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Synthesis and evaluation of a modified mucoadhesive polymer protecting from α-chymotrypsinic degradation

A. Bernkop-Schnürch*, I. Apprich

Center of Pharmacy, Institute of Pharmaceutical Technology, University of Vienna, Althanstr. 14, A-1090 Vienna, Austria

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Abstract

A number of chymostatin-poly(acrylic acid) conjugates, having the inhibitor linked via a 1,8-diaminooctane spacer, and a Bowman-Birk inhibitor (BBI)-poly(acrylic acid) conjugate were synthesised and their protective effect from enzymatic degradation caused by α-chymotrypsin as well as their mucoadhesive properties were evaluated. Whereas unmodified neutralised poly(acrylic acid) did not show any inhibitory effect under our enzyme assay conditions, 1 mg of the chymostatin-polymer conjugate exhibiting the highest protective effect was capable of inhibiting the proteolytic activity of 4 ± 0.7 U (BTEE) of α-chymotrypsin by 50% ($n = 3$; ± S.D.). This inhibitory effect was equivalent to 4.6 μg ± 0.6 μg ($n = 4$; ± S.D.) of unbound chymostatin which was added to the unmodified neutralised polymer in the same assay. Although the protective effect of the BBI-poly(acrylic acid) conjugate was even 17% higher, its mucoadhesive strength was 35.2% lower than the unmodified polymer. However, the mucoadhesive force of the chymostatin-poly(acrylic acid) conjugate exhibiting the highest protective effect was not influenced by the ligand. According to these results, the novel mucoadhesive chymostatin-poly(acrylic acid) conjugate protecting inserted therapeutic agents from α-chymotrypsinic degradation may be a useful tool for the peroral peptide and protein administration. © 1997 Elsevier Science B.V.

Keywords: Mucoadhesive polymer; α-Chymotrypsin; Chymostatin; Luminal degradation; Peroral administration of (poly)peptides

1. Introduction

Progress in bio- as well as gentechnology has produced many (poly)peptides available for pharmaceutical use. Consequently, delivery systems for the oral administration of these therapeutic agents are highly desirable. But in order to establish such systems, several problems encountered with the peroral route of application have to be solved. Beside the barrier function of the mucus covering gastrointestinal epithelia (Bernkop-

* Corresponding author. Tel.: +43 1 313368476; fax.: +43 1 31336779; e-mail: andreas.bernkop-schnuerch@univie.ac.at

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Schnürch and Fragner, 1996), proteolysis caused by membrane bound enzymes, degradation during absorption and the hepatic first pass metabolism, the rapid luminal enzymatic degradation can be seen as a main factor for a very low bioavailability. Attempts to reduce this barrier include analogues, prodrugs, formulations such as nanoparticles, microspheres and liposomes that shield (poly)peptide drugs from luminal enzymatic attack and the design of delivery systems targeting to the colon where the proteolytic activity is relatively low.

In addition, considerable interest has been shown in the use of enzyme inhibitors (Morishita et al., 1992a,b), protecting from luminal enzymatic degradation. But although these auxiliary agents promise a sufficient protective effect to exclude proteolysis of perorally administrated peptide and protein drugs, remarkable drawbacks make their practical use quite questionable. On one hand, the inhibition of luminal enzymes leads to an unintended disturbance of digestion of nutritive proteins and from case to case to an inhibitor-induced pancreatic hypersecretion caused by a luminal feedback regulation (Watanabe et al., 1992; Nitsan and Nir, 1986). On the other hand, perorally administrated enzyme inhibitors will be absorbed in the gastrointestinal tract and can therefore cause systemic toxic side effects (Grinde and Seglen, 1980; Yagi et al., 1980).

A possible solution in order to eliminate these disadvantages might be the immobilisation of enzyme inhibitors on matrix systems, which provide an intimate and prolonged contact to the absorbing membrane in the intestine. Covalently bound above all to mucoadhesive polymers (Lehr, 1994), inhibitors remain concentrated on the drug delivery system and are therefore unabsorbable (Bernkop-Schnürch and Dundalek, 1996). According to this strategy, it was the aim of this study to synthesise and evaluate a modified mucoadhesive polymer shielding inserted therapeutic (poly)peptides from enzymatic attack.

