Mucoadhesive Polymers in Peroral Peptide Drug Delivery. II. Carbomer and Polycarbophil Are Potent Inhibitors of the Intestinal Proteolytic Enzyme Trypsin

Henrik L. Lueßen,[1] J. Coos Verhoef,[1] Gerrit Borchard,[3] Claus-M. Lehr,[3] A. (Bert) G. de Boer,[2] and Hans E. Junginger[1,4]

Received March 27, 1995; accepted May 7, 1995

Purpose. The evaluation of the inhibitory action of two mucoadhesive poly(acrylates), polycarbophil and carbomer, registered by the Food and Drug Administration (FDA), on the intestinal proteolytic enzyme trypsin. ***Methods.*** The effect of the polymers on trypsin activity by measuring the degradation of a trypsin specific substrate. Binding of Ca^{2+} ions and proteins (^{125}I-BSA) to the poly(acrylates). The influence of the polymers on the secondary trypsin structure by circular dichroism. ***Results.*** Trypsin inhibition was found to be time-dependent upon addition of Ca^{2+} in the degradation experiment. Only when Ca^{2+} was added within 10 min after trypsin incubation, recovery of the enzyme could be observed. Both polymers showed a strong Ca^{2+} binding ability. Carbomer, which had a higher inhibitory effect on trypsin activity, also revealed a higher Ca^{2+} binding affinity than polycarbophil. The amount of Ca^{2+} depleted out of the trypsin structure and the reduction of enzyme activity were comparable. Immobilization of trypsin by binding to the polymers could not be observed at pH 6.7. Circular dichroism studies suggested that, under depletion of Ca^{2+} from trypsin, the secondary structure changed its conformation, followed by an increased autodegradation of the enzyme. ***Conclusions.*** The poly(acrylates) investigated may have potential to protect peptides from tryptic degradation and may be used to master the peroral delivery of peptide drugs.

KEY WORDS: peroral peptide drug delivery; mucoadhesives; trypsin; proteolytic activity; poly(acrylic acid) derivatives.

INTRODUCTION

In the last decade, mucoadhesive polymers have gained large interest in controlled drug delivery (1). They are said to prolong the residence time of a delivery system by sticking to the mucus layer at the site of drug absorption, thereby decreasing the distance between the released drug from the dosage form and the absorptive tissue which leads to a steep drug concentration gradient. In more recent studies, however, it was found that these desired aspects were difficult to achieve in the intestinal tract *in vivo,* because of the high turnover rate of the mucus layer and inactivation of the mucoadhesives by soluble mucins (2). Nevertheless, the mucoadhesive and weakly crosslinked poly(acrylate) derivative polycarbophil was able to improve the intestinal absorption of a vasopressin peptide both *in vitro* and *in vivo* in rats (3). Increased absorption was found when the peptide was applied in a dispersed liquid polycarbophil formulation. Furthermore, the peptide was stabilized from proteolytic degradation in a mucosal homogenate by polycarbophil. This suggests that polycarbophil has, besides mucoadhesion, additional properties for improved intestinal peptide drug absorption, *e.g.*, enzyme inhibition.

Current research on peroral drug delivery systems for peptide and protein drugs is focused on overcoming the proteolytic barrier of the GI-tract (4). There are three main approaches to protect peptide drugs from proteolytic degradation before they reach the site of absorption: a) designing a drug delivery system which is targeted to a particular part of the gut where the proteolytic activity is relatively low (*e.g.*, targeting to the colon (5)), b) using sophisticated carrier systems which shuttle the peptide drug to its absorption site (6), or c) lowering the proteolytic activity by using appropriate enzyme inhibitors (7). A major drawback of enzyme inhibitors used until now (*e.g.*, aprotinin, bestatin) is their risk of undesired side effects. A non-toxic, non-absorbable inhibitor which may act in a restricted area of drug absorption would, therefore, be advantageous for peroral delivery of peptide drugs.

The aim of the present study was to evaluate the inhibitory effect of the mucoadhesive polymers polycarbophil and carbomer on trypsin, a serine protease in the intestinal lumen. Moreover, the mechanisms of enzyme inactivation by these polymers were investigated.

