Drug Development and Industrial Pharmacy, 23(8), 733–740 (1997)

RESEARCH PAPER

Development and Analysis of a Polymer Protecting from Luminal Enzymatic Degradation Caused by α-Chymotrypsin

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ABSTRACT

We have been developing a modified polymer which protects inserted peptide and protein drugs from chymotrypsinic degradation. A trypsin-chymotrypsin-inhibitor was covalently bound to a bioadhesive polymer (poly(acrylic acid)) by a condensation reaction of the available amino groups of the inhibitor with the carboxyl groups of the polymer. The inhibition of α-chymotrypsin by the isolated conjugate was determined by an enzyme assay using N-benzoyl-L-tyrosine ethyl ester as substrate. It had an inhibitory effect of 2.4 CIU (= chymotrypsin inhibiting units) per mg. The protective effect was also evaluated in a drug delivery system containing 5% of this modified polymer. Under physiological α-chymotrypsin concentrations it demonstrated, in lateral parts of the carrier matrix, the remaining of 71.4% ± 14.3% (means of three experiments; ± S.D.) of undigested inserted test-protein (β-galactosidase) after 7 hr of incubation at 37 ± 0.5°C. Samples from the center of the matrix did not show any digestion of the protein. On the other hand a complete digestion in lateral parts and the remaining of only 67% ± 14.4% of undigested test-protein in the center, could be observed by the same delivery system without the conjugate. Because of this verified protective effect and the possibility to exclude toxic side effects of the inhibitor by immobilization, the system described here seems to be an important step towards a successful peroral (poly)peptide drug administration.

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INTRODUCTION

Many peptide and protein drugs, as well as many vaccines, are available for therapy and prophylaxis. The majority of such drugs and vaccines is most commonly administered by the parenteral routes that are often complex, difficult, painful, and occasionally dangerous (1). Besides so-called alternative routes of application i.e. the nasal, buccal, rectal, and vaginal routes, the peroral route also seems to be useful for peptide and protein delivery, although it requires very sophisticated approaches (2). The principle possibility of a successful peptide and even protein absorption by peroral administration has already been demonstrated by several approaches in the past decade (3,4,5). Nevertheless, up until now practical pharmaceutical use has been limited by an extremely low bioavailability mainly caused by a rapid degradation of (poly)peptides in the gastrointestinal-tract (6,7). Looking at this enzymatic barrier in detail, two presystemic components where enzymatic degradation takes place can be distinguished, and therefore this is the barrier which must be localized. First, as known from the digestion of nutritive proteins, degradation can take place in the lumen either by secreted or by membrane-bound enzymes. Secondly, cytosolic enzymes of the mucosal cells may also play a role, especially when absorption occurs via transcellular routes (8). Whether the luminal or the cytosolic barrier is limiting for the bioavailability of (poly)peptide drugs will depend on their respective structure (2).

Focusing on the luminal barrier, progress must be made in order to generate peroral drug delivery systems reducing this presystemic metabolism of peptide and protein drugs. This must be done in order to gain blood concentrations high enough for therapy or, in the case of vaccines, to obtain a sufficient immune response. With regard to reducing this barrier, considerable interest has been shown in the use of enzyme inhibitors (9,10), which protect from enzymatic degradation. But although these auxiliary agents promise a sufficient protective effect to exclude proteolysis, remarkable drawbacks, such as an unintended disturbance of digestion of dietary proteins, an inhibitor induced pancreatic hypersecretion caused by a luminal feedback regulation, as well as systemic toxic side effects, make their practical use quite questionable.

Therefore, it was the aim of this study to generate a system, exhibiting the useful protective effect of enzyme inhibitors while excluding their mentioned disadvantages. In order to achieve this goal a trypsin- chymotrypsin-inhibitor was covalently bound to a polymer (poly(acrylic acid)), which keeps it concentrated on this unabsorbable matrix. Moreover, the inhibitory efficiency from chymotrypsinic degradation of the modified polymer, as well as its influence on a possible drug delivery system, was evaluated.

