. No intact SE-ET-EP-020 was detected after few seconds of incubation with porcine small intestinal juice. SE-ET-TP-020 was also degraded in porcine gastric juice. Furthermore, SE-ET-TP-020 was extensively degraded by isolated chymotrypsin, trypsin and pepsin. Moreover, it was degraded by elastase. SE-MC-TR-020 was degraded entirely within approximately 2 h when incubated in porcine small intestinal juice, whereas no degradation was observed within a 3 h incubation period with porcine gastric juice. In presence of the isolated proteolytic enzymes, SE-MC-TR-020 was only slightly degraded by trypsin and pepsin, whereas elastase caused no degradation to SE-MC-TR-020 at all. Chymotrypsin was the protease that caused most degradation to SE-MC-TR-020. The model drug insulin was degraded extensively by chymotrypsin, elastase, pepsin and trypsin as well as by porcine gastric and porcine small intestinal juice. In conclusion, a precise characterisation of SE-ET-TP-020 and SE-MC-TR-020 degrading luminally secreted GI enzymes has been made, which is an important and substantial prerequisite for the further optimisation of these CKM.

Keywords: Cystine-knot microproteins – CKM – Oral delivery – Enzymatic degradation

Introduction

Cystine-knot microproteins (CKM) are the smallest naturally occurring proteins with a defined and stable tertiary fold. They consist of typically less than 50 amino acids and can mainly be found in plants as well as in invertebrates. CKM display unique structural features: (I) a knotted β-sheet core which is caused by the formation of three intramolecular disulfide bonds between cysteine residues and (II) β-strand connecting loops that frequently carry the pharmacophoric sequence (Craik et al., 2001). It can also be distinguished between CKM with free C- and N-termini and the so called cyclotides, which are cyclic CKM displaying a head to tail cyclized backbone (Craik et al., 1999). In previous studies it has been demonstrated that CKM are highly stable towards pH, chemical and enzymatic stability. This is believed to be due to the enormous rigidity that is introduced by covalent disulfide linkage of the knotted core (Colgrave and Craik, 2004; Kratzner et al., 2005). Natural CKMs display a plethora of pharmacologic activities ranging from protease inhibition over ion channel blockage to insecticidal activities (Craik et al., 2006). Ziconotide, an intrathecal administered CKM derived from the mollusc *Conus magnus* is already marketed to treat chronic pain (Doggrell, 2004). Moreover, since CKMs can be chemically synthesized and also produced via recombinant synthesis they are amenable to rational design modifications that introduce new or optimize already present pharmacological activities.

There are reports of African natives who use a decoct of the plant *Oldenlandia affinis* as an oral oxytocic (Grain, 1973; Pallaghy et al., 1994). The pharmacological active compound that mediates this effect is the CKM kalata B1. Therefore, an oral administration of CKM seems to be generally possible. Recently, the potential of CKM for oral administration has been investigated (Werle et al., 2006a). Three different CKM were evaluated regarding enzymatic stability, aggregation behaviour and permeation

behaviour. Two of the CKM permeated well through excised rat intestinal mucosa in comparison to other peptide drugs, however, all of them were affected by enzymatic degradation caused by gastro-intestinal (GI) proteolytic enzymes. In fact, enzymatic degradation (Woodley, 1994; Bernkop-Schnürch, 1998) is beside the absorption barrier (Bernkop-Schnürch and Fragner, 1996; Swarbrick and Boylan, 2002) the most important barrier for orally administered peptides and proteins. However, it has also been demonstrated that a substitution of theoretically preferred cleavage sites can lead to a stabilisation of CKM towards specific proteolytic enzymes (Werle et al., 2006a). Therefore, an exact knowledge of the susceptibility of CKM during GI passage is necessary to gain information regarding the feasibility of oral CKM administration. Furthermore, data gained in enzymatic degradation studies are important prerequisites for the modification of CKM in order to improve their enzymatic stability.

