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In vitro evaluation of polymeric excipients protecting calcitonin against degradation by intestinal serine proteases

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Abstract

The oral bioavailability of salmon calcitonin is strongly reduced due to the enzymatic degradation by luminally secreted serine proteases. Apart from being degraded by trypsin (EC 3.4.21.4) and chymotrypsin (EC 3.4.21.1), it was shown in this study that calcitonin is also digested by elastase (EC 3.4.21.36).

It was therefore the aim of this study to generate polymeric excipients protecting perorally administered salmon calcitonin from degradation by these enzymes. Mediated by a carbodiimide trypsin and chymotrypsin inhibitor Bowman-Birk inhibitor (BBI) and elastase inhibitor elastatinal were each covalently attached to the mucoadhesive polymer chitosan. The share of the Bowman-Birk inhibitor in the resulting conjugate was $3.5 \pm 0.1\%$ (w/w, mean $\pm$ S.D., $n = 4$) and that of elastatinal $0.5 \pm 0.03\%$ (w/w, mean $\pm$ S.D., $n = 4$). Enzyme assays with synthetic substrates demonstrated a strong inhibitory effect of the chitosan-BBI conjugate towards trypsin and chymotrypsin as well as of the chitosan-elastatinal conjugate towards elastase. In an artificial intestinal fluid containing physiological concentrations of trypsin, α-chymotrypsin and elastase, calcitonin being incorporated in unmodified chitosan (0.5%, w/v) was degraded by $99.7 \pm 0.1\%$ (mean $\pm$ S.D., $n = 3$) within 2 h at 37 °C. On the contrary, incorporating the drug in chitosan-BBI conjugate and chitosan-elastatinal conjugate (1+1, 0.5%, w/v) led to a degradation of only $36.4 \pm 0.9\%$ (mean $\pm$ S.D., $n = 3$). Hence, the chitosan-inhibitor conjugates described in this study seem to be promising tools for the oral delivery of salmon calcitonin.

Keywords: Enzymatic degradation; Calcitonin delivery; Protective effect; Chitosan derivatives

1. Introduction

In recent years, due to the great progress in biotechnology as well as gene technology a large number of peptide and protein drugs can be produced in commercial quantities. The majority of such drugs is commonly administered by the parenteral routes, which are often complex, difficult, painful and occasionally dangerous. Hence, the oral administration of peptide and protein drugs would lead to a higher patient compliance being favoured by patients, practitioners and pharmaceutical industry for reasons of ease and economics. Among these peptide drugs one of the most frequently used is calcitonin, which is beneficial in treatment of several chronical diseases such as osteoporosis or morbus Paget's. So far, calcitonin has not reached its full market potential due to the inconvenience and pain associated with the injectable dosage forms and the low patient acceptance of the nasal de-

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livery system. An oral dosage form of the drug will allow full clinical exploration of the role of calcitonin in osteoporosis or in morbus Paget's and might lead to a broader use of calcitonin as a safe and efficacious adjunct to the treatment of these diseases. However, apart from a poor absorption from the gastrointestinal-tract, the oral bioavailability of calcitonin is strongly limited by the enzymatic degradation based on luminally secreted serine proteases. One attempt to overcome this so-called enzymatic barrier is the co-administration of protease inhibitors. Although it has been demonstrated that these auxiliary agents are very efficient in improving the oral bioavailability of peptides (Yamamoto et al., 1994), their use remains questionable as they may cause several side effects, such as an unintended disturbance of digestion of nutritive proteins, an inhibitor induced pancreatic hypersecretion caused by a luminal feedback regulation as well as systemic toxic side effects. A promising strategy in order to profit from the benefits offered by enzyme inhibitors without taking their mentioned drawbacks can be seen in the covalent immobilisation of these auxiliary agents on mucoadhesive polymers used as drug carrier matrices (Bernkop-Schnürch, 1998a).

The aim of this study was therefore the development of a drug carrier system with a protective effect against those proteases, which are mainly responsible for the intestinal degradation of salmon calcitonin (see Fig. 1). On the one hand, the mucoadhesive polysaccharide chitosan was chosen as cationic carrier matrix. In contrast to anionic polymers such as polyacrylic acid incompatibilities based on ionic interactions with the positive charged peptide drug exhibiting an isoelectric point above pH 9 (Sigurjónsdóttir et al., 1999) can be excluded for chitosan. Being aware of the rapid degradation of calcitonin by trypsin and α-chymotrypsin (Dohi et al., 1993; Lang et al., 1996), on the other hand, made the development of a polymeric matrix providing a strong protective effect towards these two pancreatic serine-proteases necessary. As the Bowman-Birk inhibitor (BBI; see Fig. 2) is well