MATERIALS AND METHODS

Coupling of the Trypsin- Chymotrypsin-Inhibitor

The trypsin- chymotrypsin-inhibitor (Bowman-Birk Factor) was coupled to the bioadhesive polymer poly(acrylic acid) by a condensation reaction under the use of 1-ethyl-3-(3 dimethylaminopropyl) carbodiimide hydrochloride (EDAC), catalyzed by sulfo-N-hydroxysuccinimide (SNHS) following the procedure previously described by Staros et al. (11). The available NH_2-groups of the protein were covalently bound to the carboxyl groups of the polymer by constituting amide bindings. 10 g of poly(acrylic acid) (Carbopol 940, BF Goodrich, Cleveland, OH) were gradually added to 100 ml of a 4% (m/m) methanolic sodiumhydroxyd solution under permanent stirring. The precipitate was filtered, washed with methanol till the pH of the filtrate became neutral, and brought to dryness in an exsiccator. 65 mg of this neutralized polyacrylate, 416 mg of EDAC (Pierce, Oud-Beijerland, NL), 32.5 mg of SNHS (Pierce, Oud-Beijerland, NL), and 22 mg of previously deionized (PD10 column; Pharmacia, Uppsala, Sweden) trypsin-chymotrypsin-inhibitor (Sigma, St. Louis, MO) were dissolved in 22 ml of demineralized water and stirred for 40 hr at room temperature. 5 mg of glycine (Merck, Darmstadt, Germany) were added and the reaction was allowed to proceed for 8 hr in order to saturate the remaining activated carboxyl groups of the polymer. The mixture was then diluted with an at least 4-times the amount of demineralized water, and homogenized until viscosity reached its maximum. The resulting gel was centrifuged for 30 min at 17,000 g (Sorvall RC5C, Du Pont) and the supernatant, containing unbound trypsin- chymotrypsin-inhibitor and coupling reagents, was discarded. The remaining pellet was again diluted with demineralized water, centrifuged, and the supernatant removed as above. This purification step was repeated ten times. The isolated inhibitor-matrix conjugate was lyophilized and stored at -20°C.

Evaluation of the Inhibitory Effect of the Conjugate

Enzyme Assay

1 mg of isolated inhibitor-matrix conjugate was suspended in 0.5 ml of 80 mM Tris-HCl pH 7.8. 30 μl of a solution of α-chymotrypsin (1 mg/ml; 52 BTEE units/mg; type II: from bovine pancreas, Sigma, St. Louis, MO) in the same buffer were added and the mixture was preincubated for 2 hr at 25°C. 18.5 mg of N-benzoyl-L-tyrosine ethyl ester (=BTEE) (Sigma, St. Louis, MO) were dissolved in 31.7 ml of methanol and 18.3 ml of demineralized water. 0.5 ml of this solution was added to the preincubated mixture and immediately spectrophotometrically recorded by measuring the increase in absorbance at 256 nm ($\Delta A_{256\,nm}$) for 10 min. To determine the protective effect of the purified conjugate, it was substituted in the enzyme assay by 0.9 mg of neutralized poly(acrylic acid) with different amounts of unbound inhibitor (0–100 μg). The inhibitory efficiency of the modified polymer was then calculated by interpolation from the resulting standard curve.

SDS-Page Analysis

For SDS-Page analysis of the isolated conjugate, 0.6 mg were suspended in 270 μl 50 mM TBS (Tris-HCl buffered saline) pH 7.8 containing 0.1 mg of BSA (bovine serum albumin, initial fraction by cold alcohol precipitation, fraction V, Sigma, St. Louis, MO), and 10 μg of α-chymotrypsin (= 0.52 BTEE units). This mixture was stirred for 3 hr at 37 ± 0.5°C, 30 μl of it were withdrawn and electrophoresed (SDS-PAGE, Protean-mini II, Biorad, Veenendaal, NL). On Coomassie blue stained gels the inhibitory effect of the conjugate was determined by comparing the degree of proteolysis of the resulting bands of BSA, with bands of gradually degradated BSA, obtained by having added increasing amounts (0–100 μg) of unbound inhibitor to the enzymatic reaction. For a positive control α-chymotrypsin was omitted during the period of incubation.