Luminal enzymatic degradation is mainly caused by trypsin, chymotrypsin, elastase, carboxypeptidase A and B (Woodley, 1994). Moreover, within this group of enzymes, trypsin and α-chymotrypsin are the major proteases responsible for peptide and protein degradation. As the mucoadhesive polymer poly(acrylic acid) offers the capability to inhibit per se the intestinal proteases trypsin and carboxypeptidase A (Lueßen et al., 1994, 1995) as well as the advantage of high binding affinity to mucin-epithelial surfaces (Smart et al., 1984), it was chosen as carrier matrix. In order to extend the protective properties of this polymer, it was slightly modified to provide also an inhibitory effect towards α-chymotrypsinic degradation. Hence, chymostatin, which represents a useful inhibitor of α-chymotrypsin (Umezawa et al., 1970), was covalently immobilised to this polymer.

The development of a mucoadhesive polymer protecting from proteolysis caused by trypsin and α-chymotrypsin as well should promise new aspects in the peroral administration of therapeutic peptides and proteins, e.g. insulin (Schilling and Mitra, 1991), calcitonin (Kobayashi et al., 1994), neurotensin analogues (Aungst and Phang, 1995) and basic fibrinoblast growth factor (Fukunaga et al., 1994) which are mainly degraded by these luminal enzymes.

2. Materials and methods

2.1. Synthesis of the chymostatin-spacer conjugate

The spacer 1,8-diaminooctane (Sigma, St. Louis, MO) was coupled to chymostatin (Sigma, St. Louis, MO) by a condensation reaction under the use of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC; Sigma, St. Louis, MO) catalysed by sulfo-*N*-hydroxysuccinimide (SNHS; Pierce, Oud-Beijerland, NL) following the procedure described by Staros et al. (1986). The carboxylic acid group of the inhibitor was covalently bound to the NH_2-group of the spacer by constituting an amide binding. The molar ratio of 1,8-diaminooctane to chymostatin was 10:1 to promote the binding of chymostatin to only one primary amino group of the spacer. Whereas 7.5 mg of 1,8-diaminooctane were suspended in 2.1 ml of demineralised water, 3 mg of chymostatin were dissolved in 900 μl of THF. After the pH value of both solutions had

Table 1
Concentrations of reagents and solvents used for reaction mixtures in order to obtain poly(acrylic acid)-derivatives with increasing amounts of covalently attached chymostatin

Resulting chymostatin-polymer conjugates	Poly(acrylic acid) (mg)	*N,N*-dimethyl-formamide (μl)	SNHS + EDAC in 20 μl demin. water (mg)	Concentrated chymostatin-spacer conjugate solution (μl)
Conjugate 1:1	1.0	700	1.3 + 24	500
Conjugate 1:3	3.0	700	1.3 + 24	500
Conjugate 1:9	4.5	750	1.1 + 20	250
Conjugate 1:20	5.0	875	1.1 + 20	125
Conjugate 1:50	5.0	950	1.1 + 20	50
Conjugate 1:100	5.0	975	1.1 + 20	25

been adjusted to pH 5.5 with HCl (1 N), they were added to 60 mg of EDAC and 3.3 mg of SNHS. The reaction mixture was stirred for 12 h at room temperature and the resulting conjugate was isolated by preparative TLC [layer: aluminium sheets silica gel 60 F_{254} (Merck, Darmstadt, Germany); layer thickness: 0.2 mm; mobile phase: *n*-butanol/acetic acid/H_2O (8 + 2 + 2); migration distance: 8 cm; detection: spraying with ninhydrin-reagent (0.3 g ninhydrin, 100 ml *n*-butanol, 3 ml acetic acid) and heating to 100°C for 5 min or dipping in $AgNO_3$/$KMnO_4$-reagent (10 ml of $AgNO_3$ (0.1 N), 10 ml of NH_3 (2 N) and 20 ml of NaOH (2 N) added before used to 0.2 g of $KMnO_4$ and 0.4 g of Na_2CO_3 dissolved in 40 ml of demineralised water); R_f of the conjugate: 0.55–0.6]. Between a R_f of 0.50 and 0.65 the silica gel was scraped off the aluminium sheets. The conjugate was eluted from the separated silica gel with in all 4.5 ml of methanolic HCl (0.5 mN) and concentrated (rotavapor: Heidolph WB 2001; 25°C) up to 1.5 ml. This concentrated chymostatin-spacer conjugate solution was directly used for the coupling reaction of chymostatin to poly(acrylic acid).

