Synthesis and *In Vitro* Evaluation of Chitosan-EDTA-Protease-Inhibitor Conjugates Which Might Be Useful in Oral Delivery of Peptides and Proteins

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Purpose. To develop a novel mucoadhesive polymer that protects peptide drugs from degradation by secreted as well as membrane-bound proteases in the intestine, and to evaluate this polymer *in vitro*.

Methods. The serine protease inhibitors antipain, chymostatin and elastatinal were covalently linked to chitosan (poly-[1 → 4]-β-D-glucosamine). Thereafter, the complexing agent ethylenediaminetetraacetic acid (EDTA) was bound to the remaining primary amino groups of the polymer. The inhibitory effect of the resulting polymer-conjugate towards trypsin (EC 3.4.21.4), chymotrypsin (EC 3.4.21.1), elastase (3.4.21.36), carboxypeptidase A (EC 3.4.17.1), carboxypeptidase B (EC 3.4.17.2) and aminopeptidase N (EC 3.4.11.2) as well as its mucoadhesive properties were evaluated *in vitro*.

Results. Whereas the novel polymer-conjugate exhibited excellent swelling properties, its adhesive force was under our assay conditions 42% lower than that of unmodified chitosan. However, the polymer-conjugate showed a strong inhibitory activity towards all tested serine proteases. Due to its additional high binding affinity towards bivalent metal ions, it also inhibited the Zn^{2+}-dependent exopeptidases carboxypeptidase A, B and aminopeptidase N.

Conclusions. The novel mucoadhesive polymer-conjugate described in this study seems to be a useful tool in overcoming the enzymatic barrier to perorally administered therapeutic peptides and proteins.

KEY WORDS: chitosan-EDTA-inhibitor conjugates; peroral peptide delivery; enzymatic barrier; inhibition of intestinal proteases.

INTRODUCTION

In recent years, the use of protease inhibitors for peroral administration of peptide and protein drugs has received considerable attention, as *in vivo* studies have demonstrated that co-administration of such auxiliary agents provides a significantly increased bioavailability of therapeutic (poly)peptides (1–4). However, in overcoming the enzymatic barrier (5) by means of co-administration of protease inhibitors the risk of several side effects is taken. Inhibitors of low molecular mass such as elastatinal and pepstatin can pass the absorption membrane and cause systemic toxic side effects (6,7). Even if they are unabsorbable, disturbed digestion of nutritive proteins and pancreatic hypersecretion caused by a luminal feed-back regulation have to be expected (8,9). Hence, the practical use of such auxiliary agents seems to be questionable. A possible solution to this problem is the exclusion of this drawback by attaching protease inhibitors covalently to unabsorbable drug carrier-matrices, thereby avoiding any dilution effects of inhibitor(s) in the intestine. Such modified carrier-matrices might also promise a reduction or even exclusion of drug degradation between the delivery system and the absorbing membrane, if they exhibit additional mucoadhesive properties which ensure an intimate contact to the mucosa. Recently, our research group has developed several mucoadhesive polymer-inhibitor conjugates providing a strong protective effect for embedded peptide and protein drugs towards luminal enzymatic metabolism (10–16). As all of these systems were only capable of inhibiting one or two proteases in each case, it was the aim of the present study to integrate these subsystems into a novel polymer-conjugate providing a protective effect almost towards the entire luminal enzymatic attack.

In order to inhibit the serine proteases trypsin, chymotrypsin and elastase, on the one hand, the inhibitors antipain, chymostatin and elastatinal were covalently linked to a restricted number of amino groups of chitosan which was used as polymer-matrix. To inhibit zinc- dependent proteases such as carboxypeptidase A, B and aminopeptidase N, on the other hand, the complexing agent EDTA, representing a potent inhibitor for these enzymes, was also covalently bound to the remaining primary amino groups of the chitosan-inhibitor conjugate. The inhibitory effect of the resulting novel polymer-conjugate shown in Figure 1 towards the mentioned proteases as well as its mucoadhesive properties had to be evaluated *in vitro*.