Two CKM with clinically relevant activities have been designed recently based on the scaffold of two plant CKMs with trypsin inhibitory activity (Christmann et al., 1999; Avrutina et al., 2005). SE-ET-TP-020 is a potent inhibitor of the thrombopoietin receptor (Krause et al., submitted) and SE-MC-TR-020 inhibits human mast cell tryptase (manuscript in preparation). It was the aim of the study to provide a precise characterisation of these novel CKM towards various luminally secreted proteolytic enzymes. In a first step, the two CKM as well as the model drug insulin were incubated at 37 °C in collected porcine gastric as well as small intestinal juice. The time dependent degradation was monitored. Then the stability of the two CKM and of insulin in presence of isolated proteolytic enzymes was evaluated to identify the most important luminally secreted GI enzymes responsible for SE-ET-TP-020 and SE-MC-TR-020 degradation.

Materials and methods

Materials

The novel cystine-knot microproteins were a kind gift from SELECORE GmbH, Göttingen, FRG. All other used materials were purchased from ACROS, SIGMA and WORTHINGTON and were of analytical grade.

HPLC analyses

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 (TFA) in distilled H_2O) and B (0.1% TFA in acteonitril). For SE-ET-TP-020 and insulin the following gradient was used: 0–10.5 min (80–50% A), 10.5–12 min (50–80% A) and 12–17 min (80% A). SE-MC-TR-020 was analysed with the following gradient: 0–10.5 min (90–50% A), 10.5–12 min (50–90% A) and 12–17 min (90% A). Peptides were analysed at 220 nm using a diode array detector.

Calculations were performed using a calibration curve with 6 calibrators (0.500–0.016 mg/ml), which correspond to a range of 100.00–3.125% of the initial CKM concentration used in the experiments. The area under the curve of SE-ET-TP, SE-MC-TR and insulin at 220 nm was determined. Values were calculated using linear regression; for calibration the same buffer as for the experiment was used. All experiments were performed at least in triplicate.

Preparation of porcine small intestinal and porcine gastric juice

Preparation of porcine small intestinal and porcine gastric juice was performed as described previously (Loretz et al., 2006). In brief, gastric and small intestinal juice from a freshly slaughtered pig was collected. To 15 ml of small intestinal juice, 5 ml of distilled water was added in order to reduce viscosity. These liquids were filtered through sterile gaze. Samples were purified by centrifugation in order to remove undigested nutrient particles. The supernatants were stored at −20 °C till further use.

Enzymatic stability of CKM and insulin towards porcine gastric juice

One milligram of CKM and insulin were dissolved in one millilitre of 0.08 M HCl (respectively) and the pH was adjusted to 2. To 120 µl of this solution 120 µl of porcine gastric juice (as described in above) was added. The experiment was performed at 37 °C under shaking (300 rpm) and a final pH of 2. 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 1 M NaOH. The samples were immediately cooled to 4 °C and afterwards analyzed by HPLC as described above.

Enzymatic stability of CKM and insulin towards porcine small intestinal juice

Porcine small intestinal juice (prepared as described above) was thawed at 4 °C, centrifuged and the pH was determined to be 8.5. To 120 µl of the CKM and insulin solution (50 mM phosphate buffer, pH 7.6) 120 µl of porcine small intestinal juice (37 °C) was 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 analysed via HPLC.

Enzymatic stability of CKM and insulin towards isolated luminally secreted proteases

Studies focussing on the enzymatic stability of CKM and insulin were performed similar as described previously (Werle et al., 2006b). Enzymatic degradation tests were performed with chymotrypsin (SIGMA, 52 BTEE units/mg solid), elastase (Worthington, 4.5 *N*-succinyl-L-alanyl-L-alanyl-L-alanine-*p*-nitroanilide units/mg solid), pepsin (Sigma, 4150 hemoglobin units/mg solid) and trypsin (SIGMA, 9,820 BAEE units/mg solid).