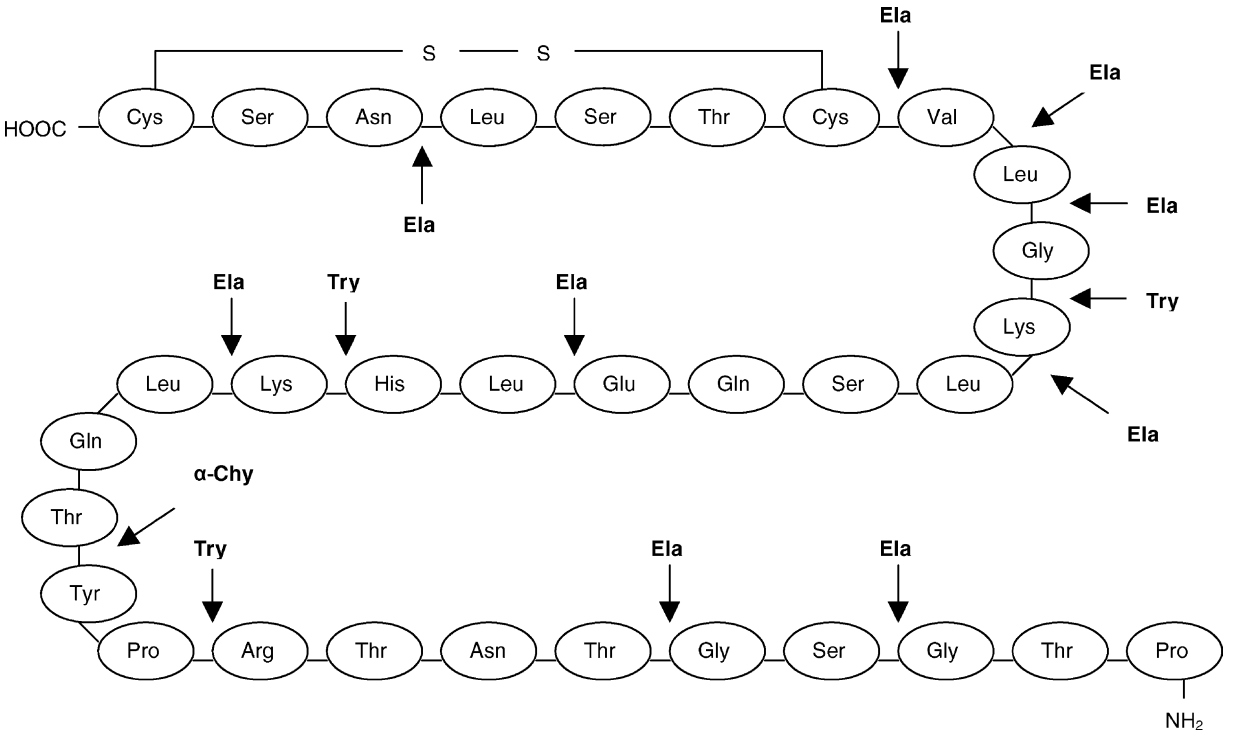

Fig. 1. Amino acid sequence of salmon calcitonin. The arrows indicate the potential cleavage sites caused by trypsin (Try), α-chymotrypsin (α-Chy) and elastase (Ela).