Preparation of the Drug Delivery System

60 mg of neutralized poly(acrylic acid), 29.5 mg of mannit (Merck, Darmstadt, Germany), 10 mg of BSA, and 0.5 mg of β-gal (β-galactosidase, grade VI: from Escherichia coli, Sigma, St. Louis, MO) were homogenized and pressed (Hanseaten, Type EI, Hamburg, Germany) to tablets (diameter: 5.0 mm; depth: ~4 mm). In order to evaluate the influence of the inhibitor-polymer conjugate on this system, the share of poly(acrylic acid) was partially substituted by the modified polymer. The pressing power was kept constant during the preparation of all tablets.

Analysis of the Drug Delivery System

Determination of Its Protective Effect

To determine the degree of enzymatic inactivation of β-gal as well as the degree of proteolysis of BSA in the drug delivery system, tablets with and without the conjugate were incubated for 7 hr with 10 ml of 50 mM Tris-HCl pH 7.8 containing 4.2 mg of α-chymotrypsin (220 BTEE units) on a waterbath-shaker (GFL 1092; 30 rpm) at 37 ± 0.5°C. After this, hydrated matrices were withdrawn and frozen for 1 hr at -20°C. 85 mg and 10 mg aliquots were separated by dividing the frozen matrices with a scalpel in well defined parts.

75 μl iced 50 mM TBS pH 7.8 containing 0.15 M $CaCl_2$ were added to the 85 mg aliquots and the precipitated polymer was removed by centrifugation (20,000 g, 4°C, Hermle Z 323K). 100 μl of the supernatant containing the isolated β-gal were transferred to the first well of a microtitration plate (96-well, non binding), and diluted in steps 1:2 with 50 mM TBS pH 7.8. 50 μl of substrate medium (8 mg of o-nitrophenyl β-D-galactopyranoside in 100 μl of N,N-dimethylformamide + 10 ml 50 mM TBS pH 7.8 containing 2 mM $MgCl_2$) were added and the enzymatic reaction was allowed to proceed at room temperature for 30 min. Optical densities were read at 405 nm with a microtitration plate reader (Anthos reader 2001). The degree of inactivation of β-gal was calculated by interpolation from a standard curve. For positive controls α-chymotrypsin was omitted during the preincubation for 7 hr.

The 10 mg aliquots were diluted with 40 μl of 1 M $CaCl_2$ and the resulting precipitate was removed by centrifugation at 4°C. 10 μl of the supernatant containing the separated BSA were diluted with 40 μl of 50 mM Tris-HCl pH 6.8 and electrophoresed (SDS-PAGE, Protean-mini II, Biorad, Veenendaal, NL). On Coomassie blue stained gels the degree of proteolysis of BSA in tablets containing the inhibitor-matrix conjugate was compared with the degree of proteolysis in tablets containing exclusively unmodified poly(acrylic acid). For a positive control α-chymotrypsin was omitted during preincubation. Marker proteins in a range from 14 to 97 kDa (Bio-Rad, Hercules, CA), α-chymotrypsin, trypsin-chymotrypsin-inhibitor, and BSA were used as references.

Analysis of the Disintegration Behavior

Disintegration of tablets containing different amounts of the conjugate, instead of the unmodified polymer, were analyzed under the same incubation conditions as those used to determine the protective effect of the delivery system. The time needed for the complete disintegration of the tablets was determined.

In Vitro Release Studies of the Unbound Inhibitor

The in vitro release rate of unbound trypsin- chymotrypsin-inhibitor from the drug delivery system was determined by a method which does not conform to the United States Pharmacopeia (USP). Tablets consisting of 60 mg of neutralized poly(acrylic acid), 5 mg of the inhibitor, and 35 mg of mannit were placed in a 25 ml beaker (Schott, Duran 25 ml, Germany) containing 10 ml release medium (50 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 enzyme inhibitor were withdrawn at 1 hr intervals, and replaced with an equal volume of release medium preequilibrated to temperature. The remaining polymer in the withdrawn samples was precipitated by adding $CaCl_2$ to a final concentration of 70 mM, and removed by centrifugation (20 000 g, Hermle Z 323K). This separation of the polyacrylate did not reduce the amount of released protein but guaranteed the exclusion of disturbing influences of the polymer during quantification. The trypsin-chymotrypsin-inhibitor was assayed by measuring the absorbance spectrophotometrically (Perkin-Elmer) at 220 nm and concentrations were determined by interpolation from a standard curve. Cumulative corrections were made for the previously removed samples in determining the total amount released.