To 120 µl of a 1 mg/ml solution of CKM in phosphate buffer (50 mM, pH 6.5), 120 µl of chymotrypsin (6.6 BTEE units) and trypsin (536 BAEE U) dissolved in phosphate 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 phosphate buffer (50 mM, pH 6.5) to obtain a concentration of 0.036 *N*-succinyl-L-alanyl-L-alanyl-L-alanine-*p*-nitroanilide U per ml. Referring to the poor solubility of insulin at neutral or slightly low pH values, 1 mg of insulin (SIGMA, from bovine pancreas; 28.5 USP units/mg) was dissolved in 1.0 ml of 50 mM phosphate buffer pH 7.5. The pH of this solution was adjusted to pH 7.5. The solution was centrifuged and the supernatant was used for the experiments. Insulin degradation studies utilizing pancreatic proteases were carried out in 50 mM phosphate buffer, pH 7.5. The concentration of the three utilized proteases chymotrpysin, trypsin and elastase was in the physiological range present in the intestinal fluid. According to the preparation instruction for artificial gastric juice of the USP 384 µg of pepsin were dissolved in 120 µl of

0.08 M HCl. According to the USP, the pH was about 1.5. This solution was added to 120 µl of the peptide solution (1 mg peptide in 1 ml 0.08 M HCl; pH 1.5). 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 for CKM; 0, 15, 60, 120 and 180 min for insulin) aliquots (30 µl) were withdrawn and the reaction was stopped immediately by the addition of 30 µl of 0.1% trifluoracetic acid solution to the intestinal protease solutions and with 30 µl of 0.1 M NaOH to the pepsin solution. The samples were cooled to 4 °C and afterwards analysed by HPLC as described in above.

Statistical data analysis

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

Results and discussion

In Fig. 1 the amino acid sequences of SE-ET-TP-020 and SE-MC-TR-020 as well as the theoretically preferred cleavage sites of chymotrypsin, trypsin and elastase are provided. According to this figure, SE-ET-TP-020 has one chymotrypsin, three trypsin, 14 elastase and two pepsin cleavage sites and SE-MC-TR-020 has one chymotrypsin, 8 trypsin, 10 elastase and two pepsin cleavage sites. Predicting the enzymatic stability of peptide drugs based exclusively on theoretically preferred protease cleavage sites might be misleading. It has been demonstrated that the theoretically preferred cleavage sites do not generally correlate with data gained in enzymatic degradation studies (Werle et al., 2006a). This might be due to the folding of peptides and proteins. Especially in the case of CKM folding is believed to play an important role; theoretical cleavages sites might be hidden in the cystine-knot or the knotted shape might just not fit into the binding site of the proteolytic enzyme. However, knowledge regarding the theoretical cleavage site is important: in a previous study the single theoretical cleavage site of a CKM, namely a phenylalanine, was substituted by isoleucine in order to remove the cleavage site. The novel CKM lacking the chymotrypsin cleavage site was not degraded anymore by this proteolytic enzyme (Werle et al., 2006a).

To gain first and meaningful data regarding the stability of the two novel CKM towards their stability in the GI tract, studies in the presence of collected porcine gastric as well as small intestinal juice were performed. As shown in Fig. 2, the model drug insulin is degraded entirely within a few minutes in gastric juice, whereas still about 60% of intact SE-ET-TP-020 could be detected within a 3 h incubation period. SE-MC-TR-020 was not significantly degraded. Interestingly, these data correlate to some extent with data of a previous study. There it has been demonstrated that CKM with 29 and 30 amino acids were not degraded by pepsin, whereas a CKM with 37 amino acids was degraded to some extent. SE-ET-TP-020 consists also of 37 amino acids, and SE-MC-TR-020 consists of 32 amino acids. In Fig. 3 the degradation of the two novel CKM as well as insulin in the presence of collected porcine small intestinal juice is shown. SE-ET-TP-020 as well as insulin is entirely degradation within a few minutes, whereas SE-MC-TR-020 is degraded within 2 h.