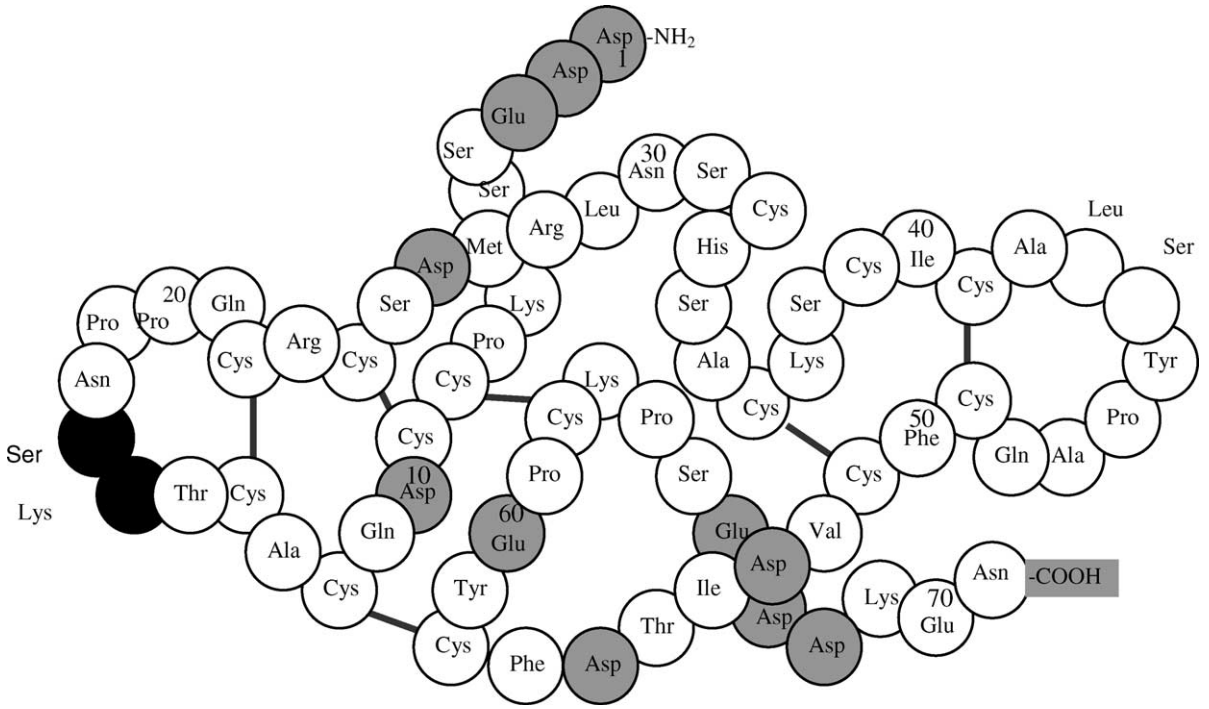

Fig. 2. Complete covalent structure of the Bowman-Birk inhibitor (assumed from Birk (Birk, 1985)). Amino acids at the trypsin-inhibitory site are shown as solid black circles, whereas amino acids at the chymotrypsin-inhibitory site are shown as white circles; available carboxylic acid moieties are marked in grey.

known for its inhibitory effect towards trypsin and α-chymotrypsin, it was covalently attached to chitosan. In addition, a so far not investigated degradation of calcitonin by elastase seemed likely, because of the high number of potential cleavage sites for elastase present in the structure of the peptide (see Fig. 1). Consequently, it was a further aim of this study to verify this theory and generate a polymeric excipient also providing a protective effect towards this enzyme. Moreover, the inhibitory efficacy and the protective effect of the polymer-enzyme inhibitor conjugates for calcitonin was investigated in vitro.

2. Materials and methods

2.1. Evaluation of the enzymatic digestion of salmon calcitonin by elastase (EC 3.4.21.36)

In order to verify that salmon calcitonin is degraded by elastase a SDS-PAGE analysis was performed. Initially, 1 mg of elastase (Type II-A: from porcine pancreas; Sigma, St. Louis, MO) was dissolved in 1 ml 50 mM acetate buffer pH 5.0. As commercially available elastase is often contaminated with trypsin and α-chymotrypsin (Walker et al., 2001) the selective inhibitors anti-pain (hydrochloride, from microbial source, Sigma, St. Louis, MO) and chymostatin (mixture of A, B and C components, from microbial source, Sigma, St. Louis, MO) were added to this solution in a concentration of each 0.01% (m/v). The mixture was incubated for 30 min at room temperature. Thereafter, 10 μl of this solution were added to 100 μl of a 50 mM acetate buffer pH 5.0 containing 100 μg of salmon calcitonin (Bachem AG, Bubendorf, Switzerland) and the obtained mixture was incubated for 60 min at 37 °C. Aliquot volumes of 15 μl were withdrawn at predetermined time points, and the enzymatic reaction was stopped by adding 5 μl of a sample buffer (1 g sodium dodecyl sulfate, 2 ml β-mercaptoethanol, 13 ml 62.7 mM Tris–HCl pH 6.8, 10 ml glycerol) and heating samples for 15 min

at 94 °C. Aliquots of 10 μl were electrophoresed in 17% SDS-PAGE (Mini Protean II, Biorad, Veenendaal, The Netherlands). The degree of proteolysis was analysed on gels stained with Coomassie blue.

2.2. Synthesis and isolation of the chitosan-Bowman-Birk inhibitor conjugate

The Bowman-Birk inhibitor (BBI; Sigma, St. Louis, MO) was covalently linked to chitosan (medium molecular mass: 400 kDa; Fluka GmbH, Buchs, Switzerland) using EDAC (1-ethyl-3,3-dimethylaminopropyl carbodiimide hydrochloride; Sigma, St. Louis, MO) to mediate the formation of amide bonds between the carboxylic acid groups of the inhibitor and the primary amino groups of the polymer. Concentrations of reagents used for the coupling reaction are listed in Table 1. Chitosan (200 mg) were suspended in 35 ml 0.01 M HCl and stirred until the polymer was completely dissolved. The pH value of this solution was then adjusted at pH 6 by adding very slowly 1 M NaOH, and demineralised water was added to a final volume of 40 ml. Bowman-Birk inhibitor (10 mg) and EDAC (820 mg) were dissolved in 2 ml of demineralised water and preincubated for 20 min at room temperature under permanent stirring in order to activate the carboxylic acid moieties of the inhibitor. The activated inhibitor solution was added to the 0.5% (w/v) chitosan HCl pH 6 solution. The reaction mixture was incubated for 15 h under permanent stirring at room temperature and the resulting polymer-conjugate was purified by dialysing four times against 50 mM HCl and then six times against demineralised water, both at 10 °C. Sample being prepared and isolated in exactly the same way as the chitosan-inhibitor conjugate but omitting EDAC during the coupling reaction served as control for evaluating the efficacy of the purification. Samples were lyophilised by drying frozen aqueous polymer solutions at −30 °C and 0.01 mbar (Christ Beta 1–8 K; Osterode am Harz, Germany). Chitosan-BBI conjugate and control were stored at 4 °C until use.