MATERIALS AND METHODS

Synthesis of Chitosan-Inhibitor Conjugates

The covalent attachment of antipain, chymostatin and elastatinal to chitosan was achieved by the formation of amide bonds between amino groups of the polymer and the terminally located carboxylic acid group of the inhibitor. One gram of chitosan obtained from crab shells (Sigma, St. Louis, USA) was suspended in 90 ml of demineralized water. The pH-value of this suspension was kept constant at pH 6 by continuously adding 1 N HCl till the polymer was completely dissolved, and demineralized water was added to a final volume of 100 ml. As listed in Table I, mixtures of antipain, chymostatin, elastatinal (Sigma, St. Louis, USA) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC; Sigma, St. Louis, USA) were dissolved in 4 ml of demineralized water and preincubated for 30 min at room temperature. Thereafter, they were added to 4.0 ml of the 1% (w/v) chitosan HCl pH 6.0 solution. The reaction mixtures were incubated for 6 h under permanent stirring at room temperature and the resulting polymer-conjugates were isolated by dialysing against 50 mM HCl for 48 h and then exhaustively against demineralized water, both at 10°C. As shown in Table I, controls being prepared and isolated in exactly the same way as the chitosan-inhibitor conjugates but omitting EDAC during the coupling reaction served as reference for the

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ABBREVIATIONS: A, L-arginine; ACE, antipain, chymostatin and elastatinal; BA, N-α-benzoyl-arginine; BAEE, N-α-benzoyl-arginine ethylester; BBM, brush border membrane; BTEE, N-benzoyl-L-tyrosine ethylester; EDAC, 1-ethyl-3-(3-dimethylamino propyl) carbodiimide hydrochloride; EDTA, ethylenediaminetetra-acetic acid; HA, hippuryl-L-arginine; HPA, hippuryl-L-phenylalanine; PA, L-phenylalanin; SNHS, sulfo-N-hydroxysuccinimide; TBS, Tris-HCl buffered saline (0.9% NaCl).

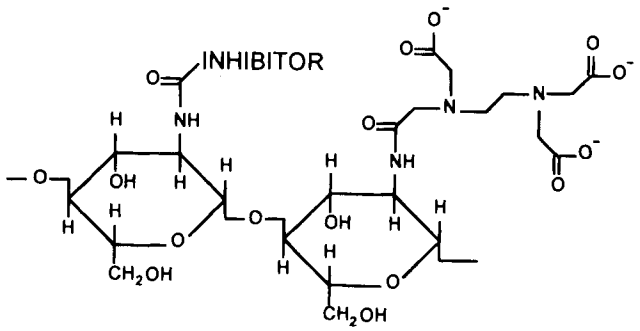

Fig. 1. Presumptive structure of chitosan-inhibitor-EDTA conjugates; covalent attachment of inhibitors (antipain, chymostatin and elastatinal) and the complexing agent (EDTA) was achieved by the formation of an amide bond between a primary amino group of the polymer and a carboxylic acid group of the inhibitor or EDTA.

following analytical studies. The purified polymer-conjugates and controls were used for the additional coupling of EDTA as described below, or lyophilized—in order to increase the surface of the resulting solid polymers and thereby providing them with excellent swelling properties—by drying frozen aqueous polymer solutions at –40°C and 0.01 mbar (Christ Beta 1–8K; Osterode am Harz, Germany). The polymer-conjugates were then stored at –20°C until evaluation.

Synthesis of Chitosan-Inhibitor-EDTA Conjugates

The covalent attachment of EDTA to chitosan or chitosan-inhibitor conjugates was achieved by the formation of amide bonds between primary amino groups of the polymer and the carboxylic acid groups of the complexing agent according to the method described by Bernkop-Schnürch *et al.* (15). 14.5 g of EDTA (Sigma, St. Louis, USA) were suspended in 60 ml of demineralized water and the pH value was adjusted to 6.0 with 5 N NaOH. Thereafter, demineralized water was added to a final volume of 100 ml. 10 ml of this solution were mixed with each dialysate obtained from the first coupling-reaction. In order to catalyze the formation of amide bonds, EDAC was dissolved in 1 ml of demineralized water and added in a final concentration of 50 mM. The reaction mixtures were incubated for 12 h under permanent stirring at room temperature and the resulting polymer-conjugates were isolated by dialysing against 0.05 N NaOH for 12 h and then exhaustively against demineralized water, both at 10°C. After adjusting the pH value of the purified polymer-conjugates to 6.0 by adding 0.05 N NaOH, the products were lyophilized as described above and stored at –20°C until evaluation. Unmodified chitosan-EDTA conjugate served as control and was synthesized as previously described for chitosan-EDTA 1:20 by Bernkop-Schnürch and Krajicek (15).