Data aquisition with collected GI fluids of animals is of particular interest because the fluids are composed of a mixture of a broad variety of proteolytic enzymes and are therefore closer to the in vivo situation in comparison to artificial GI fluids or to enzyme solutions containing only a single proteolytic enzyme. However, to get an in-depth understanding of the involved proteolytic enzymes and therefore information regarding the possible points of cleavage, studies with isolated proteolytic enzymes are important. In Fig. 4, the stability of the novel two CKM and insulin in the presence of pepsin is shown. SE-ET-TP-020 as well as insulin is rapidly degraded, whereas SE-MC-TR-020 is not significantly degraded. Theses results also correlate with the data provided in Fig. 2 where the stability of the peptides in presence of collected gastric juice is shown. Figure 5 shows the degradation of the peptides in a solution containing chymotrypsin. Also in the presence of chymotrypsin SE-ET-TP-020 and insulin are rapidly degraded. After 3 h of incubation, still about 60% of intact SE-MC-TR-020 could be detected. As shown in Fig. 1, SE-MC-TR-020 has only one single theoretical cleavage site, the tyrosine in position 30. A substitution of this amino acid with for example D-tyrosine or another non-aromatic L-amino acid is believed to completely stabilize SE-MC-TR-020 towards chymotryp-

SE-ET-TP-020:

```
                                                                    P P
G÷C–I÷E–G÷P–T–L÷R÷Q–W–L÷A÷A÷R÷A÷C–K÷Q–D–S–D–C–L÷A÷G÷C–V÷C–G–P–N–G÷F÷C–G÷S
 E   E   E     E T     E E E T E   T           E E E   E         E C   E
```

SE-MC-TR-020:

```
                                                                  P P
K÷K÷V÷G÷V÷C–P–K÷I÷L÷K÷K÷C–R÷R÷D–S–D–C–P–G÷A÷C–I÷C–R÷G÷N–G÷Y÷C–G
 T T E E E     T E E T T   T T           E E   E   T E   E C
```

Fig. 1. Amino acid sequences of SE-MC-TR-020 and SE-ET-TP-020 and the theoretically preferred cleavage sites of chymotrypsin (*C*), trypsin (*T*), elastase (*E*) and pepsin (*P*)

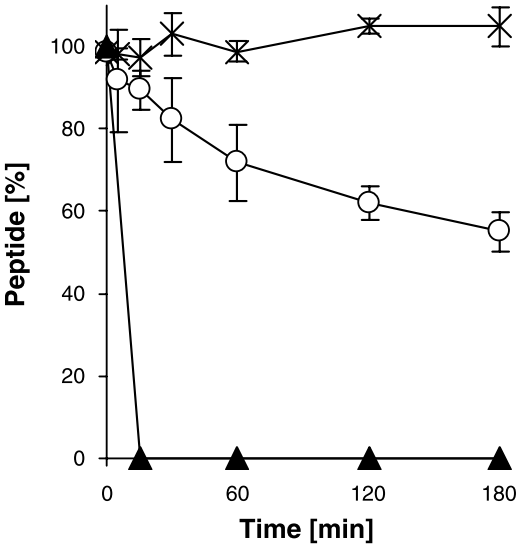

Fig. 2. Degradation of CKM and insulin by porcine gastric juice (-○- SE-ET-TP-020, - × - SE-MC-TR-020, -▲- insulin); each point represents the mean ± S.D. of at least $n = 3$ experiments