RESULTS AND DISCUSSION

Inhibitor-Polymer Conjugate

The Bowman-Birk factor has 6 available amino groups which constitute an amide binding with the available carbonic acid moieties of a polymer. It is a potent inhibitor of α-chymotrypsin and trypsin; stable against pH-changes and high temperatures, with a resistance to various luminal proteases (12). It was for these reasons that it was chosen for the development of the protective polymer. The inhibitor was immobilized to poly(acrylic acid) which has a protective effect towards enzymatic degradation caused by trypsin and carboxypetidase A (13). Moreover, poly(acrylic acid) should guarantee an intimate contact with the absorbing membrane and a prolonged residence time in the intestine due to its bioadhesive properties (14). The amount of inhibitor coupled to this polymer was gravimetrically determined. For this reason a similarly prepared and purified sample, omitting EDAC during the coupling reaction, was used as a negative control. Because of the different weight of the purified and lyophilized polymer with and without bound inhibitor, it was possible to determine the amount of coupled protein. 36% ± 6% (means of three experiments; ± S.D.) of the inhibitor were bound to the polymer, representing 10.9% (m/m) of the coupled product. Because of the molar ratio of 1:20 (carbonic acid groups of the inhibitor:carbonic acid groups of the polymer) inhibitor-inhibitor conjugations were negligible. 1 mg of the purified coupled product exhibited in the enzyme assay an inhibition of α-chymotrypsin which is comparable to 16 μg ± 4 μg (n = 4) unbound inhibitor per mg polymer. It can also be expressed by α-chymotrypsin inhibiting units (= CIU) where one BTEE unit will decrease the activity of two α-chymotrypsin BTEE units by 50%. According to this, the purified coupled product has an inhibitory effect of 2.4 CIU per mg (= 2.4 CIU/mg polymer). However, a purified polymer resulting from reaction mixtures without EDAC did not show any inhibition of α-chymotrypsin, verifying the quantitative separation of unbound inhibitor by the method described here.

The relationship between the activity of an enzyme with a synthetic substrate, as opposed to the activity with a natural protein, may be different (15). In order to verify validity of the results obtained by the enzyme assay, the inhibitory effect of the modified polymer was also evaluated with the use of a protein as substrate. However, SDS-Page analysis of this protein, which was preincubated with α-chymotrypsin in the presence of the conjugate, could confirm the results of the enzyme assay with the synthetic substrate.

With regard to a share of 10.9% of inhibitor in the coupling product a maximal inhibitory effect of 16.3 CIU per mg conjugate should be possible. However, the reduced inhibitory effect of the covalently attached inhibitor might be caused by the method of evaluation disrupting the reaction-kinetics of the immobilized inhibitor and/or by a partial steric hindrance of the polymer.

Influence of the Conjugate on a Drug Delivery System

Whether the determined protective effect of the inhibitor-polymer conjugate is sufficient for a possible formulation to shield inserted peptide and protein drugs from α-chymotrypsinic degradation under physiological conditions, was evaluated on a drug delivery system promising several advantages for the peroral administration of (poly)peptides. As poly(acrylic acid) represents a bioadhesive polymer, it should guarantee intimate contact with the absorbing membrane (I) and a prolonged residence time of the drug delivery system in the intestine (II). The matrix system allows a sustained release of peptide and protein drugs (III) and the release rate can be controlled by the share of the polymer (IV) (16).

To quantify the protective effect of this drug delivery system, a substrate for α-chymotrypsin was incorporated into the carrier matrix. The substrate should not be released from the system too early so as to ensure the possibility of quantification after several hours of incubation with α-chymotrypsin. Because of the comparably low molecular mass of N-benzoyl-L-tyrosine ethyl ester or N-acetyl-L-tyrosine ethyl ester and their fast release from the matrix system, these substrates could not be used for this assay. As an alternative we integrated β-gal which will be digested by α-chymotrypsin and remains in the delivery system. Other candidates such as peroxidase or alkaline phosphatase could not be used because of being either undigestable or released too early.

