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Development and in vitro evaluation of a drug delivery system protecting from trypsinic degradation

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Abstract

We have been developing a delivery system based on a novel polymer conjugate protecting perorally administered (poly)peptide drugs from trypsinic degradation. The trypsin inhibitor antipain was, therefore, covalently attached to the mucoadhesive polymer chitosan. The protective effect of resulting chitosan–antipain conjugates was quantified by an enzyme assay. In contrast to the unmodified polymer, chitosan–antipain conjugates exhibited a significant inhibitory effect towards enzymatic activity of trypsin (EC 3.4.21.4). Moreover, the mucoadhesive force of chitosan was not influenced by the slight modification. Based on a chitosan–antipain conjugate, a drug delivery system was generated using insulin as model drug. Tablets containing 5% polymer conjugate demonstrated after incubation with trypsin (180 spectrophotometric BAEE units/ml) for 1.5 h in lateral parts of the swelled dosage form a $13.3 \pm 2.3\%$ (mean $\pm$ S.D., $n = 3$) minor proteolysis of matrix embedded insulin compared to tablets lacking the polymer conjugate. In the inner part of the swelled dosage form containing the conjugate proteolysis was completely inhibited, whereas in control tablets $11.3 \pm 9.5\%$ (mean $\pm$ S.D., $n = 3$) insulin was degraded. Furthermore, a controlled drug release over a period of 6 h was guaranteed by the delivery system. According to these results, the novel chitosan–antipain conjugates shielding from luminal enzymatic attack may be a useful tool for the peroral administration of mainly trypsinic degraded peptide and protein drugs. © 1997 Elsevier Science B.V.

Keywords: Mucoadhesive polymer; Chitosan; Antipain; Trypsin; Peroral administration of (poly)peptides

1. Introduction

Peroral administration of peptide and protein drugs is favoured by patients, practitioners and the pharmaceutical industry for reasons of com-

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pliance, ease and economics. However, various barriers including the enzymatic (Woodley, 1994), diffusion (Bernkop-Schnürch and Fragner, 1996) and absorption barrier (Zhou, 1994) make the elaboration of an according galenic for this route of administration very difficult. The development of drug delivery systems providing a sufficient bioavailability of these therapeutic agents after oral dosing represents one of the greatest challenges for pharmaceutical technology in the years to come.

Besides various strategies, e.g. the use of formulations such as nanoparticles, microspheres and liposomes, peptide analogues and prodrugs (Bernkop-Schnürch, 1997), mucoadhesive polymers especially in combination with enzyme inhibitors have gained large interest for the development of peroral peptide and protein drug delivery systems. Mucoadhesive polymers, on the one hand, are able to localise the dosage form at the site of absorption, thereby decreasing the distance between the released drug from the delivery system and absorptive tissue (Junginger, 1990; Lehr, 1994), which leads to a reduced drug metabolism caused by luminal secreted proteases. However, although some mucoadhesive polymers, e.g. carbomer and polycarbophil, exhibit enzyme inhibitory properties towards various luminal proteases, this effect seems to be not sufficient for simple dosage forms in order to protect embedded therapeutic peptides and proteins from degradation (Akiyama et al., 1996).

On the other hand, enzyme inhibitors have received considerable attention as auxiliary agents to reduce luminal proteolysis of perorally administered peptide and protein drugs. However, it has been reported that enzyme inhibitors can lead to systemic toxic side effects (Yagi et al., 1980; Drapeau et al., 1992), 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) which make their practical use quite questionable. The immobilisation of these agents to an unabsorbable carrier matrix such as mucoadhesive polymers should therefore exclude this major drawback and provide the platform for peroral (poly)peptide delivery systems protecting from enzymatic attack and acting in a restricted area of drug absorption (Bernkop-Schnürch and Dundalek, 1996; Bernkop-Schnürch and Göckel, 1997).

As trypsin is generally regarded as one of the most abundant proteases of the intestine, it was the aim of the present study to develop a controlled drug release system, which protects embedded therapeutic peptides or proteins from trypsinic degradation. The specific trypsin inhibitor antipain was, therefore, covalently attached to the mucoadhesive polymer chitosan and the protective effect of the resulting conjugate was tested in a simple mucoadhesive dosage form using insulin as model drug.