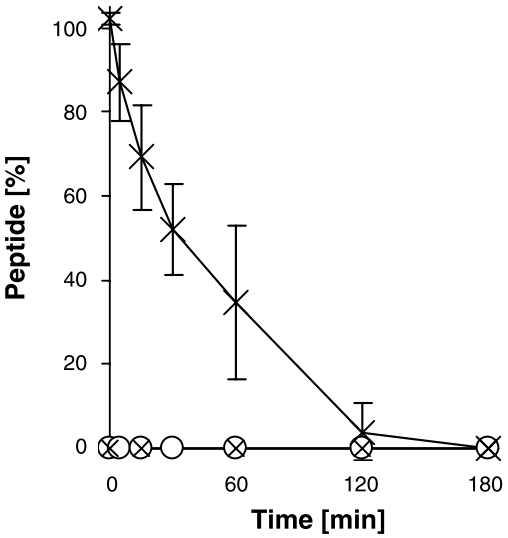

Fig. 3. Degradation of CKM and insulin by porcine small intestinal juice (-○- SE-ET-TP-020, - × - SE-MC-TR-020, ⊗ insulin); each point represents the mean ± S.D. of at least $n = 3$ experiments

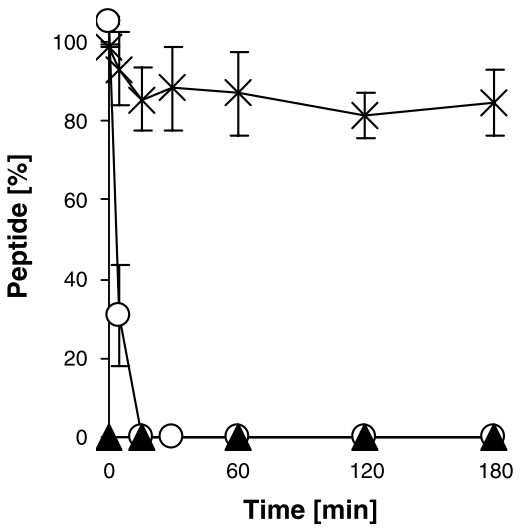

Fig. 4. Degradation of CKM and insulin by pepsin (-○- SE-ET-TP-020, - × - SE-MC-TR-020, -▲- insulin); each point represents the mean ± S.D. of at least $n = 3$ experiments

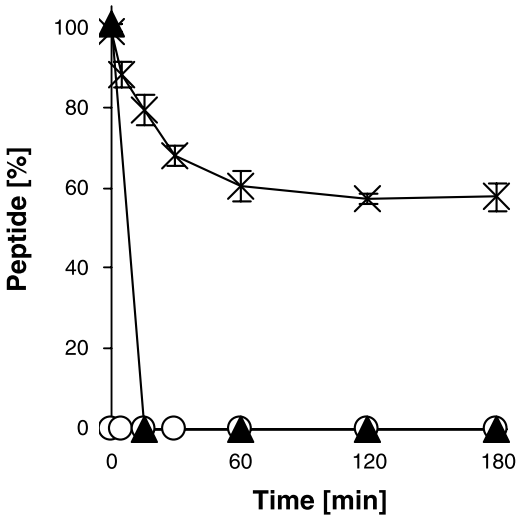

Fig. 5. Degradation of CKM and insulin by chymotrypsin (-○- SE-ET-TP-020, - × - SE-MC-TR-020, -▲- insulin); each point represents the mean ± S.D. of at least $n = 3$ experiments

sin. As mentioned above, the feasibility of the stabilisation of a CKM towards chymotrypsin by substituting the single cleavage site has already been demonstrated previously. Also trypsin degrades SE-ET-TP-020 and insulin very rapidly, whereas SE-MC-TR-020 is not significantly affected by this proteolytic enzyme (Fig. 6), although the amino acid sequence of SE-MC-TR-020 displays 8 theoretical cleavage sites for trypsin. SE-MC-TR-020 shows the highest stability towards trypsin of the CKM evaluated so far. In Fig. 7 the degradation of the two novel CKM and insulin in the presence of elastase is shown. SE-ET-TP-020 is degraded to some extent by elastase, whereas SE-MC-TR-020 is not degraded at all. Theses results also correlate with previous published results, where CKM with about 30 amino acids were not degraded by elastase, whereas the CKM with 37 amino acids was degraded by elastase.