2.2. Immobilisation of chymostatin on poly(acrylic acid)

The isolated chymostatin-spacer conjugate was coupled to poly(acrylic acid) (Carbopol 940; Goodrich, Cleveland, OH) by a condensation reaction as described above. The remaining NH_2-group of the spacer was covalently bound to a carboxyl group of the polymer by constituting an amide binding. Reaction mixtures as listed in Table 1 were incubated at room temperature for 3 h under permanent stirring. The resulting chymostatin-polymer conjugates—namely conjugate 1:1, 1:3, 1:9, 1:20, 1:50 and 1:100—were separated by centrifugation (10 min; 13000 rpm; Hermle Z 323 K). The supernatants, containing unbound inhibitor and coupling reagents, were removed. The remaining pellets were suspended in acetic acid/*N,N*-dimethylformamide (1 + 9). This purification step by suspending the pellets in the solvent, centrifugation and removing the supernatants was repeated seven times. After this, pellets were suspended in the same solvent but containing 25% demineralised water and separated by centrifugation. The share of demineralised water in the solvent was increased by 25% at each following purification step and conjugates were finally washed three times in the same way with demineralised water. The isolated inhibitor-matrix conjugates were neutralised with NaOH (1 N) and lyophilised.

2.3. Synthesis of the Bowman-Birk inhibitor-polymer conjugate

The Bowman-Birk inhibitor (BBI; Sigma, St. Louis, MO) was covalently bound to poly(acrylic acid) by a condensation reaction of the available amino residues of the protein with the carboxyl groups of the polymer as previously described by Bernkop-Schnürch and Göckel (1997). The share of the inhibitor in the coupling product was determined to be 10.9% (m/m).

2.4. Evaluation of the inhibitory effect of the conjugates towards enzymatic degradation

Then, 0.33 mg of the inhibitor-polymer conjugates or of the unmodified neutralised polymer as previously described by Bernkop-Schnürch and Göckel (1997) were hydrated in 0.3 ml of 80 mM Tris-HCl pH 7.8 containing 0.9% NaCl. In order to determine the amount of chymotrypsin which will be inhibited to 50% by tested conjugates, in steps of 0.2 U increasing amounts of chymotrypsin (52 BTEE U/mg; type II: from bovine pancreas, Sigma, St. Louis, MO) dissolved in 30 μl of the same buffer were added. After an incubation period of 30 min at 20°C, 0.3 ml of the substrate solution (18.5 mg of *N*-benzoyl-L-tyrosine ethyl ester dissolved in 31.7 ml of methanol and 18.3 ml of demineralised water) were added and the increase in absorbance ($\Delta A_{256\ nm}$) was recorded (Lambda-16; Perkin-Elmer; Vienna, A) at 1 min intervals for 10 min. In order to evaluate the amount of unbound chymostatin, which is in its protective effect equivalent to the matrix bound inhibitor, conjugates were substituted in this assay by the unmodified neutralised polymer and increasing chymostatin concentrations.

2.5. Tensile studies

Tensiometer studies were carried out in a slightly modified way as described by Mortazavi and Smart (1995). Neutralised poly(acrylic acid) (30 mg) were compressed (Hanseaten Type EI, Hamburg, Germany) into 5.0 mm diameter flat-faced discs. One side of these discs was either press coated with 10 mg of inhibitor-polymer conjugates or the unmodified polymer which was used as reference. The pressing power was kept constant during the preparation of all discs.

Porcine small intestine was obtained fresh from slaughter, longitudinally dissected and washed gently with 50 mM phosphate buffered saline (PBS) pH 7.2 to remove intestinal content. The mucosal surface was then individually mounted on a platform of 24 mm diameter in PBS pH 7.2 at 37°C and secured in place with a clamp. It was set on a balance (Mettler PC 4400) which was placed on a moving platform. The discs were individually attached with their uncoated side to this mucosal surface suspending 1.5 g weight using a cyanoacrylate adhesive. The platform was raised up till the test disc attached to the intestinal mucosa and after 2 min lowered at a rate of 2 mm/min, until the test disc pulled clear of the membrane. The maximum detachment force at which adhesive bond failed was recorded.