2. Materials and methods

2.1. Synthesis of chitosan–antipain conjugates

The covalent attachment of antipain to chitosan was achieved by forming an amide binding of one amino group of the polymer to the terminal located carboxylic acid group of the inhibitor (Fig. 1). First, 1 g of chitosan (poly-[1→4]-β-D-glucosamine; Sigma, St. Louis, MO) was suspended in 90 ml of demineralised water. The pH value of this suspension was kept constant at pH

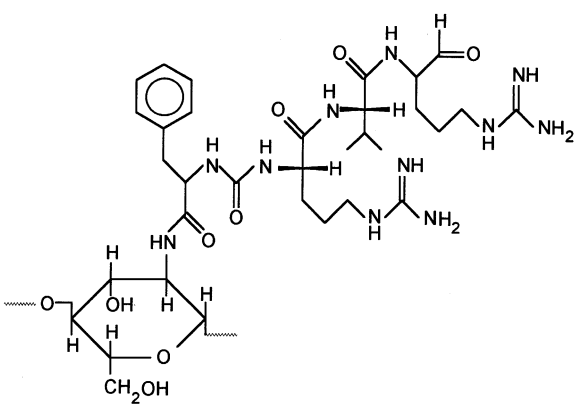

Fig. 1. Structure of the trypsin-inhibiting moiety bound to chitosan. Covalent attachment was achieved by the constitution of an amide binding of the carboxylic acid residue exhibited by antipain with an amino residue of the polymer.

Table 1
Concentrations of reagents used in reaction mixtures in order to obtain chitosan derivatives with increasing amounts of covalently attached antipain

Resulting conjugate	1% Chitosan HCl (ml)	Antipain (mg)	EDAC+SNHS (mg) dissolved in 1 ml demineralised water
Conjugate 1:15	2.0	4	80+4
Conjugate 1:60	2.0	1	80+4
Conjugate 1:240	2.0	0.25	80+4
Chitosan–antipain control	2.0	1	1 ml Demineralised water

3 by continuously adding 1 N HCl till the polymer was completely hydrated. Thereafter, the pH value was adjusted to 5.0 with 1 N NaOH and demineralised water was added to make the final volume 100 ml. Mixtures of antipain (Sigma), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC; Sigma) and sulfo-*N*-hydroxysuccinimide (SNHS; Pierce, Oud Beijerland, NL) in concentrations as listed in Table 1 were preincubated for 30 min at room temperature and added to 2.0 ml of this 1% chitosan HCl solution. Reaction mixtures were incubated for 4 h under permanent stirring at room temperature. Resulting polymer conjugates were isolated by dialysing against 1 mM HCl for 12 h and then exhaustively against demineralised water. Purified polymer conjugates were lyophilised and stored at −20°C until use.

2.2. Evaluation of the inhibitory effect of the polymer conjugates towards enzymatic degradation

First, 0.5 mg of the polymer-inhibitor conjugates or chitosan HCl pH 6.0 were hydrated in 0.7 ml of 80 mM TBS (Tris–HCl buffered saline) pH 7.8. Thereafter, trypsin (180 spectrophotometric BAEE units; United States Biochemical, Cleveland, OH) dissolved in 200 μl of 80 mM TBS pH 7.8 was added and the mixture incubated for 30 min at room temperature. After adding 0.2 mg of *N*-α-benzoyl-arginine ethylester (BAEE) dissolved in 200 μl of 80 mM TBS pH 7.8, the increase in absorbance ($\Delta A_{253\,nm}$) caused by the hydrolysis of this substrate to *N*-α-benzoyl-arginine (BA) was recorded (Lambda-16; Perkin-Elmer) at 1-min intervals for 10 min.

2.3. Tensile studies

First, 40 mg of each conjugate or lyophilised chitosan HCl pH 6.0 were compressed (Hanseaten Type EI, Hamburg, Germany) into 5.0 mm diameter flat-faced discs. The pressing power was kept constant during the preparation of all discs. Following this, tensiometer studies with these test discs were carried out on native porcine mucosa as described previously by Bernkop-Schnürch and Apprich (1997).