MATERIALS AND METHODS

Materials

Carbomer (C934P, Carbopol® 934P) and polycarbophil (PCP, Noveon® AA1) were generous gifts from BF Goodrich (Cleveland, OH, USA). Trypsin (TPCK treated, Type XIII), N-α-benzoyl-L-arginine ethylester (BAEE), N-α-benzoyl-L-arginine (BA), 2-[N-morpholino]ethane-sulfonic acid] (MES) and bovine serum albumin (BSA) were obtained from Sigma Chemie (Bornem, Belgium). Na^{121}I was purchased from Amersham (Little Chalfont, England). All other chemicals were at least of analytical grade.

Degradation Studies with Trypsin: Effect of Calcium

The effect of Ca^{2+} on the degradation activity of trypsin was studied in the following incubation media: a) 50 mM MES/KOH buffer pH 6.7 containing 250 mM mannitol (control); b) 0.35% (w/v) polycarbophil in 50 mM MES/KOH buffer pH 6.7 containing 250 mM mannitol; c) 0.25% (w/v) carbomer in 50 mM MES/KOH buffer pH 6.7 containing 250 mM mannitol.

Amounts of 1.5 mmol N-α-benzoyl-L-arginine ethylester/ml were dissolved in the incubation media. Volumes of 1 ml of the different substrate dilutions were used for one

[1] Leiden/Amsterdam Center for Drug Research, Leiden University, Division of Pharmaceutical Technology, P.O. Box 9502, 2300 RA Leiden, The Netherlands.

[2] Leiden/Amsterdam Center for Drug Research, Leiden University, Division of Pharmacology, P.O. Box 9502, 2300 RA Leiden, The Netherlands.

[3] Saarland University, Division of Pharmaceutical Technology, P.O. Box 151150, D-66041 Saarbrücken, Germany.

[4] To whom correspondence should be addressed.

0724-8741/95/0900-1293$07.50/0 © 1995 Plenum Publishing Corporation

degradation experiment. The degradation was started by adding 23.75 IU trypsin/ml to the different preparations. An appropriate volume of a 1 M $CaCl_2$ dilution was added just before, 10 min or 240 min after starting the degradation experiment, to yield a final concentration of 14.3 mM. The preparations were incubated in 4 ml polystyrene tubes (Greiner Labortechnik GmbH, Frickenhausen, Germany) at 37°C. Samples of 50 μl were withdrawn at predetermined time intervals and diluted in 1 ml 0.1 M HCl to stop trypsin activity. The formation of the metabolite N-α-benzoyl-L-arginine was measured by isocratic HPLC-UV_{253}, using a Lichrosorb RP-18 column (5 μm, 100 × 3.0 mm; Chrompack, Bergen op Zoom, The Netherlands), equipped with a RP 18 precolumn as the stationary phase. The mobile phase consisted of a solution of 86% (v/v) 10 mM ammonium acetate pH 4.2 and 14% (v/v) methanol. Using a flow rate of 0.75 ml/min, the retention time of the metabolite peak (BA) was detected at 3.9 min after 20 μl injection.

Calcium Affinity Studies

The binding affinity of poly(acrylates) toward Ca^{2+} ions was investigated at fixed pH. Polycarbophil and carbomer in concentrations of 0.25% (w/v) were dispersed in 50 mM MES/KOH buffer pH 6.7 containing 250 mmol/l mannitol. A stock solution of 1 mol calcium in 50 mM MES/KOH pH 5.2 was prepared; adjusting higher pH values in the stock solution was not possible, because of calcium precipitation. The following amounts of Ca^{2+} were added to the polymer preparations: 5, 10, 25, 50, 100, and 200 mg/g polymer. After readjusting to pH 6.7 with 10 M KOH, the preparations were incubated for 18 h at 25°C. Then the polymers were centrifuged (1,000g/3 h/25°C), and the equilibrium concentration of Ca^{2+} in the supernatant was determined by complexometric titration as described above. The slope of each curve was treated by regression analysis.

Protein Binding Studies

Carbomer and polycarbophil were dispersed in isotonic saline (pH 4.0) and isotonic 50 mM MES/KOH buffer containing 250 mM mannitol (pH 6.7), respectively. The following polymer concentrations were investigated: 0.01, 0.025, 0.05, 0.1, 0.25, 0.35 and 0.5% (w/v).

