Carbomer Inhibits Tryptic Proteolysis of Luteinizing Hormone-Releasing Hormone and N-α-Benzoyl-L-Arginine Ethyl Ester by Binding the Enzyme

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Purpose. To determine the mechanism by which Carbomer inhibits the enzymatic activity of trypsin in hydrolysis of N-α-benzoyl-L-arginine ethyl ester (BAEE) and luteinizing hormone-releasing hormone (LHRH).

Methods. Inhibition of enzymatic activity was studied by measuring the formation of metabolites from LHRH and BAEE. Binding of trypsin and substrates to 0.35% (w/v) Carbomer at pH 7.0 was studied by centrifugal filtration. Gel filtration and reverse phase HPLC was used to determine the stability of trypsin.

Results. Carbomer reduced the rate of hydrolysis of BAEE and LHRH by trypsin to 34% and 28% of the control activity, respectively. The rate of metabolite formation for both substrates followed pseudo-zero order kinetics in the presence and absence of carbomer. Binding studies showed that 68% of the trypsin protein and 10% of BAEE was bound to carbomer, but no LHRH was bound. No low molecular weight autolysis products of trypsin could be identified by gel filtration. Reverse phase HPLC analysis of the unbound carbomer-treated-trypsin suggests a number of conformational forms of trypsin. The equilibrium binding capacity was 30 μg of trypsin to 1000 μg of carbomer.

Conclusions. Decreased hydrolysis of LHRH and BAEE by trypsin in the presence of carbomer is due to enzyme–polymer interaction.

KEY WORDS: peroral peptide delivery; poly(acrylates); trypsin; proteolytic activity; luteinizing hormone–releasing hormone, protein–polymer interaction.

INTRODUCTION

The oral route is the most convenient for drug administration, however there has been only limited success in delivering peptides by this route (1). The two major barriers to successful oral peptide delivery are enzymatic degradation and absorption across the gastrointestinal epithelium. To reduce the metabolic barrier in the gut, various approaches have been tried: co-administration of peptides with protease inhibitors (2), and adsorption enhancers (3), structural modification of the peptide to prevent proteolytic attack (3,4), and carrier systems to protect the peptide from luminal proteolytic degradation and release the drug at the site of the gut most favorable for absorption (5).

Polymers of acrylic acid, known as carbomers, have been shown to enhance the bioavailability of several peptides. Aqueous gels of carbomer have been shown to improve significantly the absorption of insulin and [$Asu^{1,7}$] eel calcitonin from the rectum of rat (6,7). Polycarbophil, a weakly crosslinked polyacrylate, dispersed in saline was shown to enhance intestinal absorption of the peptide [Arg^8, des-Gly-NH_2^9]-vasopressin (DGAVP) both *in vitro* and *in vivo* in rats (8). More recently, Carbomer 934P was shown to significantly improve the intestinal absorption of buserelin, a LHRH superagonist, in rats (9).

Several mechanisms have been proposed by which polyacrylic acid polymers may improve the oral bioavailability of peptides. Ch'ng *et al.* (10), showed that the mucoadhesive properties of polyacrylic acid polymers enhance contact between the formulation and mucosal surface thereby increasing the residence time at the site of drug absorption. Secondly, polyacrylates may enhance paracellular peptide transport across the epithelial cell membrane. Carbopol has been shown to increase the permeability of epithelial cell monolayers, *in vitro* (11). It is postulated that the integrity of the epithelial tight junctions is reduced by the binding of extracellular calcium to the polyacrylic acid polymer. The reduction of extracellular calcium is presumed to result in the opening of the tight junctions (12). A number of studies have concluded that polyacrylates inhibit the proteolytic activity of enzymes present in the gastrointestinal tract (13,14). *In vitro* studies by Bai *et al.* (14), demonstrated that polyacrylic acid polymers inhibited the degradation of insulin, calcitonin and insulin-like growth factor by trypsin, chymotrypsin and luminal gut extracts from rats. The degree of inhibition was shown to correlate with the ability of the polyacrylic acid polymers to lower the pH *in vitro*. Buffered polyacrylic acid polymer dispersions at pH 6.7 have been shown to inhibit the activity of trypsin, α-chymotrypsin and carboxypeptidase A and the cytosolic leucine aminopeptidase (13). It was suggested that polyacrylic acid polymers bind the essential enzyme co-factors calcium and zinc from the enzyme, causing a conformational change resulting in enzyme autolysis and loss of enzyme activity.