2.4. Preparation of the drug delivery system

As insulin is well known to be degraded by trypsin (Schilling and Mitra, 1991) it was chosen as model drug for the delivery system. Insulin from bovine pancreas (Sigma) was, therefore, suspended in demineralised water. In order to remove the zinc content from the polypeptide, 0.01 mmol of Na_2EDTA—exhibiting in low concentrations no influence on the degradation of insulin by serine proteases (Ikesue et al., 1993)—was added to this suspension and the pH value adjusted to 7.0 with 1 N NaOH. The resulting solution was lyophilised and the so called 'pre-treated insulin' stored at −20°C until use. The polymer conjugate 1:60 was chosen for the drug delivery system as it shows an inhibitory effect which is in a middle range of all tested chitosan–antipain conjugates. As listed in Table 2, pre-treated insulin, chitosan acetate, the polymer conjugate 1:60 and mannit were homogenised and pressed (Hanseaten, Type EI, Hamburg, Germany) to tablets (diameter: 5.0 mm; depth: ~2 mm). The pressing power was kept constant during the preparation of all tablets.

Table 2
Formulations used in trypsin inhibition experiments

Tablet	Chitosan acetate (mg) pH 6.0	Conjugate 1:60 (mg)	Mannit (mg)	Insulin (mg)
Without conjugate	30	—	15	5
With 2.5% conjugate	28.75	1.25	15	5
With 5% conjugate	27.5	2.5	15	5

2.5. Evaluation of the protective effect of the drug delivery system

To determine the degree of enzymatic degradation of insulin in the drug delivery system, tablets with and without the conjugate 1:60 were incubated for 1.5 h with 10 ml of 20 mM Tris–HCl pH 7.8 containing trypsin (1800 spectrophotometric BAEE units; United States Biochemical, Cleveland, OH) on a waterbath-shaker (GFL 1092; 30 rpm) at 37 ± 0.5°C. After this, hydrated matrices were withdrawn and frozen for 1 h at -20°C. Then, 30-mg aliquots were separated by dividing the frozen matrices with a scalpel in well defined parts.

These aliquots were diluted 1:5 with 0.1% trifluoroacetic acid used as stop-solution in order to terminate the enzymatic reaction. The remaining polymer content in these samples was removed by two times centrifugation ($20\,000 \times g$, 4°C, Hermle Z 323K). A portion (7 μl) of the supernatant fluid was directly injected for HPLC analysis (series 200 LC; Perkin-Elmer). Remaining traces of polymer were hold back on a pre-column (Nucleosil 100-10C18, 40×4 mm). Insulin and/or degradation products were separated on a C_{18}-column (Nucleosil 100-5C18, 250×4 mm) at 40°C. Gradient elution was performed as follows: flow rate 1.0 ml/min, 0–16 min; linear gradient from 91% A/9% B to 39% A/61% B (eluent A: 0.1% trifluoroacetic acid in water; eluent B: acetonitrile). (Poly)peptides were detected by absorbance at 210 nm as well as 285 nm with a diode array absorbance detector (Perkin-Elmer 235C). Degradation of insulin was calculated by following the ratio of the integrated peak area of remaining insulin to the integrated peak areas of degradation products.

2.6. In vitro release studies

The in vitro release rate of insulin from the drug delivery system was determined by a method which is not conform to the United States Pharmacopeia (USP). First, 150 mg of chitosan acetate pH 6.0, 25 mg of pre-treated insulin and 75 mg of mannit were hydrated or dissolved in 15 ml of demineralised water, homogenised and lyophilised. Aliquots of 50 mg were pressed to tablets as described above. These tablets were placed in 25 ml beakers (Schott, Duran 25 ml, Germany) containing 10 ml release medium (20 mM Tris–HCl pH 7.8). The vessels were closed, placed on a waterbath-shaker (GFL 1092; 30 rpm) and incubated at 37 ± 0.5°C; sink conditions were maintained throughout the study; 1.0 ml samples of released insulin were withdrawn at 1-h intervals and replaced with an equal volume of release medium preequilibrated to temperature. The remaining polymer in withdrawn samples was removed by centrifugation ($20\,000 \times g$, Hermle Z 323K). A portion (7 μl) of the supernatant fluid was directly injected for HPLC analysis as described above. The amount of insulin released was quantified from integrated peak areas and calculated by interpolation from an according standard curve for pre-treated insulin. Linear regression analysis of the peak areas gave a correlation coefficient of 0.999665 for the standard curve ($n = 5$). Cumulative corrections were made for the previously removed samples in determining the total amount released.