BSA was radiolabelled with $Na^{125}I$ by the Iodogen®-method as described previously (8). For ^{125}I-BSA binding studies all polystyrene tubes used in the experiment were precoated with non-labelled BSA (0.1%, w/v) to avoid non-specific binding. The radiolabelled proteins were incubated at 25°C for 30 min with the polymer preparations in a concentration of 5,000 cpm/ml. The specific activity was approximately 56 nCi/mg protein. The preparations were then centrifuged, and the supernatant and pellet were separately counted for γ-radiation. In order to determine the non-specific binding, experiments without the polymer in the incubation medium (control) were performed simultaneously. The percentage of polymer-bound protein was calculated as follows:

1. free protein (f) = $cpm_{supernatant}/cpm_{total}$;
2. total protein bound (b_{total}) = $1 - f$;
3. protein bound to polymer (%) = $(b_{total} - b_{control}) * 100$.

Effects of Poly(acrylates) on Trypsin Activity and Protein and Calcium Binding

Polycarbophil and carbomer were dispersed in Ca^{2+}-free 50 mM MES/KOH buffer, pH 6.7, at a concentration of 0.35% (w/v) and 0.25% (w/v), respectively. MES/KOH buffer was used as a control. Amounts of 1 mg (11,700 IU) trypsin were added to 1 ml of these preparations. After 30 min incubation at 25°C, the samples were centrifuged (800g/4°C/30 min). The supernatants were divided into three portions and stored overnight at −20°C until analysis of the following three parameters.

Trypsin Activity Assay

An amount of 1.5 mM BAEE was dissolved in MES/KOH buffer pH 6.7. A volume of 10 μl of supernatant, containing approximately 10 μg trypsin, was added to 1 ml of the BAEE solution to start the degradation experiment. Sampling procedure and analysis were performed as described above (Degradation studies with trypsin). The area under the concentration-time curve was calculated and expressed as percentage of control incubations.

Trypsin Binding to the Polymers

Binding of trypsin was determined by measuring the protein content of the supernatants according to the Lowry protein assay (9).

Depletion of Ca^{2+} Ions

Depletion of Ca^{2+} from trypsin was determined by measuring Ca^{2+} in the supernatants by atomic absorption spectroscopy (PE 3100, Perkin Elmer, Norwalk, CT, USA). The emission was measured at λ = 422.7 nm.

Circular Dichroism Studies

Amounts of 1 mg trypsin/ml were dissolved in 0.35% (w/v) polycarbophil and 0.25% (w/v) carbomer, both prepared in 0.05 M phosphate buffer pH 6.7. As a control, 1 mg trypsin was dissolved in 1 ml phosphate buffer. One aliquot of trypsin/buffer solution was immediately stored at −20°C until performing of circular dichroism (CD) measurements ("non-treated trypsin"), whereas the other part of the sample was incubated at 37°C for 30 min ("partly autodegraded trypsin"). Buffer and polymer preparations without trypsin were used as controls. All samples were incubated for 30 min at 37°C. After centrifugation (3000g/4°C/60 min) the supernatants were stored at −20°C. CD measurements were performed using a Jobin-Yvon CD-6 spectrophotometer. Samples were scanned from 180 to 250 nm in steps of 0.25 nm. The spectrum curves were fitted using the software package "CONTIN" (10).

RESULTS

Calcium-Dependent Inhibitory Effect of the Polymers on Trypsin Activity

Both poly(acrylates) investigated, polycarbophil and carbomer, were able to inhibit completely the proteolytic activity of trypsin (Figures 1a and b). A fixed concentration

