Novel Bioadhesive Chitosan-EDTA Conjugate Protects Leucine Enkephalin from Degradation by Aminopeptidase N

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Purpose. To develop a novel bioadhesive polymer that protects peptide drugs from luminal degradation by aminopeptidase N and to evaluate the system in vitro on porcine mucosa.

Methods. EDTA was covalently bound to chitosan in order to combine the bioadhesive properties of the polymer with the well known capacity of EDTA to complexe metal ions which are essential for the enzymatic activity of proteases. The inhibitory effect of this polymer conjugate was evaluated by using leucine enkephalin (Leu enkephalin) as a model drug. The degree of Leu enkephalin degradation caused by aminopeptidase N (EC 3.4.11.2), as well as porcine mucosa, in the presence of the polymer conjugate, was quantified by HPLC analysis.

Results. The chitosan-EDTA conjugate is capable of binding 2.01 ± 0.12 mmole of zinc per gram of polymer at pH 6.5 (n = 3; ±S.D.). As zinc is an essential co-factor for aminopeptidase N, enzyme activity (48 mU/ml) could be completely inhibited under the use of 1.0% chitosan-EDTA conjugate. The inhibitory effect of 1.0% chitosan-EDTA conjugate on the degradation of Leu enkephalin on porcine mucosa within 3 h at 37°C was even 2.9-fold higher than that of a recently developed zinc complexing bacitracin-poly(acrylic acid) conjugate of the same concentration. The novel polymer conjugate is more bioadhesive than unmodified chitosan and is easily hydratable in water and basic aqueous solutions exhibiting quick swelling properties.

Conclusions. The bioadhesive polymer conjugate described here seems to be a useful tool in overcoming enzymatic degradation by aminopeptidase N.

KEY WORDS: inhibition of aminopeptidase N; chitosan; EDTA; Leucine enkephalin; brush border membrane bound enzymes.

INTRODUCTION

Peptide and protein drugs are among the most promising therapeutic agents of the decades to come, but hydrolytic instability in the gastrointestinal tract usually requires their parenteral administration, which is often complex, difficult, painful and occasionally dangerous. Therefore, it would be highly desirable to develop drug delivery systems for the peroral administration of these drugs. However, secreted and brush border membrane (BBM) bound intestinal enzymes lead to polypeptide degradation and subsequently to a very low bioavailability after oral dosing. Attempts to reduce this enzymatic barrier include analogues, prodrugs, formulations such as nanoparticles, microspheres, and liposomes that shield peptide drugs from luminal enzymatic attack, and the design of delivery systems targeting the colon where the proteolytic activity is relative low. Moreover, considerable interest has been shown in the use of bioadhesive polymers with covalently attached enzyme inhibitors in order to guarantee, on one hand, protection of inserted polypeptides and to exclude, on the other hand, side effects of these auxiliary agents (1). Although a very effective protection from enzymatic degradation caused by secreted luminal proteases can be achieved by such systems (2), they are not able to inhibit membrane bound enzymes. Immobilized on the polymer, the inhibitor cannot pass through the mucus layer covering the membrane with its enzymes (3). However, in contrast to inhibitors which are directly interacting with proteases, complexing agents do not need this contact in order to bind metal ions representing essential co-factors for proteases. Recently, our research group has demonstrated that a membrane bound enzyme can be partially inhibited by a complexing agent, even when it is covalently attached to a bioadhesive polymer (4). Following this strategy, we focused our research on the development of a bioadhesive polymer that would protect peptides from luminal degradation, in particular by aminopeptidase N which is generally regarded as the most abundant peptidase on the BBM (5). Resins containing iminodiacetic chelating groups are well known for their capability to complexe metal ions (6). EDTA–exhibiting such chelating groups–was therefore covalently bound to the bioadhesive polymer chitosan. The immobilization of the complexing agent should keep it concentrated on the polymer and subsequently also on drug delivery systems based on this novel excipient. The protective effect of the modified polymer was evaluated in vitro on porcine mucosa using Leu enkephalin as model peptide drug, since it is predominantly hydrolyzed by brush border enzymes after oral dosing (7).