3. Results

3.1. Preparation and analysis of polymer–antipain conjugates

The terminal located aldehyde function of antipain is essential for its inhibitory activity. In order to keep this moiety uninfluenced, the inhibitor was at the opposite end of the molecule bound to the polymer (Fig. 1.). The immobilisation of antipain to chitosan could be verified by the trypsin inhibiting function of all chitosan–antipain conjugates, whereas the unmodified polymer did not exhibit this property. Moreover, a polymer prepared and isolated in the same way as chitosan–antipain conjugates but omitting EDAC during the coupling reaction showed no inhibitory effect, verifying the efficiency of the purification method described here. A classification of the inhibitory effect of all conjugates was even at a conjugate concentration as low as 0.045% possible. Results of this study with the chitosan–antipain conjugates 1:15, 1:60 and 1:240 are shown in Fig. 2. Polymer conjugates

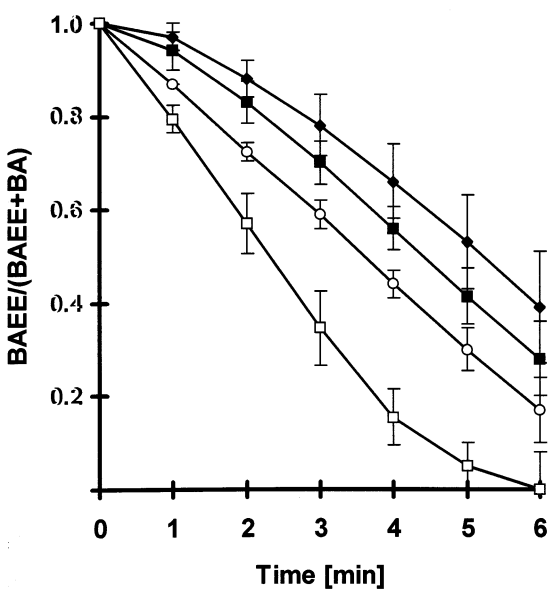

Fig. 2. Comparison of the inhibitory effect of 0.045% chitosan–antipain conjugate 1:15 (◆), conjugate 1:60 (■), conjugate 1:240 (○) and chitosan–antipain control (□) on trypsin activity. Indicated values are means of at least three experiments (± S.D.).

derived from coupling reactions with a comparable high portion of antipain displayed a stronger inhibitory effect. However, as the amount of primary amino groups which are essential for the mucoadhesive as well as absorption enhancing properties of chitosan (Artursson et al., 1994), decreases as the amount of covalently to the polymer attached antipain increases, the inhibitory effect of modified chitosan cannot be raised ad libitum.

Chitosan has been shown to be mucoadhesive at physiological pH probably mediated by an ionic interaction between the positively charged primary amino groups in chitosan and the negatively charged sialic acid residues in mucus (Lehr et al., 1992). With regard to the chitosan–antipain conjugate 1:60, theoretically at the most only 1.3% of all primary amino groups in chitosan can be modified by the covalent attachment of antipain. According to the theory for adhesion mentioned above, the slightly modification of the polymer should not have an influence on its mucoadhesive properties. In order to verify this, the mucoadhesive strength of chitosan and the slightly modified polymer was evaluated. Lyophilised chitosan HCl pH 6.0 and the polymer conjugate 1:60 showed no significant differences in their mucoadhesive properties exhibiting a maximum detachment force of 30.5 ± 8.6 mN and 32.5 ± 7.6 mN (mean ± S.D., $n = 4$), respectively.

3.2. Protective effect of the drug delivery system

The protective effect of the drug delivery system will be provided, on the one hand, by the mucoadhesiveness of the dosage form decreasing the distance between the released drug and absorptive tissue and, on the other hand, by the inhibitor which is immobilised to the carrier matrix. Tablets containing the chitosan–antipain conjugate 1:60 demonstrated a reduced trypsinic degradation of insulin in inner parts as well as in lateral parts of the dosage form. As shown in Fig. 3, proteolysis in inner parts of the swelled carrier matrix could be completely inhibited even with a share of only 2.5% polymer conjugate in the tablet, whereas $11.3 \pm 9.5\%$ (mean ± S.D., $n = 3$) insulin was degraded in tablets lacking the conjugate. In lateral parts a marked protective effect of the dosage form containing 5% polymer conjugate