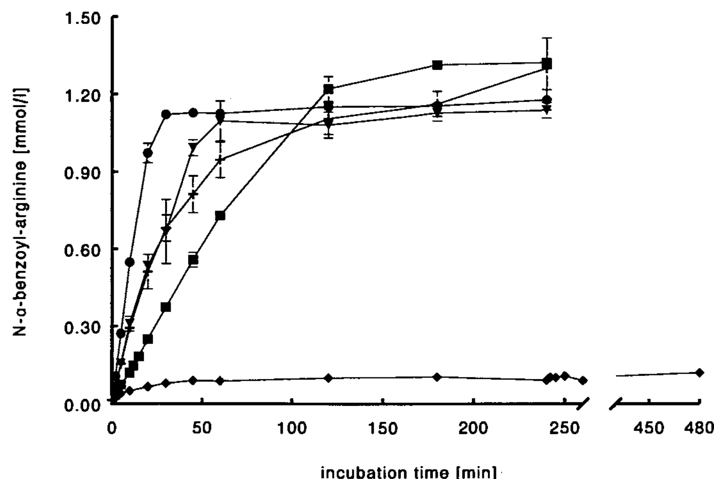

Fig. 1a. Formation of BA following incubation of BAEE with trypsin with or without 0.35% (w/v) polycarbophil (PCP). A concentration of 14.3 mM Ca^{2+} was added at predetermined time intervals (mean ± SD, n = 3). (+, control (no PCP); ●, control with Ca^{2+} (no PCP); ▲, Ca^{2+} was added just before trypsin incubation (0.35% PCP); ■, Ca^{2+} was added after 10 min incubation (0.35% PCP); ◆, Ca^{2+} was added after 240 min incubation (0.35% PCP)).

of 0.25% (w/v) carbomer and 0.35% (w/v) polycarbophil was chosen to study the effect of calcium addition at different time intervals of the degradation experiment. Without addition of Ca^{2+} to the polymer preparations, a slight metabolite formation was observed only during the first 10 to 20 min of the degradation experiment, but did not increase during the following 4 h. However, the remaining trypsin activity appeared to be dependent upon the time of Ca^{2+} addition. After addition of 14.3 mmol Ca^{2+}/l to both polymer preparations just before trypsin incubation, trypsin activity was not inhibited by the polymers. In contrast, the trypsin activity partly recovered when Ca^{2+} was added after 10 min incubation, but not when Ca^{2+} was added after 240 min.

Calcium Affinity Studies

The amount of polymer-bound calcium at pH 6.7 was plotted against the equilibrium concentration, as depicted in Figure 2. When the calcium concentrations were below the binding capacity of the polymers (5 to 200 mg Ca^{2+}/g polymer) a linear relationship for both polycarbophil (R^2 = 0.995) and carbomer (R^2 = 0.996) was found. The difference of the slopes of the curves was statistically evaluated by orthogonal regression analysis (11). The angle of each regression curve with its X axis ± SD was determined and treated by the students *t*-test (degree of freedom = n − 2). As a result, the calcium binding affinity of polycarbophil was

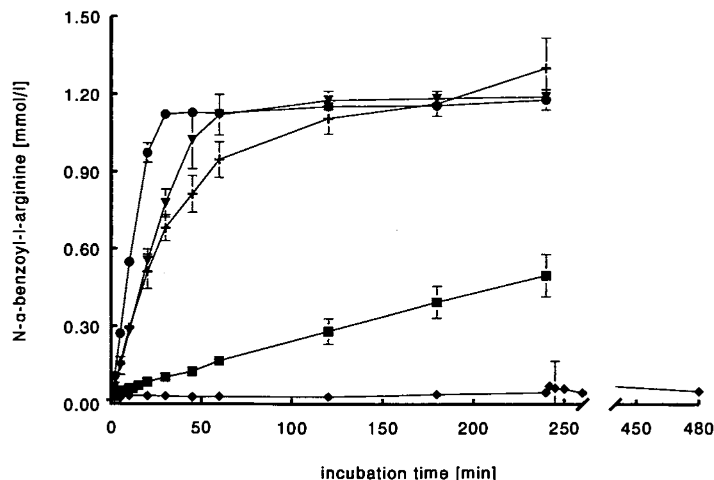

Fig. 1b. Formation of BA following incubation of BAEE with trypsin with or without 0.25% (w/v) carbomer (C934P). A concentration of 14.3 mM Ca^{2+} was added at predetermined time intervals (mean ± SD, n = 3). (+, control (no C934P); ●, control with Ca^{2+} (no C934P); ▲, Ca^{2+} was added just before trypsin incubation (0.25% C934P); ■, Ca^{2+} was added after 10 min incubation (0.25% C934P); ◆, Ca^{2+} was added after 240 min incubation (0.25% C934P).

observed to be statistically significant ($P < 0.05$) lower than for carbomer.

Protein Binding by the Polymers

To investigate possible binding of proteins to mucoadhesive polymers, binding studies with ^{125}I-BSA were performed. Both poly(acrylates) at all concentrations investigated showed a high binding of ^{125}I-BSA at pH 4, whereas at pH 6.7 no pronounced binding could be observed (Figure 3).