MATERIALS AND METHODS

Synthesis of the Chitosan-EDTA Conjugate

300 mg of chitosan (Sigma, St. Louis, USA) were suspended in 30 ml of demineralized water. The pH-value of this suspension was kept constant at pH 4–5 by continuously adding 1 N HCl until the polymer was completely hydrated (≈1 ml). Thereafter, 10.89 g of EDTA (Sigma, St. Louis, USA) were added to the hydrated polymer and the pH-value adjusted to 6.5 with 5 N NaOH (≈24 ml). In order to catalyze the formation of amide bindings between the amino groups of chitosan and the carboxyl groups of EDTA, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC) (Sigma, St. Louis, USA) was added in a final concentration of 0.1 M. The reaction mixture was incubated at room temperature under permanent stirring for 12 h. The resulting conjugate was isolated by exhaustive dialyzing against demineralized water, 0.05 N NaOH, and once more against demineralized water. The purified product was lyophilized and stored at room temperature until use.

A control, prepared and isolated in exactly the same way as the chitosan-EDTA conjugate but omitting EDAC during

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ABBREVIATIONS: BBM, brush border membrane; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodimide hydrochloride; EDTA, ethylenediaminetetraacetic acid; HPLC, high performance liquid chromatrography; TLC, thin layer chromatography; TNBS, 2,4,6-trinitrobenzenesulfonic acid.

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the coupling reaction, namely chitosan-EDTA control, was used as reference for the following analytical studies.

Degree of Modification

The degree of modification was determined by measuring the free amino groups of unmodified and modified chitosan, using 2,4,6-trinitrobenzenesulfonic acid, (TNBS) reagent (8). Eight tenths mg of the conjugate were hydrated in 200 µl demineralized water and incubated with 200 µl of 4% $NaHCO_3$ and 200 µl of 0.1% TNBS at 37°C for 2 h. Thereafter, 200 µl of 2 N HCl were added before absorbance at 344 nm was measured (Lambda 16; Perkin-Elmer). The amount of remaining free amino groups was calculated from an according standard curve of increasing amounts of unmodified polymer.

Tensile Studies

40 mg of the polymer-EDTA conjugate or lyophilized 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 previously described by Bernkop-Schnürch *et al.* (9).

Zinc Binding Studies

20 mg of the chitosan-EDTA conjugate were hydrated in 30 ml of 0.4 M ammonium chloride/ammonia buffer pH 9.0 or 50 mM phosphate buffer pH 6.5 containing 0.120 mmole zinc chloride. The amount of polymer-bound zinc was calculated by complexometric titration (0.01 M EDTA; indicator: Eriochrome Black T) of the remaining free Zn^{2+} in the polymer containing solution. The unmodified polymer was used as a reference.

Synthesis of the Bacitracin-poly(acrylic acid) Conjugate

In order to compare the inhibitory effect of the chitosan-EDTA conjugate with another polyanionic zinc complexing polymer, a bacitracin-poly(acrylic acid) conjugate was synthesized as previously described by Bernkop-Schnürch and Marschütz (4).

Inhibition of Aminopeptidase N

Chitosan-EDTA conjugate in concentrations as indicated in Fig. 3 and 1.0% of the chitosan-EDTA control were hydrated in 2.0 ml of 50 mM phosphate buffer pH 6.5. After adding aminopeptidase N (96 mU) and the substrate L-leucine-p-nitroanilide in a final concentration of 0.5 mM, samples were incubated at 37°C. Aliquot volumes of 300 µl were withdrawn at predetermined time points. The polymer content of withdrawn samples was separated by centrifugation (20,000 × g; 5 min) and absorbance of the hydrolyzed substrate in the supernatant (200 µl) immediately spectrophotometrically determined (wave length: 405 nm; Anthos reader 2001).

Preparation and Evaluation of Porcine Mucosa

The middle section of porcine small intestine was obtained fresh from slaughter, longitudinally dissected and washed gently with demineralized water to remove intestinal content. As a preliminary histological study indicated that damage to the mucosal surface from freezing and thawing was minimal, and a layer of mucus was present on the mucosal surface (10), it was frozen in a 0.9% NaCl solution at −20°C until required. After thawing at room temperature, the mucosal surface was stretched, individually mounted on a platform and fixed with a glass cylinder which was secured in place with a clamp. The cylinder was filled with 1 ml of 50 mM phosphate buffer pH 6.5 containing L-leucine-p-nitroanilide (2 mM) and incubated for 60 min at 37°C. The experimental set-up is shown in Fig. 1. At 15 min intervals, aliquot volumes of 200 µl were transferred on a microtitration plate and the amount of hydrolyzed substrate immediately spectrophotometrically determined (wave length: 405 nm; Anthos reader 2001). Fresh excised mucosa was evaluated in the same way.