There have been extensive studies describing the adsorption of proteins onto polymeric surfaces (15,16), and delivery devices (17). Protein–polymer interactions have been shown to cause structural changes and aggregation of the protein resulting in inactivation of the protein (18). More recent work has shown that peptides also interact with polymers (19,20). Thus it is also conceivable that polyacrylic acid polymers may reduce the proteolytic activity by interacting with the enzyme or the substrate. In this study we show that Carbomer 934P reduces proteolysis by interacting with trypsin, thereby identifying another mechanism by which polyacrylic acid polymers may improve the oral bioavailability of peptides. We discuss the implications of this for peptide delivery.

MATERIALS AND METHODS

Materials

Carbomer (C934P, Carbopol® 934P) was a gift from BF Goodrich (Cleveland, OH, USA). Trypsin (TPCK treated, Type

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ABBREVIATIONS: BAEE, N-α-benzoyl-L-arginine ethyl ester; BA, N-α-benzoyl-L-arginine; LHRH, luteinizing hormone-releasing hormone; I.D., internal diameter; c/(x/m), 1/ab + c/a; x, the mass of protein absorbed to carbomer (μg); m, the mass of the carbomer (μg); a, the capacity of carbomer to bind trypsin (μg/μg); b, the affinity constant of trypsin for carbomer (ml/μg); c, the concentration of free trypsin (μg/ml).

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XIII), N-α-benzoyl-L-arginine ethyl ester (BAEE), N-α-benzyol-L-arginine (BA), human luteinizing hormone-releasing hormone (LHRH) and mineral oil (heavy white oil) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ultrafree®-MC (0.45 μm, 0.2 cm^2 membrane area) centrifugal filtration units were purchased from Millipore Corporation (Bedford, MA, USA). All other chemicals were of analytical grade and were purchased from Ajax Chemicals (Auburn, NSW, Australia).

Inhibition Studies

The stability of BAEE and LHRH to trypsin was determined in 50 mM phosphate buffer adjusted to pH 7.0 with sodium hydroxide. Carbomer 0.5% (w/v) was dispersed in water by low speed stirring, disodium hydrogen phosphate was added to give a final concentration of 50 mM and the dispersion was adjusted to pH 7.0 with sodium hydroxide. Trypsin was dissolved in phosphate buffer, pH 7.0 to give a stock solution of 1 mg/ml. Trypsin stock solution (10 μl) was mixed with either 0.7 ml of the 0.5% (w/v) carbomer or 0.7 ml of phosphate buffer, and incubated at 37°C. After 30 min the substrate, either BAEE or LHRH, dissolved in the phosphate buffer pH 7.0 was added to give a final volume of 1 ml. The stability of BAEE and LHRH was determined at 20 mM and 0.4 mM, respectively. At various time intervals 50 μl samples were removed and added to 50 μl of 0.2 M HCl to stop the reaction and to precipitate the carbomer. Samples were centrifuged (20 000 g for 10 min) and the supernatants were analysed for product. BAEE stability was determined by monitoring the formation of BA from BAEE. The supernatant (50 μl) was diluted with 950 μl of phosphate buffer, pH 7.0, and the formation of BA was followed by change in absorbance at 252 nm. LHRH stability was monitored by the formation of a metabolite, using capillary electrophoresis.