Effects of Poly(acrylates) on Trypsin Activity and Protein and Calcium Binding

In this study three parameters were determined within one experiment: the influence of the poly(acrylates) on a) Ca^{2+} depletion from the trypsin structure, b) trypsin binding to the polymers, and c) enzymatic activity of trypsin. Following incubation with both polymers, Ca^{2+} was depleted from trypsin to 10–15% of the initial content, whereas the free protein concentration in the supernatants was still 60–70% of its original value. However, the remaining enzyme activity of trypsin was 7.5% compared with the non-polymer treated control (Figure 4).

Circular Dichroism Studies

The circular dichroism spectra revealed that the secondary structure of trypsin was changed under the influence of the poly(acrylates) (Figure 5). The trypsin spectrum showed a maximum at 185 nm and two minima at 194 nm and 209 nm, respectively. However, the spectrum of trypsin which was allowed to undergo autodegradation for 30 min at 37°C revealed an increased minimum in the wavelength area between 195 nm and 200 nm. This minimum was even more pronounced for the polymer-treated samples, whereby carbomer showed a stronger decrease of the minimum as compared with polycarbophil.

DISCUSSION

Trypsin is a serine protease which has a binding site for the bivalent cation Ca^{2+} (12). Calcium plays an important role in maintaining the thermodynamic stability of this enzyme (13). It has been described that depletion of Ca^{2+} from the enzyme structure by chelating agents like EDTA, can

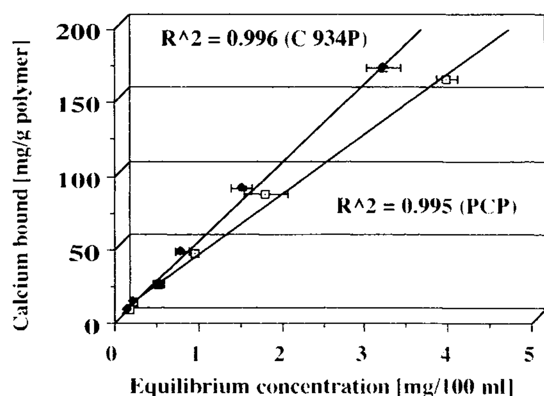

Fig. 2. Binding affinity plot of 0.25% (w/v) polycarbophil (□) and 0.25% (w/v) carbomer (♦) at pH 6.7 (mean ± SD, n = 3).

affect the activity of trypsin (14). Therefore, it is important to study whether poly(acrylic acid) derivatives, which are able to bind large amounts of bivalent cations in the dissociated state, can also inhibit trypsin activity.

As shown in the present study, the inhibitory effect of polycarbophil and carbomer is dependent upon the time interval of Ca^{2+} addition to the trypsin incubation medium. An explanation for this phenomenon may be either the time-dependent association of trypsin to the poly(acrylate) structure or time-dependent denaturation of the enzyme under the influence of the mucoadhesive polymers. The addition of Ca^{2+} at $t = 10$ min showed that due to salting-out of the polymers, no strong inclusion or adsorption of trypsin and thereby no inactivation of the enzyme occurred, which may explain the irreversible effect on trypsin inhibition by adding Ca^{2+} at $t = 240$ min. In contrast, these observations suggest that the poly(acrylates) irreversibly modify the enzyme structure itself with time.

The higher calcium binding affinity found for carbomer compared to polycarbophil may be ascribed to their different way of cross-linking. Polycarbophil is cross-linked by divinylglycol and to a lower degree than carbomer, which is cross-linked by allylsucrose. This suggests that the flexibility of the molecular structure of carbomer to avoid regions of higher charge densities, caused by proton dissociation from carboxylic groups, is reduced compared to polycarbophil. Regions of higher negative charge densities within the poly(acrylate) structure, as expected for carbomer, may explain the higher binding affinity of this polymer to bivalent cations such as calcium.

The higher Ca^{2+} binding affinity of carbomer (Figure 2) may cause a more complete depletion of Ca^{2+} out of the trypsin structure than polycarbophil. It is suggested that the dissociation constant for the polymer and Ca^{2+} should be low in comparison with the dissociation constant for trypsin and Ca^{2+} in order to reach sufficient enzyme inhibition. Such a relationship might also explain the lower carbomer concentration required for the inhibition of trypsin activity. To optimize the inhibitory effect of Ca^{2+}-binding polymers, affinity values may represent an important parameter in predicting their potency to affect the enzymatic activity of Ca^{2+}-dependent proteases.