Degradation of Leu Enkephalin by Isolated Aminopeptidase N

Two and five tenths mg of Leu enkephalin (Sigma, St. Louis, USA) or 2.5 mg of des-Tyr Leu enkephalin in 1.0 ml of 50 mM phosphate-buffer pH 6.5 were incubated for 30 min with 53.5 mU of aminopeptidase N (EC 3.4.11.2; Sigma, St. Louis, USA) at 37°C. Aliquot volumes of 20 µl were withdrawn and degradation fragments separated by 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 (3 + 1 + 4); migration distance, 8.5 cm; detection, spraying with ninhydrin-reagent (0.3 g ninhydrin, 100 ml n-butanol, 3 ml acetic acid)]. Leu enkephalin, des-Tyr Leu enkephalin, Gly-Phe-Leu, Phe-Leu, glycine and tyrosine were used as references.

Degradation of Leu Enkephalin on Porcine Mucosa

Leu enkephalin (1 mM) in 2 ml of 50 mM phosphate buffer pH 6.5 was incubated with porcine mucosa (surface: 0.785 cm^2) at 37°C. In order to evaluate the influence of polymer conjugates on Leu enkephalin degradation, 1.0% chitosan-EDTA conjugate or 1.0% bacitracin-poly(acrylic acid) conjugate were added. Aliquot volumes of 300 µl were withdrawn at predetermined time points and the reaction terminated by

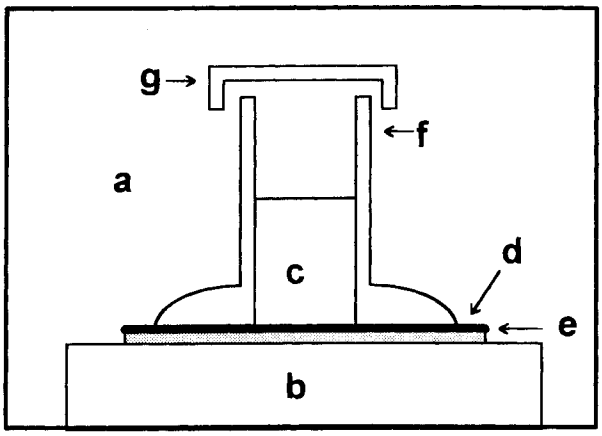

Fig. 1. Experimental set-up to study peptide degradation by BBM-bound enzymes. a. Incubation cell with constant temperature of 37 ± 1°C, b. Platform, c. Incubation medium, d. Mucus layer, e. Porcine intestinal mucosa, f. Glass cylinder of 10 mm inside diameter, g. cap.

boiling samples for 5 min, followed by the addition of 10 µl of 20% trifluoroacetic acid and 30 µl of acetonitrile. The polymer was separated by centrifugation (20,000 × g; 5 min) and a portion (7 µl) of the supernatant fluid was directly injected for HPLC analysis (series 200 LC; Perkin-Elmer). Remaining traces of polymer were held back on a precolumn (Nucleosil 100-10C18, 40 mm × 4 mm). Leu enkephalin and its degradation fragments were separated on a C_{18}-column (Nucleosil 100-5C18, 150 mm × 3 mm) at 40°C. Gradient elution was performed as follows: flow rate 0.5 ml/min; 0–22.5 min; linear gradient from 90% A/10% B to 52.5% A/47.5% B (eluent A: 0.1% trifluoroacetic acid in water; eluent B: acetonitrile). Peptides and amino acids were detected by absorbance at 205 nm as well as 280 nm with a diode array absorbance detector (Perkin-Elmer 235C) and were identified by comparison and comigration with authentic peptide standards.