Beside luminally secreted proteolytic enzymes, also membrane bound proteolytic enzymes are important for an intended oral administration of the CKM. Membrane bound proteolytic enzymes such as aminopeptidase N, dipeptidyl peptidase IV or carboxypeptidase M and P

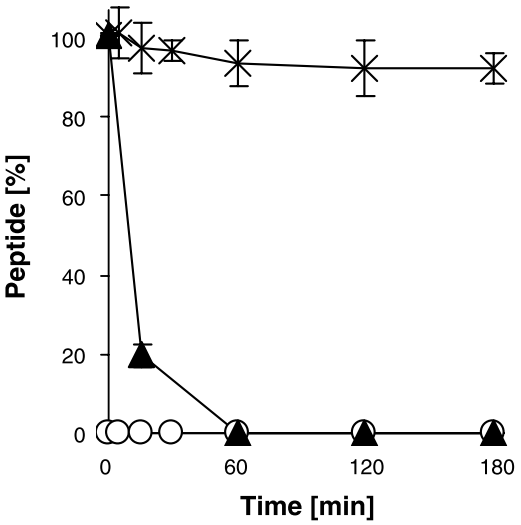

Fig. 6. Degradation of CKM and insulin by trypsin (-○- SE-ET-TP-020, -×- SE-MC-TR-020, -▲- insulin); each point represents the mean ± S.D. of at least $n = 3$ experiments

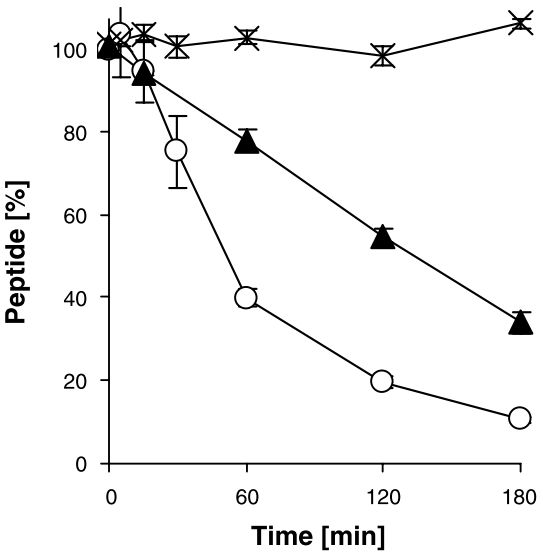

Fig. 7. Degradation of CKM and insulin by elastase (-○- SE-ET-TP-020, -×- SE-MC-TR-020, -▲- insulin); each point represents the mean ± S.D. of at least $n = 3$ experiments

are exopeptidases. Strategies to protect the termini of peptides and proteins have been discussed previously (Werle et al., 2006c). However, detailed studies addressing the susceptibility of the novel CKM towards membrane bound enzymes seem to be necessary.

Regarding the enzymatic stability of SE-MC-TR-020, an oral delivery of this CKM seems feasible, especially when taking into account the possibility of a substitution of the single amino acid tyrosine at position 30. Oral delivery of SE-ET-TP-020 seems to be more challenging. However, within this study the degradation products were not identified, so it might be possible that certain degradation products might also exhibit pharmacological activity. Due to the multitude of potential CKM, it seems more important to first focus on the most promising candidates for oral delivery and to evaluate degradation products in further studies.

Conclusion

Within the current study the stability of two novel CKM – SE-ET-TP-020 and SE-MC-TR-020 – towards luminally secreted proteolytic enzymes of the GI tract has been investigated. SE-ET-TP-020 was degraded by chymotrypsin, trypsin, elastase and pepsin and consequently also in collected porcine gastric as well as small intestinal juice. An oral delivery of SE-ET-TP-020 therefore seems to be very challenging. In contrast, SE-MC-TR-020 was relatively stable in presence of collected porcine gastric juice, trypsin, elastase and pepsin. The degradation observed in collected porcine small intestinal juice was in first instance caused by chymotrypsin. It seems feasible to modify SE-MC-TR-020 in order to stabilise it towards this specific proteolytic enzyme and therefore the development of highly stable SE-MC-TR-020 analogues for oral administration seems to be possible.