The present study showed that polycarbophil and carbomer are able to bind protein molecules such as BSA at pH 4.0. This can be explained by the adhesive properties of these polymers toward hydrophilic macromolecules. It has been observed previously that the adhesion force of poly(acrylates) toward intestinal mucosae is dependent upon pH (15). At pH values lower than 4, where the poly(acrylates) are mainly undissociated, the adhesion force is the strongest. In a neutral environment, however, the chemical properties change due to dissociation of the carboxylic groups, and highly negatively charged polymers are obtained. Glycoproteins like intestinal mucins, which contain a large quantity of sulphuric acid and carboxylic acid groups, will cause repulsive effects on dissociated poly(acrylates). Consequently, the adhesion force is strongly reduced by such interactions.

High binding values between poly(acrylates) and BSA could only be observed at pH 4.0 (Figure 3). Under the chosen conditions of the present degradation experiments at pH 6.7, the polyanionic poly(acrylate) structure showed a rela-

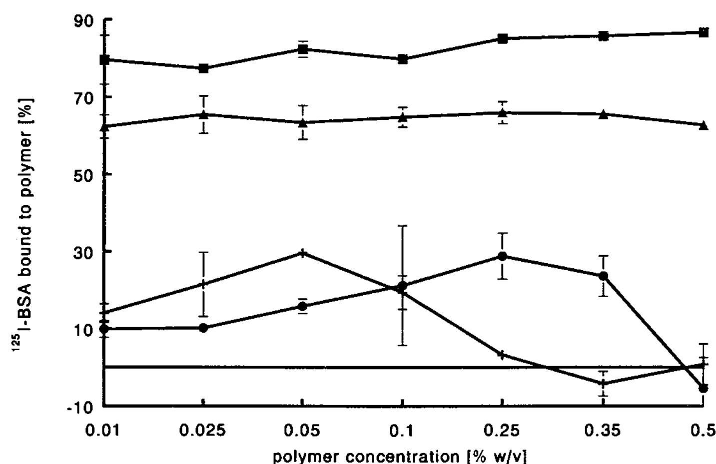

Fig. 3. Binding of radiolabelled bovine serum albumin (^{125}I-BSA) to polycarbophil (PCP) and carbomer (C934P) at different polymer concentrations. Protein binding to the polymers was studied at pH 4.0 and 6.7, respectively. The following symbols were used: ■, PCP at pH 4.0; ▲, C934P at pH 4.0; ●, PCP at pH 6.7; +, C934P at pH 6.7 (mean ± SD, n = 3).

tively low binding tendency towards BSA, which therefore cannot explain their pronounced inhibitory effect on trypsin activity.

The extent of trypsin inactivation by polycarbophil and carbomer seems to be comparable with the reduced Ca^{2+} content in the supernatant under influence of the polymers. The Ca^{2+} content in the supernatant of the control samples can be attributed to trypsin, which contains Ca^{2+} to build up its tertiary structure (12). The reduced Ca^{2+} concentration in the supernatant after polymer treatment may be attributed to depletion of Ca^{2+} out of the trypsin structure. The free protein content in the same supernatants was still 60–70% of the control samples, indicating that only a small amount of trypsin was adsorbed to the polymers, which can hardly explain the almost complete inhibition of enzyme activity. Furthermore, these results are in accordance with the ^{125}I-BSA binding studies, in which (despite a low total protein concentration) no pronounced binding could be observed at neutral pH values. However, the remaining amount of protein