Degradation of Leu enkephalin was evaluated by following the disappearance of Leu enkephalin and the appearance of tyrosine from the reaction mixture by HPLC. Peak areas were directly proportional to the mass of standards injected and peptide hydrolysis was quantified from integrated peak areas and molar absorbance values calculated from standards for Leu enkephalin and tyrosine.

RESULTS

Chitosan-EDTA Conjugate

The covalent attachment of EDTA to chitosan was achieved by the formation of amide bindings of primary amino groups of the polymer with carboxylic acid groups of the complexing agent. After the covalent coupling of EDTA, the amount of remaining primary amino groups on the polymer was determined to be 0.1% ± 0.03% (mean of three experiments; ±S.D.). According to the ratio of 1:80 (NH_2- groups of chitosan:carboxylic acid groups of EDTA) during the coupling reaction, cross-linking caused by covalent binding of one EDTA molecule to more than one NH_2- group of the polymer should be negligible. However, a ratio of 1:20 during the coupling reaction led to a significant increase in viscosity of the resulting conjugate (data not shown) which could be explained by a cross-linking due to a lower excess of EDTA. Because of its primary amino groups, unmodified chitosan is easily hydratable in acid aqueous solutions. In contrast, caused by the covalent binding of EDTA, exhibiting three remaining carboxylic acid groups on the polymer (Fig. 2), the conjugate is almost unhydratable in acid aqueous solutions. However, the polymer conjugate is hydratable in water and basic aqueous solutions forming transparent gels and exhibiting quick swelling properties.

Tensile studies demonstrated a significant increase in bioadhesive properties of the chitosan-EDTA conjugate compared to the unmodified polymer. Whereas chitosan showed a maximal detachment force of 29.0 ± 12.5 mN under our experimental conditions, it was 43.8 ± 10.9 mN for the conjugate (mean of seven experiments; ±S.D.).

Inhibition of Aminopeptidase N

On one hand, the pH optimum of aminopeptidase N is between pH 6.5–8.5 (11) and, on the other hand, the binding capacity of EDTA decreases as the concentration of H^+-ions

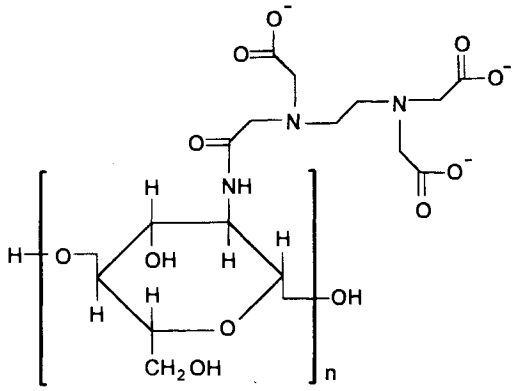

Fig. 2. Presumptive structure of the chitosan-EDTA conjugate; covalent attachment of the complexing agent was achieved by the constitution of an amide binding of a primary amino group of the polymer to a carboxylic acid group of EDTA.

increases. In order to simulate the presumptive worst case situation for the inhibitory effect of the conjugate, we therefore chose a pH-value of 6.5 for the incubation medium. Results of inhibition studies obtained at this pH-value are shown in Fig. 3. The complete removal of all unbound EDTA by the isolation method described by us could be verified by the chitosan-EDTA control showing no marked inhibitory effect. As the inhibitory effect of 0.25% conjugate was still higher than that of 1.0% chitosan-EDTA control, inhibition of aminopeptidase N activity caused by a possible immobilization effect due to the viscous properties of the polymers should be negligible. The inhibition of aminopeptidase N activity by EDTA is caused by the complexation of Zn^{2+} which is an essential co-factor within the enzyme structure. Zinc binding studies of the chitosan-EDTA conjugate demonstrated that one gram of the modified polymer