Analysis of the protective effect of the drug delivery system containing 5 mg of inhibitor-matrix conjugate demonstrated under stimulated physiological α-chymotrypsin concentrations in samples withdrawn from lateral parts of the matrix system the remaining of 71.4% ± 14.3% (means of three experiments; ± S.D.) of activity after 7 hr of incubation at 37 ± 0.5°C. Samples withdrawn from the center of the matrix system did not show any decrease in β-gal activity. On the other hand a complete inactivation of β-gal in lateral parts and the remaining of only 67% ± 14.4% (means of three experiments; ± S.D.) of activity in the center could be observed by the delivery system without the modified polymer (Fig. 1). This demonstrates that the matrix per se has an insufficient protective effect against proteolytic degradation caused by α-chymotrypsin. SDS-Page analysis of BSA incorporated into the matrix stand in good correlation with these results and could therefore confirm the protective effect of the delivery system.

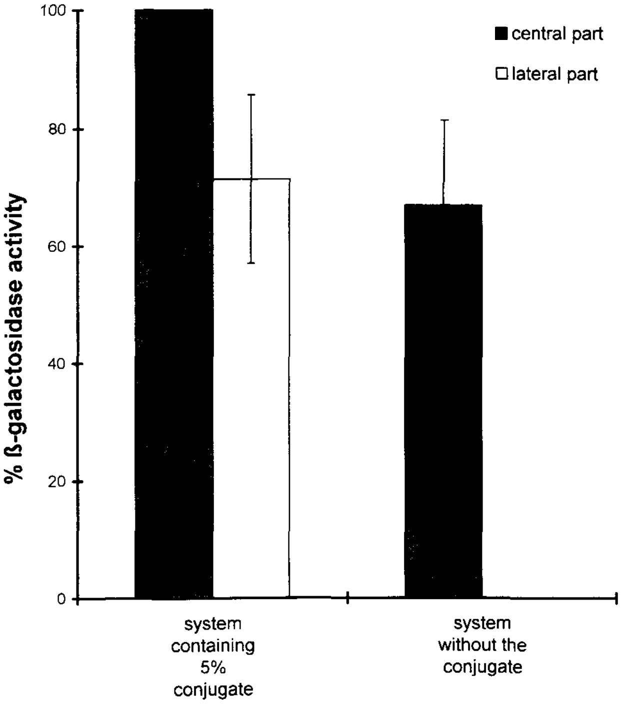

Figure 1. Protective effect of the drug delivery system with and without 5% inhibitor-matrix conjugate, under stimulated physiological α-chymotrypsin concentrations, after 7 hr of incubation at 37 ± 0.5°C (means of three experiments, ± S.D.).

Despite these results, the share of the inhibitor-matrix conjugate in the bioadhesive drug delivery system had a significant influence on the disintegration behavior of the matrix system. Within the first 7.5 hr the modification of the polymer by the covalent attachment of the inhibitor led to a complete disintegration of matrix systems containing more than 15 mg synthesis product instead of the unmodified polymer. This disintegration behavior depending on the concentration of the conjugate in the delivery system is illustrated in Fig. 2. It demonstrates that the share of the inhibitor-polymer conjugate in the delivery system cannot be highered ad libidum, as the cohesion of the matrix system represents a prerequisite for the sustained release as well as for the protective effect. Considering that the protective effect