2.3. Synthesis and purification of the chitosan-elastatinal conjugate

The covalent attachment of elastatinal (Sigma, St. Louis, MO) to chitosan was obtained by the formation of amide bonds between amino groups of the polymer and the terminally located carboxylic acid group of the inhibitor. The conjugation was achieved as described previously by our research group (Bernkop-Schnürch and Scerbe-Saiko, 1998b). Thereby, 200 mg of chitosan (medium molecular mass: 400 kDa; Fluka GmbH, Buchs, Switzerland) obtained from crab shells were suspended in 15 ml 0.01 M HCl and stirred until the polymer was completely dissolved. The pH value was then adjusted at pH 6 by adding 1 M NaOH, and demineralised water was added to a final volume of 20 ml. Elastatinal (5 mg) and EDAC (820 mg) (Sigma, St. Louis, MO) were dissolved in 1 ml of demineralised water and preincubated for 30 min. This solution was added to the 1% (w/v) chitosan HCl pH 6 solution. The reaction mixture was incubated for 10 h under permanent stirring at room temperature and the resulting polymer-inhibitor conjugate was purified by dialysing as described above. Sample being prepared and isolated in exactly the same way as the chitosan-elastatinal conjugate but omitting EDAC during the coupling reaction served as control for evaluating the efficacy of the purification. Samples

Table 1
Concentrations of reagents used for reaction mixtures in order to form chitosan-inhibitor conjugates with covalently attached Bowman-Birk inhibitor and elastatinal

	Chitosan (mg/ml)	EDAC (mg/ml)	Bowman-Birk inhibitor (mg/ml)	Elastatinal (mg/ml)	Share of inhibitor on the polymer (%; w/w; $n = 4$; ±S.D.)
Chitosan-BBI conjugate	4.8	19.5	0.24	–	3.5 ± 0.1
Chitosan-elastatinal conjugate	9.5	39	–	0.24	0.5 ± 0.03
Control (BBI)	4.8	–	0.24	–	<0.3
Control (Elastatinal)	9.5	–	–	0.24	<0.05

were lyophilised and stored in the same way as described above.

2.4. Determination of the degree of modification of the chitosan-BBI conjugate

The amount of immobilised Bowman-Birk inhibitor on the chitosan backbone was determined on the one hand by spectrophotometric analysis and on the other hand enzymatically via trypsin inhibition test.

2.4.1. Determination of the coupling rate by spectrophotometric analysis

First, 30 mg of the chitosan-BBI conjugate were hydrated in 3 ml demineralised water to obtain a 1% (w/v) solution. Then, the absorbance of aliquots of 500 μl was determined between 210 and 320 nm (Hitachi U-3000 Spectrophotometer, Tokyo, Japan). The amount of bound BBI was quantified from integrated peak areas (between 250 and 290 nm) and calculated by interpolation from an according standard curve obtained by determination of the peak area (250–290 nm) of a series of aqueous solutions containing increasing concentrations of BBI.

2.4.2. Determination of the coupling rate by trypsin inhibition test

First, 0.2 mg of chitosan-Bowman-Birk inhibitor conjugate or of unmodified chitosan were swelled in 0.4 ml of 50 mM phosphate buffer pH 6.8. Thereafter, trypsin (EC 3.4.21.4) (9.82 units; Sigma, St. Louis, MO) dissolved in 20 μl of 50 mM phosphate buffer pH 6.8 was added and the mixture was incubated for 30 min at room temperature. After addition of 0.34 mg of *N*-α-benzoylarginine ethylester (BAEE) dissolved in 100 μl of 50 mM phosphate buffer pH 6.8, the increase in absorbance ($\Delta A_{253\,nm}$) caused by the hydrolysis of the substrate to *N*-α-benzoylarginine (BA) was recorded (Lambda-16, Perkin-Elmer) at 1 min intervals for 14 min.

Thereafter, the inhibitory efficacy of the modified polymer was compared with the inhibitory effect of gradually increasing Bowman-Birk inhibitor concentrations and the amount of bound BBI was quantified by interpolation.

2.5. In vitro evaluation of the inhibitory effect of the chitosan-BBI conjugate towards trypsin (EC 3.4.21.4) and α-chymotrypsin (EC 3.4.21.1)

The evaluation of the inhibitory effect of the chitosan-Bowman-Birk inhibitor conjugate towards trypsin was carried out as described above.