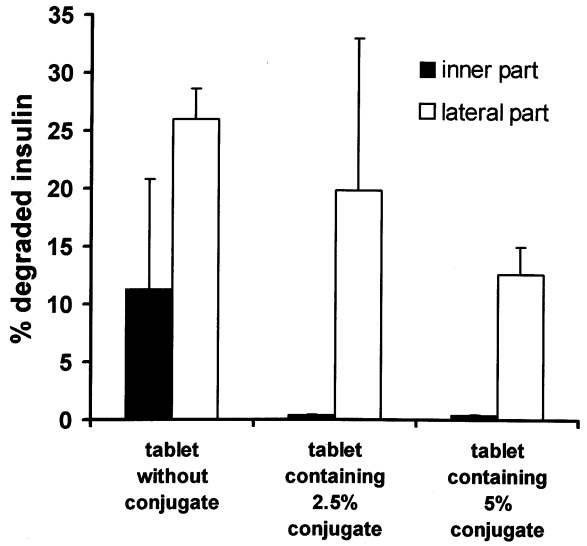

Fig. 3. Amount of degraded insulin (%) in drug delivery systems without (control) and with 2.5 or 5% polymer conjugate 1:60. Tablets were incubated for 1.5 h in 20 mM Tris–HCl pH 7.8 containing 0.2 mg of trypsin (180 BAEE units) per ml at 37 ± 0.5°C. Each bar represents the mean $\pm$ S.D. of three experiments.

could be observed, exhibiting a $13.3 \pm 2.3\%$ (mean $\pm$ S.D. $n = 3$) minor proteolysis of insulin.

3.3. Sustained drug release

Considering the small intestinal transit time of the order of 3 h (Davis et al., 1986) and taking a prolonged residence time of the dosage form due to its mucoadhesive properties in account, a sustained release of insulin over a period of approximately 6 h should be guaranteed. Therefore, we investigated the release profile of our system with chitosan acetate pH 6.0 concentrations between 30 and 80% (data not shown). A 60% portion of the polymer on the whole drug delivery system showed a release rate of approximately 5% insulin per h within the first 3 h and then of 25% per h. The release profile of insulin from this drug delivery system is illustrated in Fig. 4.

4. Discussion

In order to generate peroral drug delivery systems that shield inserted peptide and protein drugs from enzymatic attack by trypsin, it is essential to be well-versed about the proteolytic activity of trypsin in the small intestine. However, no detailed information is given about that in literature, which can be explained by the influence of various parameters, e.g. pancreatic stimulation as well as type and quantity of nutrients leading to high variable values of enzymatic activity. Hence, data are only given in amounts of trypsin secreted from human pancreas. On the one hand, the stimulated trypsin secretion of 25 adults was determined to be 16–61 titrimetric BAEE units per min, when one unit will hydrolyse 1.0 μmol of BAEE per min at 25°C (Rick, 1970). On the other hand, the intestinal flow rate in the duodenum can reach several ml per min and was determined to be 2.16 and 0.67 ml/min in the jejunum and ileum, respectively (Wissenschaftliche Tabellen Geigy, 1983). Therefore, the enzymatic activity of trypsin

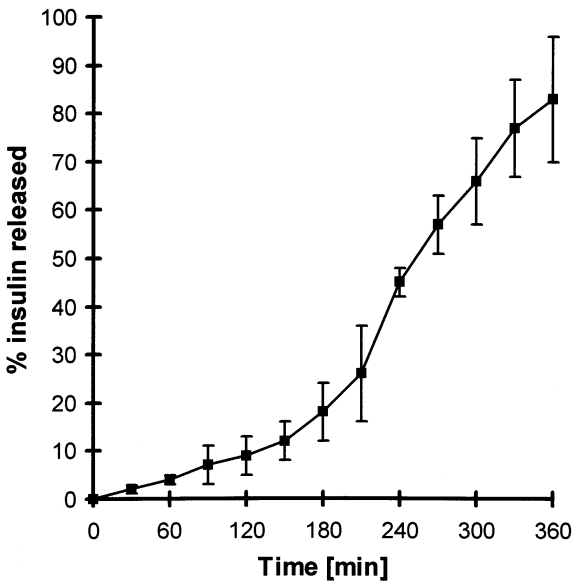

Fig. 4. Release profile of insulin from the drug delivery system containing 30 mg of chitosan acetate pH 6.0, 15 mg of mannit and 5 mg of insulin. The dosage form was incubated with 10 ml release medium (20 mM Tris–HCl pH 7.8) at 37 ± 0.5°C. Each point represents the mean $\pm$ S.D. of three experiments.