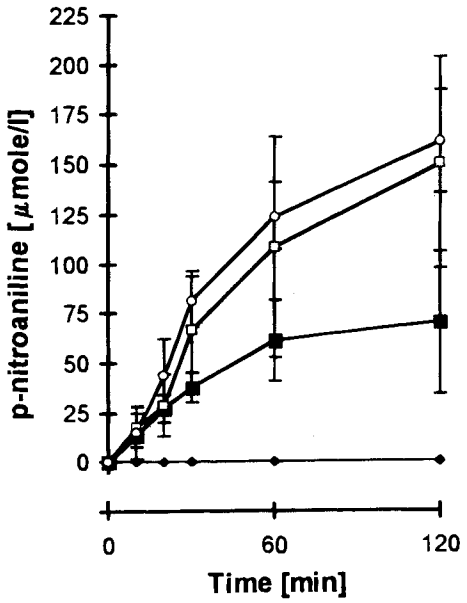

Fig. 3. Hydrolysis of L-leucine-p-nitroanilide by aminopeptidase N in the presence of the chitosan-EDTA conjugate. ◆, 1.0% (m/m) conjugate; , ■ 0.5% (m/m) conjugate, 0.25% (m/m) conjugate; ○, 1.0% (m/m) chitosan–EDTA control (mean of three experiments; ±S.D.).

is able to bind 2.01 ± 0.12 mMole Zn^{2+} at pH 6.5 and 2.00 ± 0.05 mMole Zn^{2+} at pH 9.0 (mean of three experiments ±S.D.). The zinc binding capacity of according references was marginal.

Evaluation of the BBM

Within one hour the enzymatic activity of fresh excised, as well as previously frozen, porcine mucosa was almost constant. Fresh excised mucosa (0.785 cm^2) hydrolyzed 3.28 nmole of L-leucine-p-nitroanilide to L-leucine and p-nitroaniline per min at pH 6.5 at 37°C. Evaluation of mucosa which had been previously frozen at −20°C demonstrated a hydrolyzation of 2.24 nmole per min under the same assay conditions.

Inhibition of BBM-Bound Enzymes

As aminopeptidase N is generally regarded as the most abundant peptidase on the BBM (5), it was important to analyze first of all the enzymatic degradation of Leu enkephalin by this enzyme. A TLC demonstrated that not only N-terminally located tyrosine but subsequently also glycine is split off by this exopeptidase. Results are shown in Fig. 4. HPLC analysis of Leu enkephalin, which had been incubated with porcine mucosa, also showed the predominant hydrolysis of Leu enkephalin to tyrosine and des-Tyr Leu enkephalin which could be identified by according references (Fig. 5). However, HPLC profiles of additional degradation products caused, on one hand, by incubation with porcine mucosa and, on the other hand, by isolated aminopeptidase N were different (data not shown). Leu enkephalin degradation was therefore exclusively quantified by the cleavage of tyrosine, representing in both cases, the first step of enzymatic hydrolysis. An earlier study demonstrated

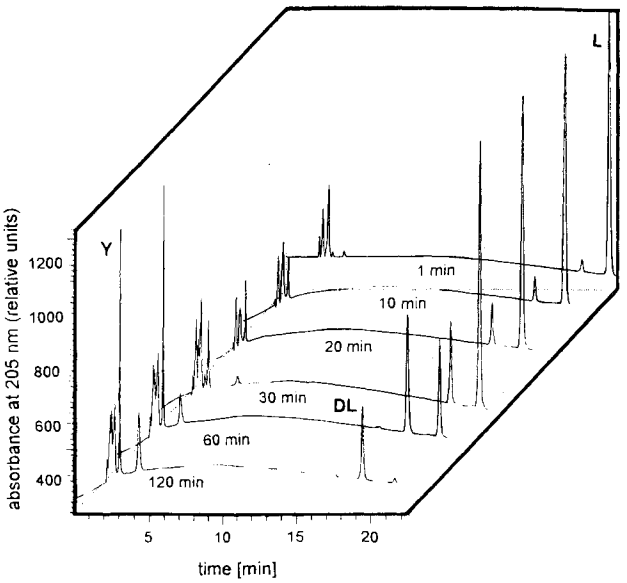

Fig. 5. HPLC profile of Leu enkephalin (L) and its degradation products (DL = des-Tyr Leu enkephalin; Y = tyrosine). Leu enkephalin (1 mM) in 2 ml of 50 mM phosphate buffer pH 6.5 were incubated with porcine mucosa (surface: 0.785 cm^2) at 37°C and aliquot volumes of 300 µl analyzed by HPLC at indicated time points.