3. Results

3.1. Preparation of chymostatin-polymer conjugates

Since chymostatin exerts its inhibitory activity through the terminal located aldehyde residue, the 1,8-diaminooctane spacer providing the free availability of the subsequently matrix attached inhibitor was bound at the opposite end of the molecule (Fig. 1). This was achieved by forming an amide binding of one amino group of the spacer to the terminal COOH-group of the inhibitor. The isolated chymostatin-spacer conjugate had a R_f of 0.55–0.6, which is between the R_f of chymostatin (0.66–0.7) and 1,8-diaminooctane (0.2–0.3). Although pure chymostatin was not detectable with ninhydrin spraying reagent, the band of the conjugate was ninhydrin positive. Because of the selectivity of this reagent for primary amines, the successful coupling of the spacer

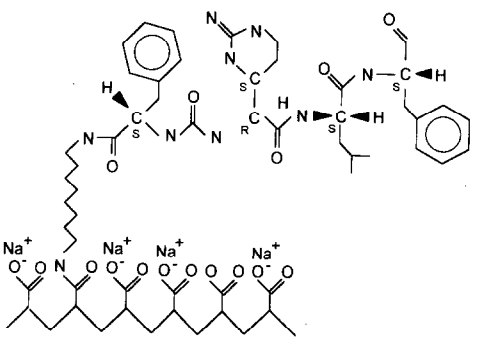

Fig. 1. Structure of the chymotrypsin inhibiting moiety bound to poly(acrylic acid); covalent attachment was achieved by the constitution of an amide binding of the available amino residue exhibited by the chymostatin-spacer conjugate with a carboxylic acid residue of poly(acrylic acid).

to the inhibitor as well as the free availability of the remaining NH_2-group after the coupling reaction could be verified. Moreover, the band of the conjugate was $AgNO_3/KMnO_4$ positive, demonstrating the remaining of the aldehyde-function on the inhibitor. TLCs of reaction mixtures prepared at −20°C were used as negative control and did not exhibit the band of the conjugate. After isolation by preparative TLC, the chymotrypsin-spacer conjugate appeared as a ninhydrin—as well as $AgNO_3/KMnO_4$-reagent positive band on the gel. It could be dissolved in a methanolic solution of HCl (1 mM) and showed a chymotrypsin inhibiting activity which was comparable to the unbound pure inhibitor. The constitution of a stable imine-structure of the primary amine of the spacer and the aldehyde could therefore be excluded.

This inhibitor-spacer conjugate was covalently attached to poly(acrylic acid) by a condensation reaction as described above. The successful coupling of the inhibitor to the polymer could be verified by the strong inhibitory effect as described below and the $KMnO_4$ reducing capability of the isolated conjugates, whereas the unmodified polymer did not exhibit these properties.

3.2. Inhibitory efficiency of the conjugates

In the concentration used for the enzyme assay developed by us, unmodified poly(acrylic acid) did not exhibit any inhibitory effect against α-chymotrypsinic degradation. However, chymostatin-polymer conjugates showed strong inhibitory properties towards α-chymotrypsin. A polymer prepared and purified in the same way as conjugates but using chymostatin instead of the chymostatin-spacer conjugate during the coupling reaction showed no inhibitory effect, verifying the successful immobilisation of the inhibitor and the efficiency of our method of isolating conjugates as well. The evaluation of the protective effect of conjugates resulting from different polymer inhibitor ratios during the coupling reaction, demonstrated an increase in the protective effect as the ratio of the inhibitor was raised. Whereas conjugate 1:100 and 1:50 did not show any inhibitory effect, conjugate 1:20 was capable of inhibiting the proteolytic activity of 4 ± 0.7 U

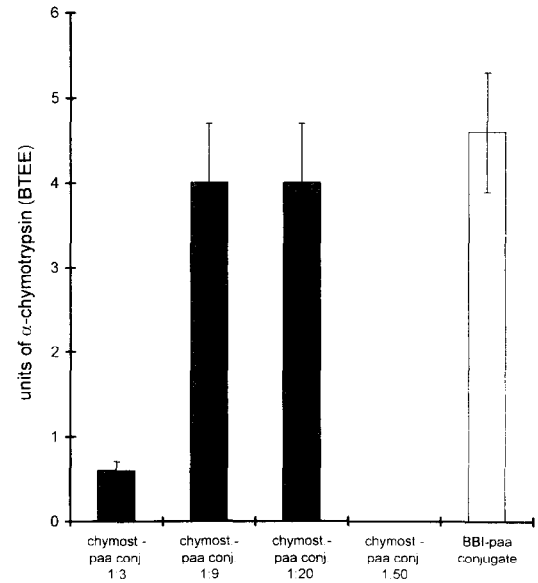

Fig. 2. Comparison of the protective effect of different chymostatin-poly(acrylic acid) (paa) conjugates (hatched bars) with the BBI-paa conjugate (blank bar). The protective effect was determined by units of α-chymotrypsin (BTEE), which have to be added to 1 mg of derivative in order to obtain a 50% inhibition of proteolytic activity. Each bar represents the mean protective effect of at least three experiments ± S.D.