In Vitro Evaluation of the Inhibitory Effect of the Polymer-Conjugates Towards Enzymatic Degradation

Trypsin (EC 3.4.21.4)

First, 0.5 mg of chitosan-inhibitor conjugates and—taking the increase in weight due to additionally bound EDTA into account—1.2 mg of chitosan-EDTA-inhibitor conjugates as shown in Fig. 2 were swelled in 0.7 ml of 50 mM TBS (Tris-HCl

Table I. Concentrations of Reagents Used for Reaction Mixtures in Order to Form Chitosan-Inhibitor Conjugates with Increasing Amounts of Covalently Attached Antipain, Chymostatin and Elastatinal

Resulting chitosan-inhibitor conjugate	EDAC	chitosan HCl pH 6.0	antipain	chymostatin	elastatinal
chitosan-antipain control	—	40 mg	2 mg	—	—
chitosan-antipain conjugate 10:1	160 mg	40 mg	4 mg	—	—
chitosan-antipain conjugate 20:1	160 mg	40 mg	2 mg	—	—
chitosan-antipain conjugate 40:1	160 mg	40 mg	1 mg	—	—
chitosan-chymostatin control	—	40 mg	—	2 mg	—
chitosan-chymostatin conjugate 10:1	160 mg	40 mg	—	4 mg	—
chitosan-chymostatin conjugate 20:1	160 mg	40 mg	—	2 mg	—
chitosan-chymostatin conjugate 40:1	160 mg	40 mg	—	1 mg	—
chitosan-elastatinal control	—	40 mg	—	—	2 mg
chitosan-elastatinal conjugate 10:1	160 mg	40 mg	—	—	4 mg
chitosan-elastatinal conjugate 20:1	160 mg	40 mg	—	—	2 mg
chitosan-elastatinal conjugate 40:1	160 mg	40 mg	—	—	1 mg
chitosan-ACE control	—	40 mg	2 mg	2 mg	2 mg
chitosan-ACE conjugate 20:1	160 mg	40 mg	2 mg	2 mg	2 mg

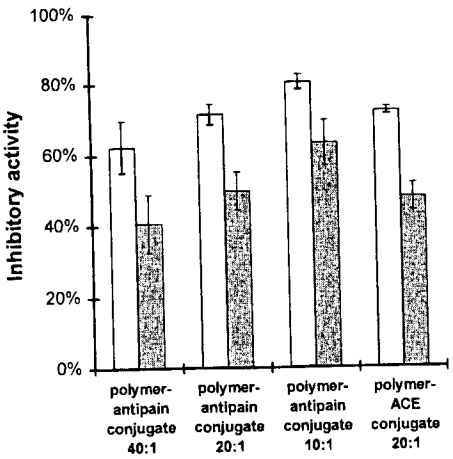

Fig. 2. Comparison of the inhibitory activity of polymer-antipain conjugates 40:1, 20:1 and 10:1 as well as polymer-ACE conjugate 20:1 with and without additionally bound EDTA (chitosan-EDTA-inhibitor conjugates: grey bars; chitosan-inhibitor conjugates: blank bars). Inhibitory activity towards trypsin (82 BAEE units/ml) is shown by the percentage of remaining unhydrolyzed substrate after an incubation period of 9 min with 0.045% chitosan-inhibitor conjugates and 0.11% chitosan-EDTA-inhibitor conjugates (mean ±SD; n = 3).