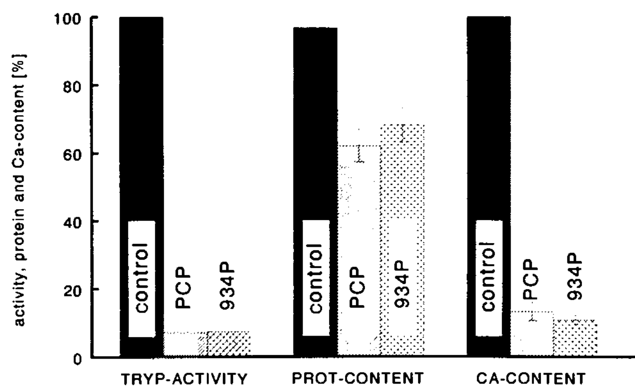

Fig. 4. Effects of 0.35% (w/v) polycarbophil (PCP) and 0.25% (w/v) carbomer (934P) on trypsin activity, protein content and Ca^{2+} depletion from trypsin following 30 min incubation at 25°C (mean ± SD, n = 3). Control incubations contained trypsin without poly(acrylates).

showed a strongly reduced trypsin activity which fits with the observed Ca^{2+} depletion out of the trypsin structure.

The minimum at 194–209 nm of the circular dichroism spectra of polymer-treated trypsin was comparable to the spectrum of trypsin which was allowed to undergo autodegradation for 30 min. This suggests that the observed irreversible inhibition of trypsin activity by poly(acrylic acid) derivatives, which deprives the enzyme of Ca^{2+}, is in parallel with structural changes similar to autodegradation. Calcium has an essential role in the thermodynamic stability and in the resistance against degradation and autodegradation of trypsin (16–18). Hence, the release of Ca^{2+} from trypsin leads also to changes of the tertiary structure of the enzyme (12). It may be suggested that exposure of arginine and lysine bonds, which are normally protected from the external environment by being embedded inside the trypsin structure, increases the chance for enhanced autodegradation. This im-

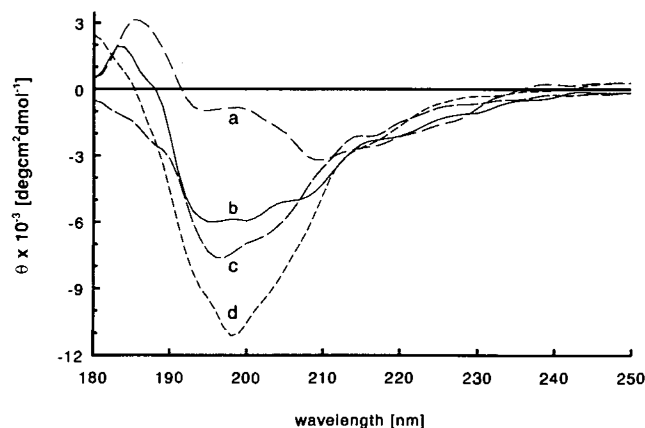

Fig. 5. Circular dichroism spectra of polycarbophil (c) and carbomer (d) treated trypsin preparations. Two trypsin control samples were also included: (a) "non-treated trypsin": trypsin dissolved in buffer, stored at −20°C until use and then thawed directly before CD measurements; (b) "treated trypsin": trypsin incubated for 30 min at 37°C.

plies that the mechanism of trypsin inhibition does not require a direct interaction between polymer and enzyme, which would be necessary in the sense of classical Michaelis-Menten kinetics. In the case of trypsin, the inactivation of the enzyme is not directly related to the "inhibitor," but to the degree of degradation of the enzyme itself as a consequence of Ca^{2+}-deprivation. This makes it difficult to predict the time course of trypsin inactivation by compounds such as poly(acrylates), because many different parameters influence the inhibitory profile. Some of these are the degree of Ca^{2+}-binding affinity of the polymers, pH, ionic strength, trypsin activity, type and amount of nutrients, as well as the concentration and type of different cations in the intestinal lumen.

CONCLUSIONS

The inhibitory effect of poly(acrylates) on the proteolytic activity of the luminal enzyme trypsin can be explained by the pronounced Ca^{2+}-binding affinity of the polymers. There are also indications that, by depletion of Ca^{2+} from the trypsin structure, the enzyme may undergo structural changes similar to autodegradation. Furthermore, these findings suggest that the poly(acrylic acid) derivatives polycarbophil and carbomer may have potential in the development of peroral peptide drug delivery systems for these peptides which are prone to tryptic degradation.

ACKNOWLEDGMENTS

The authors wish to thank the German National Scholarship Foundation (Bonn, Germany), Lohmann Therapie Systeme (Neuwied, Germany), and the Saal van Zwanenberg Stichting (Oss, The Netherlands) for financial support as well as Dr. I. E. Smit-Kingma and Mr. H. Meder from Unilever Research Laboratories (Vlaardingen, The Netherlands) for performing CD measurements at their company.