Binding Studies

Carbomer 934P 0.5% (w/v) dispersed in 50 mM phosphate buffer pH 7.0 was mixed with trypsin (12.5 μg), LHRH (0.1 μmol) or BAEE (5 μmol) to give a final carbomer concentration of 0.35% (w/v) and a total volume of 0.25 ml. In the control experiment the carbomer was replaced with phosphate buffer pH 7.0. Following incubation at 37°C for 30 min, 0.2 ml samples of the suspensions were removed, placed in a centrifugal filtration unit and centrifuged at 5 000 g for 2 h at room temperature. The trypsin incubates were cooled on ice before centrifugation at 4°C. The filtrates were analysed for unbound material. BAEE was measured by the change in absorbance at 252 nm and LHRH was measured by capillary electrophoresis. The trypsin filtrates were assayed for protein content by the modified Lowry assay (21) and analysed by gel filtration and reverse-phase HPLC. The filtrates were also assayed for BAEE hydrolytic activity by incubating 0.1 ml of the filtrate with BAEE (0.7 mM) in phosphate buffer pH 7.0 at 37°C. BA formation was measured by monitoring the change in absorbance at 252 nm (22).

The effect of concentration on binding trypsin to 0.35% (w/v) carbomer was evaluated at trypsin concentrations (35, 50, 70, 80, 150, 285, and 525 μg/ml) in 50 mM phosphate buffer pH 7.0. Carbomer 0.5% (w/v) in 50 mM phosphate buffer pH 7.0 (0.7 ml) was pre-incubated at 37°C for 10 min, then the appropriate volume of trypsin (2 mg/ml) was added with phosphate buffer pH 7.0 to give a total volume of 1 ml. The dispersion was vortexed and incubated at 37°C for 30 min, the incubates were put on ice for 10 min then a 0.4 ml sample was transferred to the centrifugal filtration unit and centrifuged at 5 000 g for 2 h at 4°C. The filtrates were assayed for protein content (21). The data obtained were fitted to the linearised form of the Langmuir equation (23):

$$c/(x/m) = 1/ab + c/a$$

where x is the mass of trypsin absorbed to carbomer, m is the mass of the carbomer, a is the capacity of carbomer to bind trypsin, b is the affinity constant of trypsin for carbomer, c is the concentration of free trypsin.

Capillary Electrophoresis

Samples (20 μl) were transferred to a Dionex sample vial and diluted with 60 μl of water. A 1 mm layer of mineral oil was placed on top of the sample to prevent sample evaporation. Capillary zone electrophoresis was carried out in a Dionex Model CES-1 capillary electrophoresis instrument (Dionex, Sunnyvale, CA, USA). Data were collected via a Dionex advanced computer interface at 5 Hz and integrated using the Dionex A-450 Chromatography Automation Software v 3.3.2. A 55 cm × 50 μm I.D. bare fused silica capillary was installed and conditioned as described in the Dionex CES I users manual. The capillary was cooled by circulating water at 20°C from a Neslab (RTE-111). The running buffer, 10 mM NaH_2PO_4/H_3PO_4, pH 2.5 containing 75 mM NaCl was made fresh each day from stock solutions, filtered through a 0.45 μm filter and sonicated for 5 min under reduced pressure. Capillaries were cleaned at the start of the day by rinsing the capillary and buffer reservoirs with 0.1 M NaOH, Milli-Q water and then running buffer. Prior to each run the capillary was rinsed with buffer for 120 sec and the source and destination reservoirs were rinsed with buffer for 7 sec. Hydrodynamic injections were made by raising the sample vial containing the capillary inlet end to a height of 150 mm for 60 sec. Electrophoresis was carried out at constant current by ramping the current from 0 to 60 μA over 1 min and holding the current at 60 μA for the remainder of the run. The peptides were detected by a UV detector set at 215 nm.

Gel Filtration

The instrumentation consisted of a Spectra-Physics (SP8810) HPLC pump, a Rheodyne injector with a 100 μl loading loop, Shimadzu SPD-6A UV detector set at 215 nm and a Hewlett Packard (3390A) integrator. Samples (50 μl) were applied to the gel filtration column (Waters Protein-Pak™ SW, 7.8 × 300 mm), using a mobile phase of 10 mM phosphate buffer pH 6.5, containing 150 mM sodium chloride at 1.00 ml/min.