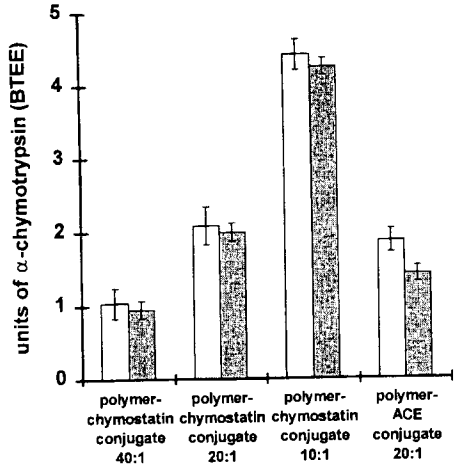

Fig. 3. Comparison of the inhibitory activity of polymer-chymostatin conjugates 40:1, 20:1 and 10:1 as well as polymer-ACE conjugate 20:1 with and without additionally bound EDTA (chitosan-EDTA-inhibitor conjugates: grey bars; chitosan-inhibitor conjugates: blank bars) towards α-chymotrypsin. Inhibitory activity was measured in units of α-chymotrypsin (BTEE) which have to be added to 1.0 ml of a substrate solution containing 0.05% chitosan-inhibitor conjugate or 0.12% chitosan-EDTA-inhibitor conjugate in order to obtain a 50% hydrolysis of the substrate within a period of 9 min. Units, which had to be added to the pure substrate solution in order to obtain a 50% hydrolysis, were deducted from all obtained values (mean ± SD; n = 3).

buffered saline) pH 6.8. Thereafter, trypsin (90 spectrophotometric BAEE units; United States Biochemical, Cleveland, OH) dissolved in 200 µl of 50 mM TBS pH 6.8 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 50 mM TBS pH 6.8, 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 one minute intervals. For positive controls trypsin was omitted. In order to evaluate the amount of unbound antipain exhibiting the same protective effect as the polymer-conjugates, the inhibitory effect of these modified polymers was compared with gradually increasing inhibitor concentrations.

Chymotrypsin (EC 3.4.21.1)

Polymer conjugates shown in Fig. 3 were swelled as described above and increasing amounts of α-chymotrypsin (52 BTEE units/mg; type II: from bovine pancreas, Sigma, St. Louis, MO) dissolved in 30 µl of 50 mM TBS pH 6.8 were added in steps of 0.2 units. 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 ethylester dissolved in 31.7 ml of methanol and 18.3 ml of demineralized water) were added and the increase in absorbance ($\Delta A_{254\ nm}$) was recorded at one minute intervals. For positive controls chymotrypsin was omitted. The amount of unbound chymostatin exhibiting the same protective effect as the polymer-conjugates was evaluated according to the method described for trypsin.

Elastase (EC 3.4.21.36)

Polymer-conjugates as shown in Fig. 4 and 6.7 µg of elastase (Type II-A: from porcine pancreas; Sigma, St. Louis, USA) in 180 µl 50 mM TBS pH 6.8 were transferred to the wells of a microtitration plate (96-well, not binding) and incubated for 30 min at 37°C. 130 µl of the substrate medium (0.2 mg of succinyl-(L-alanyl)$_3$-4-nitroanilide (Sigma, St. Louis, USA)/ml 50 mM TBS pH 6.8; filtered before use) were added and the increase in absorbance ($\Delta A_{405\ nm}$) caused by the enzymatic reaction at 20°C was recorded at one minute intervals with a microtitration plate reader (Anthos reader 2001;

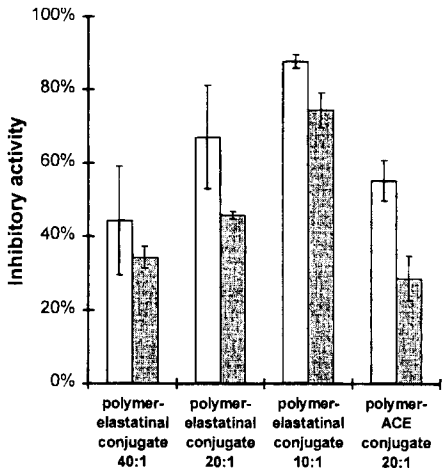

Fig. 4. Comparison of the inhibitory activity of polymer-elastatinal conjugates 40:1, 20:1 and 10:1 as well as polymer-ACE conjugate 20:1 with and without additionally bound EDTA (chitosan-EDTA-inhibitor conjugates: grey bars; chitosan-inhibitor conjugates: blank bars). Inhibitory activity towards elastase (21.6 µg/ml) is shown by the percentage of remaining unhydrolysed substrate after an incubation period of 9 min with 0.16% chitosan-inhibitor conjugates and 0.39% chitosan-EDTA-inhibitor conjugates (mean ±SD; n = 3).