($n = 3$; ± S.D.) of α-chymotrypsin by 50% (Fig. 2). However, below a ratio of 1:20 no further increase in protective effect could be observed. Conjugates with a higher share of chymostatin than obtained at conjugate 1:9 had a reduced and no inhibitory efficiency, respectively.

Comparing the inhibitory effect of conjugates with increasing concentrations of unbound chymostatin in the same enzyme assay, showed the equivalent amounts of inhibitor, which have to be added to the unmodified neutralised polymer in order to obtain the same protective effect. Results are shown in Table 2.

3.3. Mucoadhesive properties of the conjugates

The mucoadhesive strength of neutralised poly(acrylic acid), the chymostatin-polymer conjugate 1:20 and the BBI-polymer conjugate as well were estimated by the determination of the maximum detachment force calculated by multiplying

Table 2
Mean amounts of unbound chymostatin which have to be added to 1 mg of neutralised poly(acrylic acid) (paa) in order to obtain the same protective effect as of 1 mg of the according inhibitor-paa conjugate ($n = 4$)

Test material	Chymostatin (μg)	S.D.
BBI-paa conjugate	5.4	0.8
Chymost.-paa conjugate 1:1	0.0	—
Chymost.-paa conjugate 1:3	0.7	0.2
Chymost.-paa conjugate 1:9	4.6	0.6
Chymost.-paa conjugate 1:20	4.6	0.6
Chymost.-paa conjugate 1:50	0.0	—
Chymost.-paa conjugate 1:100	0.0	—

the measured decrease of weight in grams (minimum displayed weight minus displayed weight after complete detachment) by acceleration due to gravity. Results are listed in Table 3. Comparing press coated with uncoated test discs of the same weight (40 mg) did not show any measurable differences in the mucoadhesive strength.

4. Discussion

Compared with other serine protease inhibitors, e.g. elastatinal and antipain, chymostatin is due to an iso-butyl- and two benzyl-residues as shown in Fig. 1 a lipophilic agent (Kambara et al., 1982), exhibiting poor solubility in water. Hence, in order to obtain a high coupling rate of the inhibitor to the polymer, the use of a suitable solvent was necessary, which guarantees sufficient solubility of chymostatin as well as poly(acrylic acid) during the coupling reaction. *N,N*-Dimethylformamide can be diluted with water and is a good solvent for both reactants especially at low pH values. Moreover, it has no disturbing influence on the enzyme assay described here, as other organic solvents do, e.g. DMSO. For these reasons it was chosen as solvent for the second coupling reaction and for isolation of conjugates.

Table 3
Comparison of the adhesive strength of neutralised poly(acrylic acid) with the BBI- and chymostatin-polymer conjugate

Test material	Maximum detachment force (mN)	S.D.
Control (no disc)	1.30	0.14
Neutralised poly(acrylic acid)	44.19	5.88
BBI-polymer conjugate	28.63	7.57
Chymost.-polymer conjugate 1:20	44.00	3.38

Maximum detachment force was determined in a pH 7.2 isotonic phosphate buffer at 37°C ($n = 4–8$)

The lipophilic character of the inhibitor also influenced the hydration behaviour of the chymostatin-polymer conjugate. Whereas the pH value has no influence on the hydration behaviour of unmodified neutralised poly(acrylic acid) in aqueous solutions, it was significant for conjugates. On one hand, due to ionisation of the basic capreomycidine ([*S,S*]-α-(2-Iminohexahydro-4-pyrimidyl)glycine) moiety of the covalently to the polymer bound inhibitor (Fig. 1), conjugates were hydratable at pH values below 5.5. On the other hand, conjugates were also hydratable at a pH above 7.0, due to the ionised remaining carboxylic acid residues of the polymer. Conjugates resulting from reaction mixtures with a high share of chymostatin (conjugate 1:1 and 1:3) were unhydratable or slightly unhydratable. With regard to the pH situation of the small intestine, the amount of inhibitor linked to the polymer therefore cannot be raised ad libidum, as the resulting poor hydratability of the conjugate leads to a significant reduction of its protective efficiency.