Reverse Phase HPLC

The HPLC system consisted of a Spectra-Physics ternary pump (SP8800), Phenomenex on-line mobile phase degasser

(DS-4000), Rheodyne injector with a 50 μl loop, Spectra-Physics Spectra 100 variable wavelength detector and a Spectra-Physics ChromJet integrator. The column, Jupiter C4 (150 × 4.6 mm, 5 μm) was equilibrated in 5% acetonitrile in 0.08% trifluoroacetic acid at 1.00 ml/min. The column was eluted with a linear gradient of acetonitrile (5–65%, 30 min) in 0.08% trifluoroacetic acid 1.00 ml/min. The eluate was monitored at 215 nm.

RESULTS

Inhibition Studies

Carbomer, 0.35% (w/v) dispersed in phosphate buffer pH 7.0 inhibited the tryptic digestion of both BAEE and LHRH. The rate of the formation of BA from BAEE in the presence and absence of carbomer was constant over the period assayed. In the presence of carbomer the rate of BAEE hydrolysis was 34% of the control (Fig. 1). Using capillary electrophoresis, the proteolysis of LHRH was monitored by measuring the formation of a principal metabolite 12.5 ± 0.1 min, n = 12 from LHRH 18.34 ± 0.34 min, n = 15. Figure 2 shows capillary electropherograms of LHRH extracts after 24 h digestion with trypsin in the presence and absence of carbomer. The rate of metabolite formation was constant over 24 h in the presence and absence of carbomer. In the presence of carbomer the rate of LHRH metabolite formation was 28% of the control (Fig.3).

Binding Studies

In preliminary studies we were unable to pellet the carbomer by centrifugation as described by Lueßen *et al.* (24). Consequently the binding of LHRH (0.4 mM), BAEE (20 mM) and trypsin (50 μg/ml) to 0. 35% (w/v) carbomer at pH 7.0 was investigated using centrifugal filtration. Non-specific binding to the membrane was not observed during filtration of either BAEE or LHRH, and less than 5% of the trypsin protein was bound to the membrane during filtration. The carbomer was completely retained. There was no significant binding of LHRH to carbomer and only 10 ± 2% of BAEE was bound to carbomer. Analysis of the trypsin filtrate for protein content showed that 32 ± 5% of the protein was filtered implying 68% binding to the carbomer (Table I). Only 11 ± 2% of the tryptic activity was filtered after incubation with carbomer and this had a specific activity of only 34% of the control activity.

The binding of trypsin to carbomer was well described by the linearised form of the Langmuir isotherm with $r^2 = 0.997$ (Fig. 4). The binding capacity of carbomer for trypsin was 30 mg/g with an affinity constant of 0.028 ml/μg.