that membrane bound aminopeptidase N which is covered with a mucus layer, could not be inhibited by poly(acrylic acid) but partially by a bacitracin-poly(acrylic acid) conjugate (4). The comparison of the inhibitory effect of the novel polymer described here with a recently developed, also polyanionic, complexing polymer is shown in Fig. 6. It demonstrates an

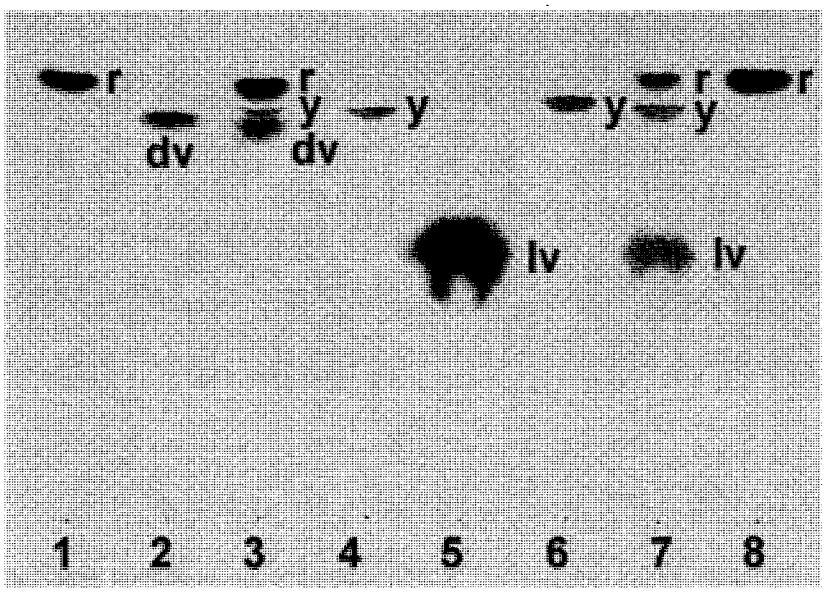

Fig. 4. Degradation of Leu enkephalin after 30 min incubation with aminopeptidase N. Leu enkephalin and degradation products were developed in n-butanol/acetic acid/water (3 + 1 + 4) and stained for protein with ninhydrin-reagent (appearing colors: r = reddish brown, dv = dark violet, lv = light violet, y = yellow): lane 1, Leu enkephalin; lane 2, tyrosine; lane 3, Leu enkephalin incubated with aminopeptidase N; lane 4, des-Tyr Leu enkephalin; lane 5, glycine; lane 6, Gly-Phe-Leu; lane 7, des-Tyr Leu enkephalin incubated with aminopeptidase N; lane 8, Phe-Leu;

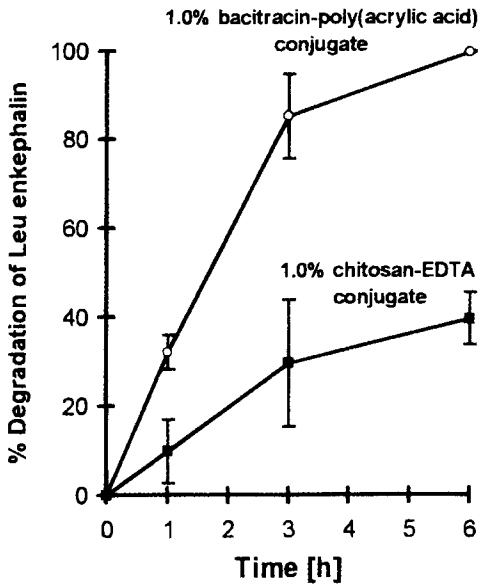

Fig. 6. Comparison of the inhibitory effect of the chitosan-EDTA conjugate with an other bioadhesive polyanionic polymer on degradation of Leu enkephalin by membrane bound enzymes.

approximately 3-fold higher inhibitory effect of the chitosan-EDTA conjugate.