in the small intestine can theoretically reach a maximum of almost 100 titrimetric BAEE units/ml or approximately 100 000 spectrophotometric BAEE units/ml, when 1 unit will cause an increase in absorbance (ΔA_{253}) of 0.001 per min at 25°C. With regard to this presumptive in vivo situation, the protective effect of the delivery system described here which has been evaluated at a trypsin activity of 180 spectrophotometric BAEE units/ml, seems to be quite insufficient. However, the development of new methods allowing the production of comparable high amounts of polymer-inhibitor conjugates would supply the development of drug delivery systems with higher conjugate concentrations as described here leading to an improved protective effect. The functionality of according systems in principle could be verified in the present study.

The effectiveness of a dosage form containing a trypsin inhibitor for the peroral administration of insulin has already been demonstrated by a significant continuous hypoglycemic effect in both normal and diabetic rats when compared with controls (Morishita et al., 1992). Hence, similar in vivo effects can be expected for the delivery system described here, but have to be verified by further studies. In vivo studies with the elastase inhibitor elastatinal and the chymotrypsin inhibitor chymostatin demonstrated also an enhanced absorption of insulin out of the intestine (Fujii et al., 1985). As the chemical structure of antipain is very similar in comparison with chymostatin (Bernkop-Schnürch and Apprich, 1997) as well as elastatinal (Bernkop-Schnürch et al., 1997a), on the one hand, the stability of the aldehyde moiety of antipain in the intestine should therefore also be guaranteed. On the other hand, the possibility of a successful coupling of elastatinal and chymostatin to chitosan seems to be very likely. It would allow the development of systems protecting towards the entire enzymatic attack of luminal serine proteases. Moreover, our research group could recently demonstrate that also membrane bound luminal proteases even when they are covered with a mucus layer can be inhibited by mucoadhesive polymers. As these polymers act by the deprivation of bivalent cations which are essential co-factors for the enzymatic activity of various brush border membrane bound proteases, this effect can be enhanced by the covalent attachment of strong complexing agents to these polymers (Bernkop-Schnürch and Marschütz, 1997). Accordingly, the combination of the chitosan–antipain conjugate described here with, e.g. a chitosan–EDTA conjugate, might allow the development of systems exhibiting an additional protective effect against brush border membrane bound proteases (Bernkop-Schnürch et al., 1997b).

In conclusion, the cationic polysaccharide chitosan is well known for its mucoadhesive (Lehr et al., 1992) as well as absorption enhancing properties (Artursson et al., 1994) which makes this polymer to an interesting pharmaceutical excipient for the non-parenteral administration of peptides (Illum et al., 1994). However, in contrast to poly(acrylate) derivatives such as carbomer and polycarbophil exhibiting an inhibitory effect towards enzymatic degradation by various luminal proteases (Lueßen et al., 1995, 1996), chitosan does not offer this property. In the present study we demonstrated, that this major drawback of chitosan for the peroral peptide and protein administration can be eliminated by a slightly modification. The immobilisation of antipain to an unabsorbable polymer might promise the exclusion of toxic side-effects, but has to be verified by additional toxicological studies. Moreover, unintended dilution effects of the inhibitor can be excluded due to its covalent attachment to the polymer. Especially in combination with polymers inhibiting further luminal proteases, chitosan–antipain conjugates should help to overcome the luminal enzymatic barrier. The novel polymer conjugates offering following advantages represent a useful tool for the peroral administration of peptide and protein drugs:

(I) Chitosan–antipain conjugates have a protective effect towards enzymatic degradation caused by trypsin.

(II) As the chemical structure of antipain is similar to inhibitors of other luminal proteases, the successful immobilisation of these additional auxiliary agents to chitosan seems to be very likely and should make the inhibition of further luminal proteases possible.

(III) As shown for the chitosan–antipain conjugate 1:60, modified chitosan displays high mucoadhesive properties.

(IV) Chitosan exerts penetration enhancing properties which should not be influenced by the slightly modification.

(V) As shown in this study chitosan and, therefore, also slightly modified chitosan, can be used as a vehicle in sustained release systems.

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