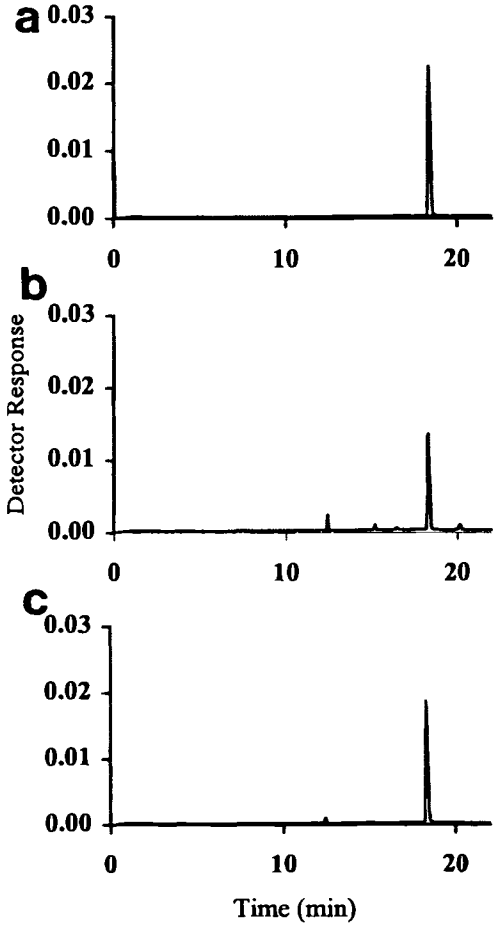

Fig. 2. Capillary electropherograms of LHRH incubations with trypsin (10 μg/ml), at time 0 min in the presence of carbomer (a), at time 24 h in the absence of carbomer (b), and at time 24 h in the presence of 0.35% (w/v) carbomer (c) in 50 mM phosphate buffer pH 7.0 at 37°C.

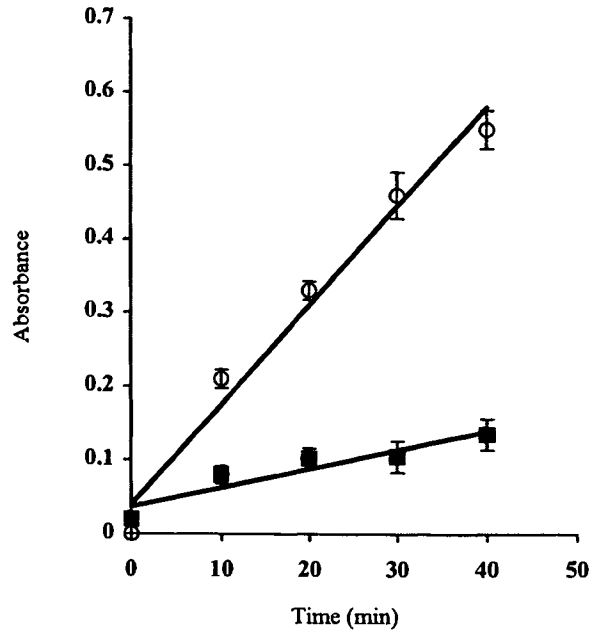

Fig. 1. Formation of BA from BAEE by trypsin (10 μg/ml) in the presence (■) and absence (○) of 0.35% (w/v) carbomer in 50 mM phosphate buffer pH 7.0 (mean ± s.d, n = 3).

Gel Filtration

Trypsin (50 μg/ml) was autolysed by heating at 60°C for 45 min. Samples of the trypsin before and after heating were analysed by gel filtration and assayed for protein content by the modified Lowry assay (21). Gel filtration (Fig. 5) showed a dramatic reduction in the peak area and height for the autolysed trypsin with respect to control. A shoulder of lower molecular

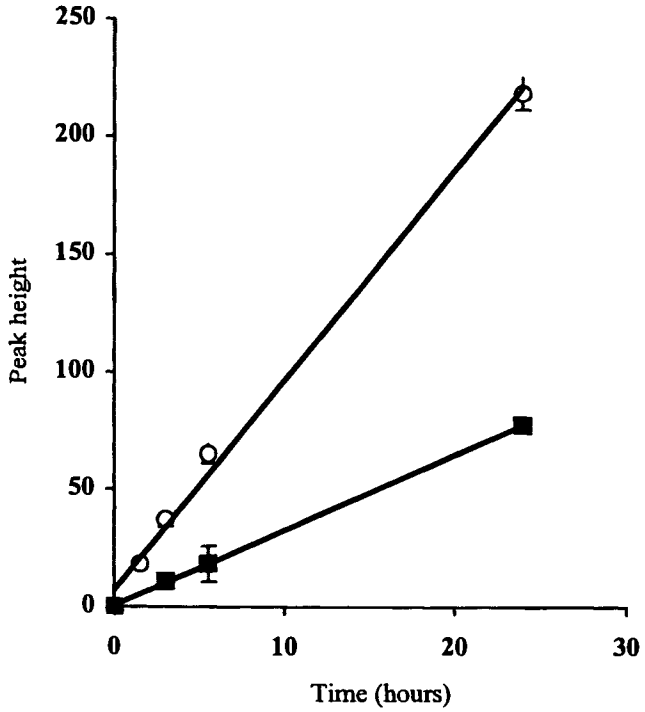

Fig. 3. Formation of the principal metabolite of LHRH following incubation of LHRH with trypsin (10 μg/ml) in the presence of 0.35% (w/v) carbomer (■) and absence of carbomer (○) in 50 mM phosphate buffer pH 7.0 (mean ± s.d, n = 3).