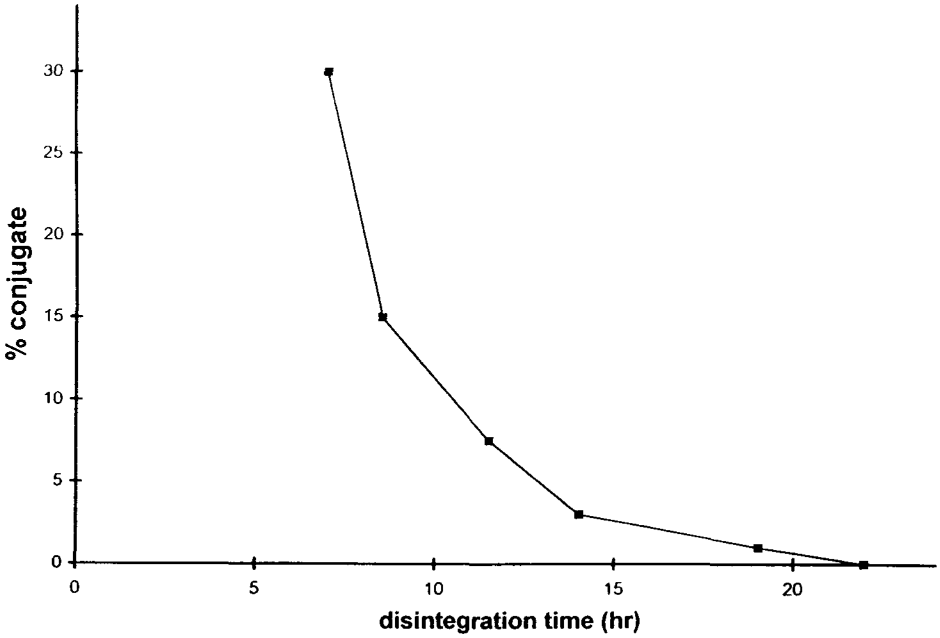

Figure 2. Disintegration behavior based on the concentration of the conjugate in the drug delivery system. The share of the polymer in tablets containing neutralized poly(acrylic acid), BSA and mannit (60 + 10 + 30) was substituted by different amounts of the conjugate. Tablets were incubated in 10 ml 50 mM Tris-HCl pH 7.8 containing 4.2 mg of α-chymotrypsin on a waterbath-shaker (30 rpm) at 37 ± 0.5°C. Studies were repeated three times and did not show any deviations in the results.

will also be reduced by a partial disintegration of lateral parts of the matrix system, a concentration of 5% conjugate in the delivery system should promise an optimized protection.

Sustained Protective Effect

Because of being immobilized on the matrix, dilution effects of the inhibitor could be excluded. However, the protective effect of the matrix system with unbound inhibitor is limited by its release from the delivery system. Studies of unbound trypsin- chymotrypsin-inhibitor showed a release of 94% ± 4% (means of three experiments; ± S.D.) after an incubation period of 13 hr. The release profile of the inhibitor from the delivery system is illustrated in Fig. 3.

With regard to the comparably high molecular mass (MM: 8 kDa) of the Bowman-Birk inhibitor and its tendency of self-association to dimer forms (12), the release rate of low sized protease inhibitors e.g. elastatinal (MM: 512.6 Da), antipain (MM: 604.7 Da), chymostatin (MM: 607.71 Da), bestatin (MM: 344.8 Da), leupeptin (MM: 475.6 Da) amastatin (MM: 511 Da), and pepstatin (MM: 685.9 Da) has to be much higher than the approximately 9% per hour, as determined for the Bowman-Birk inhibitor. Bernkop-Schnürch and Dundalek (17) determined for example a pepstatin liberation of 98 ± 1.9% within the first 3 hr from a comparable drug delivery system. But matrix bound low sized inhibitors will also remain concentrated on the drug delivery system which should lead to a more effective protection from luminal enzymatic degradation.

Reduction of Toxic Side Effects

With regard to the peroral administration of trypsin-chymotrypsin-inhibitor, raised cholecystockinin blood-concentrations have to be expected which can cause a hyperplasie of endocrine pancreas. In vivo studies with the trypsin- chymotrypsin-inhibitor could not exclude a higher risk of pancreas cancer (18,19). Because of the lack of dilution effects and therefore the high inhibitor concentration in the matrix, it should be possible to reduce the necessary amount of inhibitor providing drug stability. Moreover, the immobilization on the unabsorbable polymer should help to exclude systemic toxic