The inhibitory effect toward α-chymotrypsin was performed as follows. First, 0.05 mg of chitosan-Bowman-Birk inhibitor conjugate or of unmodified chitosan were swelled in 0.25 ml of 50 mM phosphate buffer pH 6.8. Thereafter, α-chymotrypsin (0.104 BTEE units; type II, from bovine pancreas, Sigma, St. Louis, MO) dissolved in 20 μl of 50 mM phosphate buffer pH 6.8 was added and the mixture was incubated for 30 min at room temperature. After addition of 0.25 ml of substrate solution (18.5 mg of *N*-benzoyl-L-tyrosine ethylester (BTEE) dissolved in 31.7 ml of methanol and 18.3 ml of demineralised water) the increase in absorbance ($\Delta A_{254\,nm}$) caused by the hydrolysis of the substrate to *N*-α-benzoyltyrosine (BT) was recorded at 1 min intervals for 14 min.

2.6. Determination of the degree of modification and inhibitory effect of the chitosan-elastatinal conjugate

Initially, 0.1 mg of chitosan-elastatinal conjugates or unmodified chitosan and 10 μg of elastase (Type II-A: from porcine pancreas; Sigma, St. Louis, MO) in 170 μl of 50 mM phosphate buffer pH 6.8 were transferred to the wells of a microtitration plate (96-well, not binding) and incubated for 20 min at room temperature. Then, 130 μl of the substrate medium (0.2 mg of succinyl-(L-alanyl)$_3$-4-nitroanilide (Sigma, St. Louis, MO)/ml 50 mM phosphate buffer pH 6.8; filtered before use) were added and the increase in absorbance ($\Delta A_{405\,nm}$) caused by the enzymatic reaction at room temperature was recorded at 2 min intervals for 14 min and then after 30 min with a microtitration plate reader (Anthos reader 2001; Salzburg, Austria).

The inhibitory activity of the modified polymer was compared with the inhibition caused by increasing elastatinal concentrations and the quantity of linked elastatinal was calculated by interpolation.

2.7. Evaluation of the protective effect for calcitonin

First, 2.5 mg of the chitosan-BBI conjugate and 2.5 mg of the chitosan-elastatinal conjugate were

hydrated in 500 μl of 50 mM acetate buffer pH 5.5. Then, 100 μl of a calcitonin solution (2 mg of salmon calcitonin (Bachem AG, Bubendorf, Switzerland)/100 μl of 50 mM acetate buffer pH 5.5) and 400 μl of the same buffer but containing trypsin (EC 3.4.21.4; 196.4 units), chymotrypsin (EC 3.4.21.1; 2.08 units) and elastase (EC 3.4.21.36; 0.042 units) were added. Immediately after the addition of the enzyme solution aliquots of 60 μl were withdrawn and diluted with the same volume of 1% trifluoroacetic acid (TFA) used as stop solution in order to avoid any enzymatic degradation of the peptide. These first samples were used as reference values (time point zero).

The remaining mixture was incubated for 2 h at 37 ± 0.5 °C. Thereafter aliquots of 60 μl were withdrawn and the enzymatic reaction was terminated as described above. All samples were centrifuged (13 500 rpm, 6 min, Hermle Z 323 K) in order to remove remaining polymer contents. For determining the remaining undigested calcitonin, 20 μl of the supernatant fluid was directly injected for HPLC-analysis (series 200 LC; Perkin-Elmer). The HPLC-analysis was carried out according to a method previously described by our research group (Marschütz and Bernkop-Schnürch, 2000a). Concentrations of calcitonin were quantified from integrated peak areas and calculated by interpolation from an according standard curve. Samples prepared in the same way but containing 5 mg of unmodified chitosan instead of chitosan-inhibitor conjugates served as control.

2.8. Statistical data analysis

Statistical data analysis was performed using the student *t* test with $P < 0.05$ as the minimal level of significance.

3. Results

3.1. Synthesis of the chitosan-Bowman-Birk inhibitor conjugate

As shown in Fig. 2 the Bowman-Birk inhibitor has 12 available carboxylic acid moieties to constitute an amide binding with the available amino groups of the polymer. The amount of covalent bound BBI on the chitosan backbone was determined by two different test systems. On the one hand spectrophotometric analysis were used and the quantity of linked inhibitor calculated by this method was of 3.3 ± 0.2% (w/w; $n = 4$). On the other hand, the degree of modification was calculated enzymatically by a trypsin inhibition test. As displayed in Table 1 the coupling rate acquired from this evaluation was of 3.5 ± 0.1% (w/w; $n = 4$) and evidenced a very good correlation with data obtained by the previously described analysis. Control being prepared in exactly the same way as the conjugate but omitting EDAC during the coupling reaction showed neither a significant increase in the absorbance nor in the inhibitory effect towards trypsin compared to unmodified chitosan. These data showed that only a negligible amount of unbound BBI remained on the polymer after purification.