References

Avrutina O, Schmoldt HU, Gabrijelcic-Geiger D, Le Nguyen D, Sommerhoff CP, Diederichsen U, Kolmar H (2005) Trypsin inhibition by macrocyclic and open-chain variants of the squash inhibitor MCoTI-II. Biol Chem 386: 1301–1306

Bernkop-Schnürch A (1998) The use of inhibitory agents to overcome the enzymatic barrier to perorally administered therapeutic peptides and proteins. J Control Rel 52: 1–16

Bernkop-Schnürch A, Fragner R (1996) Investigations into the diffusion behaviour of polypeptides in native intestinal mucus with regard to their peroral administration. Pharm Sci 2: 361–363

Christmann A, Walter K, Wentzel A, Kratzner R, Kolmar H (1999) The cystine knot of a squash-type protease inhibitor as a structural scaffold for *Escherichia coli* cell surface display of conformationally constrained peptides. Protein Eng 12: 797–806

Colgrave ML, Craik DJ (2004) Thermal, chemical, and enzymatic stability of the cyclotide kalata B1: the importance of the cyclic cystine knot. Biochemistry 43: 5965–5975

Craik DJ, Daly NL, Bond T, Waine C (1999) Plant cyclotides: a unique family of cyclic and knotted proteins that defines the cyclic cystine knot structural motif. J Mol Biol 294: 1327–1336

Craik DJ, Daly NL, Waine C (2001) The cystine knot motif in toxins and implications for drug design. Toxicon 39: 43–60

Craik DJ, Cemazar M, Daly NL (2006) The cyclotides and related macrocyclic peptides as scaffolds in drug design. Curr Opin Drug Discov Devel 9: 251–260

Doggrell SA (2004) Intrathecal ziconotide for refractory pain. Expert Opin Investig Drugs 13: 875–877

Grain L (1973) Isolation of oxytocic peptides from Oldenlandia affinis by solvent extraction of tetraphenylborate complexes and chromatography on sephadex LH-20. Lloydia 36: 207–208

Kratzner R, Debreczeni JE, Pape T, Schneider TR, Wentzel A, Kolmar H, Sheldrick GM, Uson I (2005) Structure of Ecballium elaterium trypsin inhibitor II (EETI-II): a rigid molecular scaffold. Acta Crystallogr D Biol Crystallogr 61: 1255–1262

Loretz B, Föger FA, Werle M, Bernkop-Schnürch A (2006) Oral gene delivery: strategies to improve stability of pDNA towards intestinal digestion. J Drug Targ 14: 311–319

Pallaghy PK, Nielsen KJ, Craik DJ, Norton RS (1994) A common structural motif incorporating a cystine knot and a triple-stranded beta-sheet in toxic and inhibitory polypeptides. Protein Sci 3: 1833–1839

Swarbrick J, Boylan JC (2002) Encyclopedia of pharmaceutical technology, 2nd ed. Marcel Dekker, Inc., New York, p 885

Werle M, Schmitz T, Huang H, Wentzel A, Kolmar H, Bernkop-Schnürch A (2006a) The potential of cystine-knot microproteins as novel pharmacophoric scaffolds in oral peptide drug delivery. J Drug Targ 14: 137–146

Werle M, Samhaber A, Bernkop-Schnürch A (2006b) Degradation of teriparatide by gastro-intestinal proteolytic enzymes. J Drug Targ 14: 109–115

Werle M, Bernkop-Schnürch A (2006c) Strategies to improve plasma half life time of peptide and protein drugs. Amino Acids 30: 351–367

Woodley JF (1994) Enzymatic barriers for GI peptide and protein delivery. Crit Rev Ther Drug Carrier Syst 11: 61–95

Authors' address: Martin Werle, Ph.D., Department of Pharmaceutical Technology, Institute of Pharmacy, Innrain 52, 6020 Innsbruck, Austria, Fax: +43-512-507-2933, E-mail: m.werle@thiomatrix.com