Salzburg, Austria). The concentration of the hydrolyzed substrate was calculated by interpolation from an according standard curve. For positive controls elastase was omitted. The amount of unbound elastatinal exhibiting the same protective effect as the polymer-conjugates was evaluated according to the method described for trypsin.

Carboxypeptidase A (EC 3.4.17.1)

First, 1.5 mg of the polymers shown in Fig. 5 and carboxypeptidase A (0.5 units; from bovine pancreas; Sigma, St. Louis, USA) in 750 µl of 25 mM Tris-HCl pH 6.8 containing 2.9% NaCl were incubated for 30 min at room temperature. After adding 750 µl of the substrate hippuryl-L-phenylalanine (1 mM) dissolved in the same buffer, the increase in absorbance was measured at 254 nm at one minute intervals at 20°C.

Carboxypeptidase B (EC 3.4.17.2)

Five milligrams of the chitosan-EDTA-ACE conjugate 20:1 or 3 mg of the chitosan-EDTA conjugate in 800 µl of 1 M Tris-HCl pH 6.8 were incubated with carboxypeptidase B (0.16 units; from bovine pancreas; Sigma, St. Louis, USA) for 30 min at room temperature. After adding 200 µl of the substrate hippuryl-L-arginine (2 mM) dissolved in the same buffer, the increase in absorbance was measured at 258 nm at one minute intervals at 20°C.

Aminopeptidase N (EC 3.4.11.2)

First, 1 mg of the polymers shown in Fig. 7 and 12 mU of aminopeptidase N (Sigma, St. Louis, USA) in 125 µl of 50 mM TBS pH 6.8 were incubated in the wells of a microtitration plate for 30 min at 37°C. Thereafter, 50 µl of L-leucine-p-nitroanilide in a final concentration of 1 mM were added and the increase in absorbance was spectrophotometrically determined as described under the assay for elastase.

Tensile Studies

Forty milligrams of chitosan-EDTA-ACE conjugate 20:1, chitosan-EDTA conjugate or lyophilized chitosan HCl pH 6.0 were compressed (Hanseaten Type El, 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. After a contact time between test disc and mucosa of 2 min in 50 mM phosphate buffered saline pH 7.2 at 37°C, the maximum detachment force at which the adhesive bond between test disc and mucosa failed, was recorded by pulling the mucosa at a rate of 2 mm/min off the disc (10).

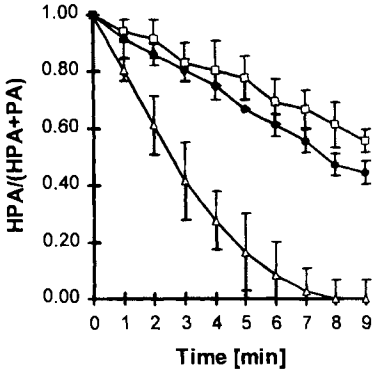

Fig. 5. Hydrolysis of hippuryl-L-phenylalanine (**HPA**) to L-phenylalanine (**PA**) and hippuric acid by carboxypeptidase A (0.33 units/ml) in presence of 0.1% chitosan-EDTA conjugate (❑—❑), 0.1% chitosan-EDTA-ACE conjugate 20:1 (●—●), and without polymer-conjugate (△—△). Each point represents the mean ±SD of at least three experiments.