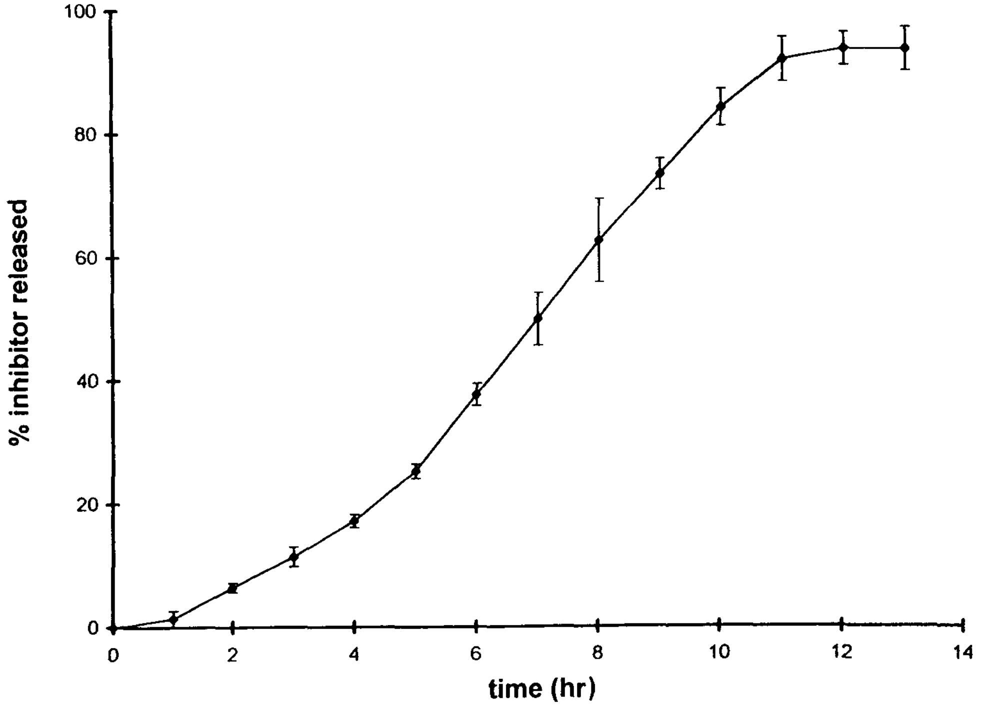

Figure 3. Release profile of unbound trypsin- chymotrypsin-inhibitor from tablets containing 60 mg of neutralized poly(acrlyic acid), 5 mg of the inhibitor and 35 mg of mannit. Tablets were incubated in 10 ml release medium (50 mM Tris-HCl pH 7.8) at 37 ± 0.5°C. Studies were repeated three times to determine represented means (±S.D.).

side effects of the inhibitor (20). The immobilization of inhibitors on the drug delivery system might also guarantee an undisturbed luminal digestion of dietary peptides and proteins.

Because of these advantages covalently bound protease inhibitors seem to be an important step toward a successful peroral (poly)peptide drug administration. In this work we demonstrated the protective effect of one matrix attached inhibitor but to ensure a complete protection against all luminal proteolytic enzymes a combination of different types of inhibitors will become necessary. Whether such immobilized inhibitors are able to protect therapeutic (poly)peptides against membrane bound proteases as well is subject to further investigations.

CONCLUSION

Although covalently attached to a carrier matrix, the Bowman-Birk inhibitor guarantees a protective effect for inserted therapeutic (poly)peptides from enzymatic degradation caused by α-chymotrypsin. The immobilization keeps it concentrated on the matrix system and prevents its absorption followed by systemic toxic side effects. Because of these advantages, the system developed in this study should offer new aspects on the way to a successful peroral (poly)peptide drug administration.

ABBREVIATIONS

BSA: bovine serum albumin; BTEE: N-benzoyl-L-tyrosine ethyl ester; CIU: chymotrypsin inhibiting units; EDAC: 1-ethyl-3-(3dimethylaminopropyl) carbodiimide hydrochloride; β-gal: β-galactosidase; MM: molecular mass; SNHS: sulfo-N-hydroxysuccinimide; TBS: Tris-HCl buffered saline.

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