The depletion of Ca^{2+} by polyacrylates might inhibit several Ca^{2+} dependent luminal proteases. Poly(acrylic acid) is able to reduce the activity of the proteolytic enzyme trypsin as reported by Lueßen et al. (1994, 1995). Although chymotrypsin is also a Ca^{2+} containing serine protease exhibiting high similarities in its tertiary structure with trypsin (Hedstrom et al., 1994), we could not observe any inhibitory properties of the unmodified polymer towards this enzyme. In contrast, Lueßen et al. (1996) could determine a weak inhibitory effect of unmodified poly(acrylic acid) under their assay conditions. However, in their approach, using the same concentration but of the unneutralised polymer, α-chymotrypsinic activity (0.08 BTEE U/ml) was below the lowest activity (0.2 BTEE U/ml) tested by us.

According to theories proposed to explain mucoadhesion on the basis of hydration and swelling of polymers followed by chain interpenetration with a hydrated mucus layer (Ponchel et al., 1987; Leung and Robinson, 1990; Jabbari et al., 1993), a reduced force of adhesion for the chymostatin-poly(arcylic acid) conjugate due to its reduced hydratability had been expected by us. However, no differences in adhesion between the conjugate and the unmodified polymer could be observed, indicating that the slightly modification had no significant influence on mucoadhesive properties. In this connection, Sanzgiri et al. (1994) showed, that a 50% benzyl esterfication of hyaluronic acid leads only to a 27% reduction of the force of adhesion. According to this result, the theoretical maximum of only 0.7% modified carboxylic acid residues of the conjugate 1:20 might explain the uninfluenced adhesive properties of the slightly modified polymer. But beside hydrophilic functional groups other factors also, e.g. molecular mass, chain length, conformation and molecular flexibility have an important influence on mucoadhesive properties of polymers (Junginger, 1990). The covalent attachment of the Bowman-Birk inhibitor with a share of 10.9% in the conjugate caused a decrease of 35% in the force of adhesion. As this hydrophilic inhibitor has a molecular mass of 8 kDa (Birk, 1985) which is 13 times higher than the molecular mass of chymostatin (607.7 Da), the significant loss of mucoadhesion of the BBI-polymer conjugate might be explained by a reduced molecular motility and flexibility. Comparing the chymostatin-polymer conjugate 1:20 with the BBI-polymer conjugate, demonstrates on one hand a stronger mucoadhesion of the chymostatin-polymer conjugate and on the other hand a 17% stronger inhibitory effect of the BBI-polymer conjugate towards enzymatic degradation. Accordingly, the practical use of either the chymostatin- or the BBI-polymer conjugate in order to generate drug delivery systems for the peroral (poly)peptide application, will mainly depend on the priority demand made on the delivery system.

Out of its relative low production costs, 3-biphenylboronic acid also seems to be a promising candidate as inhibitor of α-chymotrypsin. Covalently bound to mucoadhesive polymers it should shield from chymotrypsinic attack. Recently, Suenaga et al. (1995) demonstrated, that the inhibitory effect of biphenylboronic acids could even be intensified by added saccharides. Whether this effect can also be achieved by modified polysaccharides, e.g. the mucoadhesive polymer sodium carboxymethyl cellulose used as carrier matrix and whether such systems subsequently lead to a comparable higher protective effect under uninfluenced mucoadhesive properties, should be subject of further investigations.

In summary, the modification of poly(acrylic acid) by the covalent attachment of chymostatin offers potential advantages in the peroral administration of mainly chymotrypsinic degradable therapeutic (poly)peptides. The immobilisation keeps the inhibitor concentrated on the matrix system, excludes an unintended disturbance of digestion of nutritive proteins and prevents its absorption followed by possible toxic side effects. Moreover, the mucoadhesive properties of the polymer were not influenced by the slightly modification. According to these results, the novel mucoadhesive polymer shielding from luminal enzymatic attack may be a useful tool for the peroral administration of mainly chymotrypsinic degradable therapeutic (poly)peptides.

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