3.2. Inhibitory efficacy of chitosan-BBI conjugate

The conjugate demonstrated a strong inhibitory activity towards trypsin resulting in a significant decrease in the degradation of the trypsin substrate *N*-α-benzoyl-L-arginine-ethylester (BAEE). As shown in Fig. 3 after 14 min of enzymatic reaction only 5.8% of the substrate were digested in presence of 0.04% chitosan-BBI conjugate, whereas at this reference point 56.4% of BAEE were degraded in presence of the same concentration of unmodified chitosan. Also α-chymotrypsin was strongly inhibited by the conjugate (Fig. 4), but the effect displayed by the decrease of the degradation of the substrate *N*-benzoyl-L-tyrosine ethylester (BTEE) was not as pronounced as in the case of trypsin. This difference may be caused on the one hand by the higher inhibitory effect of BBI towards trypsin. On the other hand a sterical hindrance of the chymotrypsin inhibiting site of BBI in consequence of the formation of the amide bonds during the coupling reaction may be responsible. The chitosan-BBI conjugate was also evaluated with regard to its inhibitory activity towards elastase, but no significant inhibition of this enzyme was found (data not shown). These results are in good agreement with former studies showing that the Bowman-Birk inhibitor itself has a comparatively low effect towards elastase (Seidl and Liener, 1972).

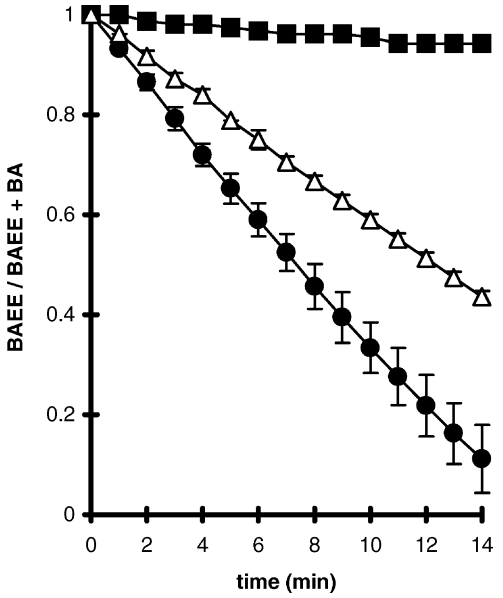

Fig. 3. Comparison of the decrease in enzymatic activity of trypsin (18.88 spectrophotometric BAEE units/ml) in presence of chitosan-BBI conjugate 0.04% (■) unmodified chitosan 0.04% (△) and buffer only (●). Hydrolysis of the substrate *N*-α-benzoyl-arginine ethyl ester (BAEE) to *N*-α-benzoyl-arginine (BA) was determined at pH 6.8 and 20 °C. Indicated values are the means of at least three experiments (±S.D.).

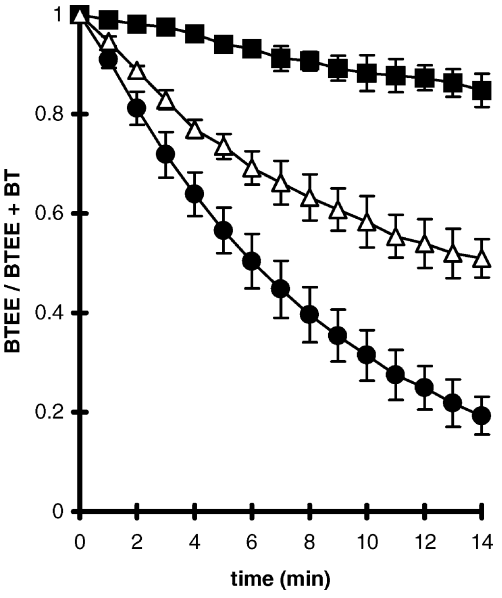

Fig. 4. Comparison of the decrease in enzymatic activity of α-chymotrypsin (0.2 spectrophotometric BTEE units/ml) in presence of chitosan-BBI conjugate 0.01% (■), unmodified chitosan 0.01% (△) and buffer only (●). Hydrolysis of the substrate *N*-α-benzoyl-L-tyrosine ethyl ester (BTEE) to *N*-α-benzoyl-L-tyrosine (BT) was determined at pH 6.8 and 20 °C. Indicated values are the means of at least four experiments (±S.D.).

3.3. Proteolytic effect of elastase on calcitonin

As this study intended to develop a drug carrier system protecting salmon calcitonin from enzymatic digestion in the intestine, the structure of this peptide (see Fig. 1) was analysed and the potential cleavage sites caused by the most important proteases were considered. Apart from expected potential cleavage sites for trypsin and α-chymotrypsin also further cleavage sites for elastase were identified, as this latter protease is known for digesting proteins on the carboxylic side of small amino acids like glycine or leucine (Bernkop-Schnürch, 1998a). In order to prove the enzymatic degradation of salmon calcitonin caused by elastase a SDS-PAGE analysis was carried out. The polyacrylamide gel depicted in Fig. 5 demonstrated the high enzymatic efficacy of elastase in decomposing salmon calcitonin.