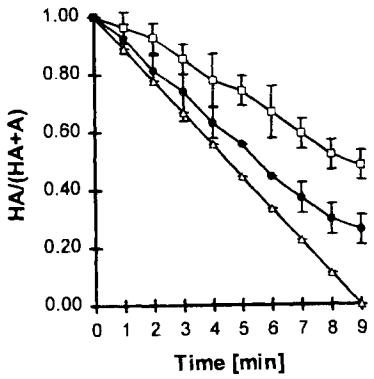

Fig. 6. Hydrolysis of hippuryl-L-arginine (**HA**) to L-arginine (**A**) and hippuric acid by carboxypeptidase B (0.16 units/ml) in presence of 0.3% chitosan-EDTA conjugate (❑—❑), 0.5% chitosan-EDTA-ACE conjugate 20:1 (●—●), and without polymer-conjugate (△—△). Each point represents the mean ±SD of at least three experiments.

Statistical Data Analysis

Statistical data analysis were performed using the *t* test with $p < 0.05$ as the minimal level of significance.

RESULTS

Chitosan-Inhibitor Conjugates

The terminally located aldehyde function of antipain, chymostatin and elastatinal is essential for their inhibitory activity.

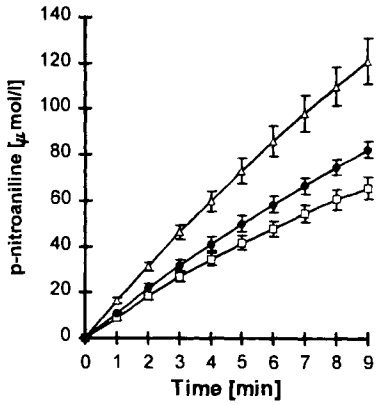

Fig. 7. Hydrolysis of L-leucine-p-nitroanilide by aminopeptidase N (69 mU/ml) in presence of 0.57% chitosan-EDTA conjugate (❑—❑), 0.57% chitosan-EDTA-ACE conjugate 20:1 (●—●), and without polymer-conjugate (△—△). Each point represents the mean ±SD of at least three experiments.

In order to keep this moiety uninfluenced, the inhibitors were bound to the polymer at the opposite end of the molecule. The immobilization of inhibitors on chitosan could be verified by the enzyme inhibiting effect of all chitosan-inhibitor conjugates, whereas the unmodified polymer did not exhibit this property. Moreover, polymers prepared and isolated in the same way as chitosan-inhibitor conjugates but omitting EDAC during the coupling reaction—namely chitosan-inhibitor controls—showed no inhibitory effect, verifying the efficacy of the method used for purification. Results of inhibition-studies with the chitosan-inhibitor conjugates 40:1, 20:1 and 10:1 are shown in Figures 2, 3 and 4. Polymer-conjugates derived from coupling reactions with a comparably high portion of inhibitor displayed a stronger inhibitory effect. However, since the amount of primary amino groups, which are essential for the mucoadhesive as well as absorption enhancing properties of chitosan (17), decreases with an increasing amount of covalently attached inhibitors, the inhibitory effect of chitosan-inhibitor conjugates cannot be raised ad libitum.

Chitosan-EDTA-Inhibitor Conjugates

The covalent attachment of EDTA to chitosan or chitosan-inhibitor conjugates was achieved according to the method which has already been described by our research group (15). Due to the covalently bound EDTA, exhibiting three remaining carboxylic acid groups on the polymer (Fig. 1), all conjugates were hydratable in water and basic aqueous solutions forming transparent gels and showing quick swelling properties. Although EDTA is covalently attached to the polymer, it still displays its complexing capability towards bivalent cations, as recently demonstrated by our research group (15). Hence, all chitosan-EDTA-inhibitor conjugates were able to inhibit zinc-dependent proteases. However, compared to the chitosan-EDTA conjugate, chitosan-EDTA-inhibitor conjugates showed a markedly lower inhibitory effect towards carboxypeptidase A, B and aminopeptidase N. As the percentage of primary amino groups on the polymer was reduced due to covalently bound inhibitors, this reduced inhibitory effect can be explained by the lower amount of EDTA bound to chitosan-inhibitor conjugates. Results of the inhibitory effect of the chitosan-EDTA-ACE conjugate 20:1 towards the targeted protease(s) in comparison with the chitosan-EDTA conjugate are shown in Fig. 5, 6 and 7.