REFERENCES

1. J.-M. Gu, J. R. Robinson, and S.-H. S. Leung. Binding of acrylic polymers to mucin/epithelial surface: structure-property relationships. *Crit. Rev. Therap. Drug Carrier Sys.* 5:21–67 (1988).
2. C.-M. Lehr, F. G. J. Poelma, H. E. Junginger, and J. J. Tukker. An estimate of turnover time of intestinal mucus gel layer in the rat in situ loop. *Int. J. Pharm.* 70:235–240 (1991).
3. C.-M. Lehr, J. A. Bouwstra,, W. Kok, A. G. de Boer, J. J. Tukker, J. C. Verhoef, D. D. Breimer, and H. E. Junginger. Effects of the mucoadhesive polymer polycarbophil on the intestinal absorption of a peptide drug in the rat. *J. Pharm. Pharmacol.* 4:402–407 (1992).
4. V. H. L. Lee, R. D. Traver, and M. E. Taub. Enzymatic barriers to peptide and protein drug delivery. In V. H. L. Lee (ed.), *Peptide and Protein Drug Delivery*. Marcel Dekker, Inc., New York, 1991, pp. 303–358.
5. M. Saffran, G. S. Kumar, D. C. Neckers, J. Peña, R. H. Jones, and J. B. Field. Biodegradable azopolymer coating for oral delivery of peptide drugs. *Biochem. Soc. Trans.* 18:752–754 (1990).
6. C. Damgé, C. Michel, M. Aprahamian, and P. Couvreur. New approach for oral administration of insulin with polyalkylcyanoacrylate nanocapsules as drug carrier. *Diabetes* 37:246–251 (1988).
7. I. Morishita, M. Morishita, K. Takayama, Y. Machida, and T. Nagai. Hypoglycemic effect of novel oral microspheres of insulin with protease inhibitor in normal and diabetic rats. *Int. J. Pharm.* 78:9–16 (1992).
8. C.-M. Lehr and V. H. L. Lee. Binding and transport of some bioadhesive plant lectins across Caco-2 cell monolayers. *Pharm. Res.* 10:1796–1799 (1993).
9. O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193:265–275 (1951).
10. S. W. Provencher. Contin: A general purpose constrained regularization program for inverting noisy linear algrebraic and integral equations. *Comput. Phys. Commun.* 27:229–242 (1982).
11. E. A. van der Velde. Orthogonal regression equation. In A. M. H. P. van den Besselaar, H. R. Gralnick, and S. M. Lewis (eds.), *Thromboplastin calibration and oral anticoagulant control*. Martinus Nijhoff, Amsterdam, 1984, pp. 25–39.
12. H. D. Bartunik, L. J. Summers, and H. H. Bartsch. Crystal structure of bovine β-trypsin at 1.5 Å resolution in a crystal form with low molecular packing density—active site geometry, ion pairs and solvent structure. *J. Mol. Biol.* 210:813–828 (1989).
13. M. Delaage and M. Lazdunski. The binding of Ca^{2+} to trypsinogen and its relation to the mechanism of activation. *Biochem. Biophys. Res. Commun.* 28:390–394 (1967).
14. G. Salvesen and H. Nagase. Inhibition of proteolytical enzymes. In R. J. Beynon and J. S. Bond (eds.), *Proteolytical enzymes: a practical approach*. IRL Press, Oxford, 1989, pp. 83–104.
15. C.-M. Lehr, H. E. Boddé, J. A. Bouwstra, and H. E. Junginger. A surface energy analysis of mucoadhesion. II. Prediction of mucoadhesive performance by spreading coefficients. *Eur. J. Pharm. Sci.* 1:19–30 (1993).
16. W. R. Rypniewski, A. Perrakis, C. E. Vorgias, and K. S. Wilson. Evolutionary divergence and conservation of trypsin. *Protein Eng.* 7:57–64 (1994).
17. R. H. Kretsinger. Calcium-binding proteins. *Annu. Rev. Biochem.* 45:239–266 (1976).
18. D. Gabel and V. Kasche. Autolysis of β-trypsin—influence of calcium ions and heat. *Acta Chem. Scand.* 27:1971–1981 (1973).