3.4. Synthesis and inhibitory efficacy of chitosan-elastatinal conjugate

As calcitonin is on the one hand rapidly degraded by elastase and the chitosan-BBI conjugate on the other hand did not display any protective effect towards elastase, the synthesis of a chitosan-enzyme inhibitor conjugate capable of decreasing the activity of elastase was necessary. The protease inhibitor chosen for the coupling reaction with chitosan was elastatinal, a selective elastase inhibitor. As the terminal located aldehyde function of elastatinal is essential for its inhibitory activity, the inhibitor was bound to the polymer at the opposite end of the molecule. The amount of covalently linked elastatinal was determined enzymatically by an elastase inhibition test to be $0.5 \pm 0.03\%$ (w/w; $n = 4$). Control prepared in the same way as chitosan-elastatinal conjugates but omitting EDAC during the coupling reaction showed no difference in the inhibition behaviour compared to unmodified chitosan,

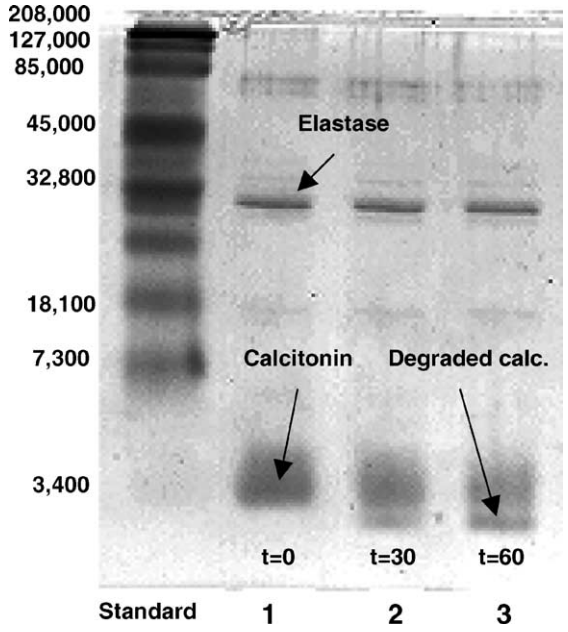

Fig. 5. SDS-PAGE 17% analysis of salmon calcitonin degradation by elastase: 100 μg of calcitonin and 10 μg elastase (preincubated with 0.01% antipain and 0.01% chymostatin) dissolved in 50 mM acetate buffer pH 5 were incubated for 60 min at 37 °C. Degradation was analysed after 0 (lane 1), 30 (lane 2) and 60 min (lane 3) of incubation.

verifying the efficiency of the purification method used.

The inhibitory efficacy of the chitosan-elastatinal conjugate towards elastase displayed by the decrease of the degradation of the substrate *N*-succinyl-(L-alanyl)$_3$-4-nitroanilide is shown in Fig. 6. After 30 min of enzymatic reaction only 17.2% of the substrate were degraded in presence of 0.27% of chitosan-elastatinal conjugate, whereas in presence of 0.27% of unmodified chitosan the substrate was completely digested. A comparatively higher enzymatic activity of elastase in presence of unmodified chitosan was observed.

3.5. Protective effect for calcitonin

A mixture of chitosan-BBI and chitosan-elastatinal (1+1, w/w) was evaluated regarding its protective effect on the enzymatic degradation of calcitonin. The mixture of the two chitosan derivatives showed a strong protective efficacy for calcitonin towards trypsin, α-chymotrypsin and elastase. After

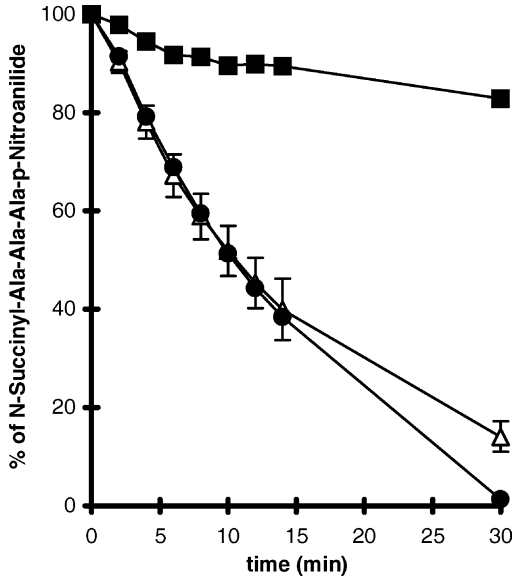

Fig. 6. Comparison of the decrease in enzymatic activity of elastase (33.3 μg/ml) in presence of chitosan-elastatinal conjugate 0.27% (■), unmodified chitosan 0.27% (△) and buffer only (●). Hydrolysis of the substrate medium *N*-succinyl-(L-alanyl)$_3$-4-nitroanilide was determined at pH 6.8 and 20 °C. Indicated values are the means of at least four experiments (±S.D.).