Whereas due to the additional attachment of EDTA the polymer-conjugates were also able to inhibit zinc- dependent proteases, the inhibitory effect towards the serine proteases trypsin, chymotrypsin and elastase was slightly reduced. This effect can be explained by a restricted accessibility of the immobilized inhibitors for the corresponding protease caused by the additional linkage of EDTA, which might lead to a partial sterical hindrance. Results are shown in Fig. 2, 3 and 4. In order to be able to compare the inhibitory activity of polymer-conjugates described in this study with other mucoadhesive polymers displaying inhibitory activity *per se*, as well as with already established polymer-inhibitor conjugates, amounts of unbound antipain, chymostatin and elastatinal exhibiting the same protective effect as one milligram of the corresponding polymer-inhibitor conjugate were determined. The results are summarized in Table II.

Tensile Studies

Tensile studies revealed significantly reduced mucoadhesive properties of the chitosan-EDTA-ACE conjugate compared to chitosan-EDTA conjugate. Whereas the maximum detachment force for the chitosan-EDTA-ACE conjugate was determined to be 16.7 ± 2.9 mN, it was 43.8 ± 10.9 mN for the chitosan-EDTA conjugate and 29.0 ± 12.5 for chitosan HCl (mean ± SD; n = 3–8). In order to avoid a further decrease in mucoadhesive properties of chitosan-EDTA-ACE conjugates, the amount of covalently attached antipain, chymostatin and elastatinal should therefore not be raised.

DISCUSSION

Delivery systems which are based on mucoadhesive polymers are supposed to provide an intimate contact with the intestinal mucosa, thereby reducing the degradation of perorally administered therapeutic peptides and proteins between delivery system and absorbing membrane (18). However, the efficacy of such systems is limited by the fact that luminally secreted proteases penetrate in the mucoadhesive polymer causing degradation of embedded (poly)peptide drugs (11,16). Attempts to avoid this degradation led to the development of mucoadhesive polymers, which guarantee a protective effect towards luminally secreted proteases. In this study, we were for the first time able to synthesize a mucoadhesive polymer, which displays a strong protective effect towards all proteases secreted from the pancreas. However, the inhibitory activity of the chitosan-EDTA-ACE conjugate towards elastase was markedly weaker than that of the recently developed poly(acrylate) derivative-elastatinal conjugates and carboxymethylcellulose-elastatinal conjugates (19). A reason for the comparably stronger inhibitory effect of these already established polymer-conjugates can be seen in the additional use of a spacer, providing an easy accessibility of the immobilized inhibitor for the corresponding protease. In contrast, the inhibitory activity of the chitosan-EDTA-ACE conjugate towards trypsin and chymotrypsin was in the range of chitosan-antipain conjugates (14), and poly(acrylic acid)-chymostatin as well as poly(acrylic acid)-Bowman-Birk inhibitor conjugates, respectively (10,11). Whether this inhibitory activity will be sufficient to guarantee a protective effect for embedded (poly)peptide drugs, will mainly depend on the therapeutic agent and on the type of dosage form. The enzymatic activity used to evaluate the inhibitory effect of chitosan-EDTA-ACE conjugates, however, was comparably higher than it has to be expected *in vivo* (20).

Moreover, the additional inhibition of brush border membrane bound enzymes, even being separated from the mucoadhesive polymer by a mucus layer, should be feasible using the chitosan-EDTA-ACE conjugate. Recently our research group could demonstrate the inhibition of a membrane bound protease by a poly(acrylic acid)-bacitracin conjugate exhibiting high binding affinity towards Zn^{2+} -ions without any direct contact to the enzyme (12). This so called 'far distance inhibitory effect' (21) through the mucus layer covering gastrointestinal epithelia could also be shown for chitosan-EDTA conjugates (13) and can therefore be expected for the chitosan-EDTA-ACE conjugate as well. Compared to the chitosan-EDTA conjugate, the slightly reduced inhibitory effect of this polymer-conjugate towards aminopeptidase N, as shown in Figure 7, is a good evidence for this possi-

Table II. Mean Amounts of Unbound Inhibitors Exhibiting the Same Protective Effect as 1 mg of the Corresponding Conjugate (mean ± SD; n = 3)