DISCUSSION

It is well known that poly(acrylate) derivatives display inhibitory effects on proteases by deprivation of bivalent cations from the enzyme structure (12). The significant inhibition of trypsin by poly(acrylate) derivatives caused by the deprivation of Ca^{2+}-ions is a good example for this mechanism (13). However, this inhibitory effect depends very much on whether the polymer or the enzyme has a stronger binding affinity to the according bivalent cation. As the inhibition of aminopeptidase N was comparably very weak by poly(acrylate) derivatives but high using strong complexing agents like EDTA (14); it may be concluded that the capability of these polymers to extract Zn^{2+}-ions out of the enzyme structure is insufficient. Hence, the combination of strong complexing agents with bioadhesive polymers should lead to systems exhibiting a sufficient binding affinity to bivalent cations. Recently, our research group demonstrate a significantly improved inhibitory effect of a bacitracin-poly(acrylic acid) conjugate in comparison with the unmodified polymer (4). As the association constant of zinc bacitracin and zinc EDTA was estimated to be 2.5×10^3 and 1.6×10^{16}, respectively (15,16), EDTA was substituted for bacitracin in this study. Accordingly, the approximately 3-fold higher inhibitory effect of the chitosan-EDTA conjugate in comparison with the bacitracin-poly(acrylic acid) conjugate (Fig. 6) could be explained, on one hand, by the much higher association constant of zinc EDTA and, on the other hand, by the higher amount of EDTA compared to bacitracin which was bound to the according polymer.

The covalent attachment of EDTA to chitosan opens the door to a new generation of bioadhesive polymers exhibiting a strong binding affinity to the bivalent cation Zn^{2+} as already demanded by Lueßen et al. (17). Hence, inhibition of BBM-bound aminopeptidase N even covered by a mucus layer became possible. Moreover, as EDTA is able also to bind other bivalent cations, the inhibition of further proteases seems to be very likely and should be subject of further investigations. The inhibition of Leu enkephalin degradation by entire enzymes of the BBM using the chitosan-EDTA conjugate is a good evidence for this possibility.

Covalenty attached to the polymer, EDTA looses at least one of its carboxylic acid groups as shown in Fig. 2. However, this change in its structure does not seem to have a significant influence on its complexing capability. Under the assumption that to each amino group of the polymer one EDTA molecule is covalently linked and that the complexing capability of coupled EDTA remains uninfluenced, one gram of this polymer should be able to bind 2.00–2.30 mMole Zinc, as calculated for the sodium salt and free acid, respectively. The conjugate described here is able to bind 2.0 mM Zinc per gram polymer. As this complexing capability of the novel polymer correlates directly with the same amount of unbound EDTA, the binding capacity of EDTA attached to the polymer does not seem to be reduced due to the amide binding.

Tensile studies revealed enhanced bioadhesive properties of the novel polymer as compared to unmodified chitosan. This observation is in agreement with earlier investigations of polymers' ability to bind to mucin-epithelial surfaces, suggesting that a polyanionic polymer is preferred over a polycationic polymer. Moreover, it is also suggested that within polyanionic polymers, carboxylic acid polymers are the most promising candidates (18). In contrast to unmodified chitosan, the chitosan-EDTA conjugate should be easily hydratable in the alkaline environment of the intestine.

With regard to overcoming enzymatic barriers to perorally administrated protein and peptide drugs the novel polymer described here, offers several advantages compared to already established systems:

(I) The novel polymer can be easily produced under comparably low costs.

(II) It exerts an excellent inhibitory effect on aminopeptidase N and it is very likely that further membrane bound enzymes will be inhibited by this polymer.

(III) As already shown for similar polyanionic bioadhesive polymers (2, 19), the chitosan-EDTA conjugate also should be useful in different pharmaceutical formulations as a vehicle in sustained release systems.

(IV) Compared to unmodified chitosan, the novel polymer offers improved bioadhesive properties.

(V) As the complexing agent is immobilized to the unabsorbable polymer, unintended dilution effects as well as systemic toxic side effects of the inhibitor (20) can be excluded.

CONCLUSIONS

The chitosan-EDTA conjugate described here, displays a high binding affinity for the bivalent cation zinc which enables a complete inhibition of enzymatic activity of aminopeptidase N. Moreover, it is more bioadhesive than unmodified chitosan. As it is likely that further, at least zinc-containing, peptidases will be inhibited by the chitosan-EDTA conjugate, this novel polymer seems to be a very useful tool in protecting peptide drugs from luminal degradation by membrane-bound peptidases.

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