2 h incubation with the proteases only 0.3 ± 0.1% (mean ± S.D., $n = 3$) of undegraded salmon calcitonin was remaining in a 0.5% solution of unmodified chitosan. Instead the combination of 0.25% chitosan-BBI and 0.25% chitosan-elastatinal provided a protection for 63.6 ± 0.9% (mean ± S.D., $n = 3$) of the deployed calcitonin. The activity of a protease with a synthetic substrate can probably not be directly compared to the activity of the same enzyme with a protein. However, because of the protective effect of the chitosan-inhibitor conjugates on salmon calcitonin, the validity of the results obtained by the enzyme assays with mentioned synthetic substrates could be confirmed as well.

4. Discussion

Within this study novel polymeric excipients for the peroral administration of calcitonin, providing a strong protective effect towards an enzymatic attack of intestinal proteases have been generated. Among these enzymes, luminally secreted serine proteases

seem to be mainly responsible for the digestion of salmon calcitonin. Sakuma et al. (1997), for instance, could demonstrate a high cleavage rate of calcitonin caused by trypsin. Furthermore, Dohi et al. (1993), and Lang et al. (1996), showed that the peptide is digested not only by trypsin but also by α-chymotrypsin. Within this study the ability of elastase in decomposing salmon calcitonin was shown as well. The presystemic metabolism of perorally administered calcitonin caused by luminally secreted proteases should be strongly reduced by the following properties of the polymers generated within this study.

(a) A protection towards an enzymatic attack should occur at least to some extent due to the incorporation of calcitonin even in an unmodified chitosan carrier matrix, as the proteases must, first of all, penetrate into the polymeric network of the hydrophylic matrix before degrading the incorporated peptide drug. This protecting effect of unmodified chitosan should be particularly manifested towards trypsin and α-chymotrypsin like it can be evinced from Figs. 3 and 4. A much weaker protection, however, has to be expected towards elastase.

(b) In order to improve the protective effect of such a polymeric matrix system, chitosan has been substituted by chitosan-BBI and chitosan-elastatinal conjugate. As shown in Figs. 3, 4 and 6 the protective properties of the carrier system could be remarkably advanced. This improvement in the features of chitosan should occur already by addition of a low percentage of these simple polymer modifications.

(c) As already anticipated, chitosan is well known for its mucoadhesive features (Lehr et al., 1992). This mucoadhesiveness can even be maintained in acceptable extent after the covalent attachment of low amounts of enzyme inhibitors (Bernkop-Schnürch et al., 1997a). Because of this property, such polymers are able to provide an intimate contact between the intestinal mucosa and a matrix system made of the same macromolecules. This should therefore avoid a degradation of the embedded drug on the way between the carrier system and the absorption membrane. Kawashima et al. (2000), for instance, could demonstrate that an improvement in the mucoadhesive properties of nanospheres containing elcatonin by coating them with chitosan increased the oral bioavailability of the peptide significantly. Similar results were obtained by Takeuchi et al. (1996), with orally administered liposomes containing insulin.

Although all these considerations are based on in vitro data, a strong support for these theories was provided recently. Orientating in vivo studies with orally given calcitonin matrix tablets containing the polymer-inhibitor conjugates described here led to a significantly improved bioavailability versus control formulations based on unmodified chitosan (Guggi et al., 2002). Moreover, Marschütz et al. (2000b) could achieve a significantly increased oral bioavailability of insulin using a system based on sodium carboxymethylcellulose (Na-CMC)-enzyme inhibitor conjugates, thus leading to the presumption that the polymeric excipients described within this study should be responsible for the mentioned increased oral bioavailability of calcitonin . Furthermore, a sustained drug release from such formulations can be easily controlled by the share of polymer added (Bernkop-Schnürch and Göckel, 1997b).

5. Conclusion

Within this study novel polymeric excipients for peroral administration of calcitonin have been generated and the enzymatic digestion of salmon calcitonin by elastase was demonstrated for the first time. Chitosan was selected as basis polymer because of its well known mucoadhesive properties which should provide an intimate contact of the matrix system to the mucosa, thus allowing to avoid the digestion of calcitonin on the way between the polymeric network and the absorption site. A further decrease of the degradation of the peptide drug during this process should be achieved by the covalent attachment of protease inhibitors. The polymer-inhibitor conjugates described, showed excellent inhibitory efficacy towards trypsin, α-chymotrypsin and elastase, respectively, and were also able to reduce significantly the digestion of calcitonin caused by these proteases. Therefore, the mentioned conjugates seem to be promising components for drug carrier systems protecting salmon calcitonin from intestinal degradation caused by pancreatic serine proteases.

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