Polymer-conjugate	antipain in µg	chymostatin in µg	elastatina in µg	Polymer-conjugate	antipain in µg	chymostatin in µg	elastatinal in µg
chitosan-antipain control	0.0 ± 0.0	—	—				
chitosan-antipain conjugate 10:1	24.6 ± 0.5	—	—	chitosan-EDTA-antipain conjugate 10:1	4.9 ± 0.3	—	—
chitosan-antipain conjugate 20:1	17.8 ± 0.5	—	—	chitosan-EDTA-antipain conjugate 20:1	2.7 ± 0.1	—	—
chitosan-antipain conjugate 40:1	11.6 ± 0.8	—	—	chitosan-EDTA-antipain conjugate 40:1	2.2 ± 0.2	—	—
chitosan-chymostatin control	—	0.0 ± 0.0	—				
chitosan-chymostatin conjugate 10:1	—	35.6 ± 1.8	—	chitosan-EDTA-chymostatin conjugate 10:1	—	14.3 ± 0.4	—
chitosan-chymostatin conjugate 20:1		18.0 ± 1.8	—	chitosan-EDTA-chymostatin conjugate 20:1	—	7.2 ± 0.3	—
chitosan-chymostatin conjugate 40:1		10.3 ± 1.2	—	chitosan-EDTA-chymostatin conjugate 40:1	—	4.0 ± 0.3	—
chitosan-elastatinal control	—	—	0.0 ± 0.0				
chitosan-elastatinal conjugate 10:1	—	—	1.5 ± 0.1	chitosan-EDTA-elastatinal conjugate 10:1	—	—	0.4 ± 0.0
chitosan-elastatinal conjugate 20:1	—	—	0.9 ± 0.1	chitosan-EDTA-elastatinal conjugate 20:1	—	—	0.2 ± 0.0
chitosan-elastatinal conjugate 40:1	—	—	0.6 ± 0.1	chitosan-EDTA-elastatinal conjugate 40:1	—	—	0.2 ± 0.0
chitosan-ACE control	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	chitosan-EDTA conjugate	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
chitosan-ACE conjugate 20:1	18.0 ± 0.2	16.0 ± 1.0	0.7 ± 0.0	chitosan-EDTA-ACE conjugate 20:1	2.5 ± 0.1	5.1 ± 0.3	0.2 ± 0.0

bility. However, compared to the chitosan-EDTA conjugate, the mucoadhesive force of the chitosan-EDTA-ACE conjugate was markedly reduced. The results are in accordance with the observation that additional basic moieties on chitosan-EDTA conjugates, such as exhibited by covalently attached antipain, chymostatin and elastatinal, cause a significant decrease in mucoadhesion. For example, a percentage of only 3.5% primary amino groups on a chitosan-EDTA conjugate causes an approximately 50% decrease in mucoadhesiveness compared to the same polymer without any remaining basic moieties (15). A reason for this effect can be seen in the reduced polyanionic character of polymers, which is supposed to be responsible for adhesion due to such cationic moieties on the polymer.

Beside the chitosan-EDTA-ACE conjugate, the chitosan-ACE conjugate exhibiting a strong inhibitory activity towards trypsin, chymotrypsin and elastase is also a promising novel polymer. Recently, we could demonstrate a strong protective effect of a drug delivery system containing a chitosan-antipain conjugate towards trypsinic degradation using insulin as a model drug (14). An according similar protective effect towards chymotrypsin and elastase can therefore be expected for the chitosan-ACE conjugate. As unmodified chitosan is reported to display penetration enhancing properties (17), according effects can also be expected for the chitosan-ACE conjugate. Moreover, as already shown for the chitosan-antipain conjugate (14), the mucoadhesive properties of chitosan, representing in contrast to the chitosan-EDTA conjugates a polycationic polymer, will not be markedly influenced by the covalent attachment of inhibitors with basic moieties.

CONCLUSIONS

The novel mucoadhesive polymer-conjugates described in this study, showing a strong inhibitory activity towards trypsin, chymotrypsin, elastase, carboxypeptidase A, B and aminopeptidase N, seem to be a useful tool in protecting therapeutic (poly)peptides from presystemic metabolism in the intestine, with regard to their peroral administration.

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