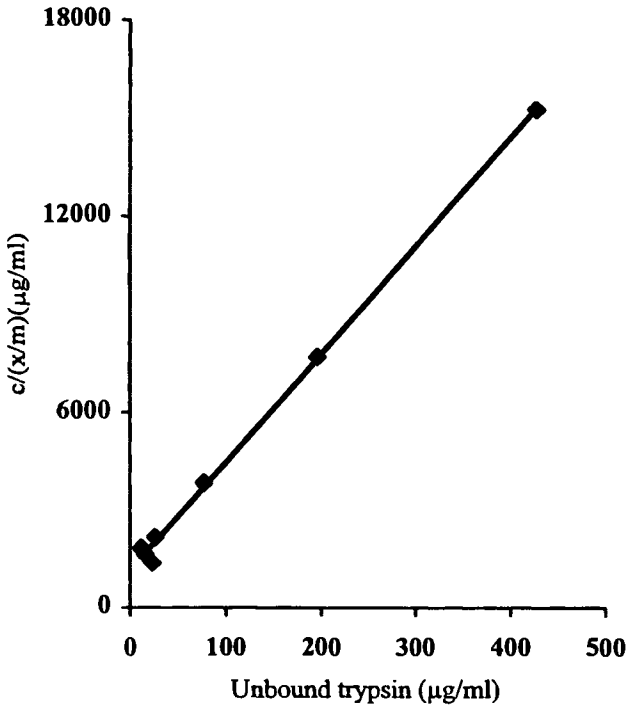

Fig. 4. Linearised Langmuir adsorption isotherm of trypsin onto 0.35% (w/v) carbomer incubated in 50 mM phosphate buffer pH 7.0 at 37°C for 30 min (mean, n = 3).

weight material was also observed for the autolysed trypsin peak. Protein estimations of control and autolysed trypsin showed no significant difference ($p > 0.05$) because the modified Lowry assay (21) estimates reactive tyrosines and is not influenced by autolysis of the protein. Figure 6 shows the gel filtration analysis of centrifugal filtrates of trypsin only, trypsin in the presence of carbomer and carbomer only. No peaks were observed for phosphate buffer pH 7.0 only (result not shown), suggesting that the peak observed for carbomer only is a low molecular weight polymer in the carbomer. The peak area of the treated trypsin was corrected by subtracting the peak area of the carbomer contaminant. The corrected peak area of the trypsin filtrate in the presence of carbomer was 32 ± 4% of the area of the control trypsin. The carbomer trypsin filtrate peak had no low molecular weight shoulder. The protein content of the trypsin-only filtrate and the trypsin carbomer filtrate determined by the modified Lowry assay (21) was 48 ± 3 μg/ml and 14 ± 2 μg/ml, respectively, implying that 29% only of the protein was filtered in the presence of carbomer.

Table I. The Protein Concentration, Activity and Specific Activity of Trypsin Solutions Filtered After Incubation with pH 7 Buffer Alone (Control) and After Incubation with Carbomer (mean ± s.d, n = 3)

	Control	Carbomer	% Filtered
Protein concentration (μg/ml)	50 ± 2.0	16 ± 1.86	32 ± 5
Trypsin activity[a] (μmol/min/ml)	0.118 ± 0.015	0.0129 ± 0.0012	11 ± 2
Trypsin specific activity[a] (μmol/min/mg)	2.36 ± 0.26	0.81 ± 0.074	34 ± 2[b]

[a] Tryptic activity for BAEE.
[b] Relative specific activity.

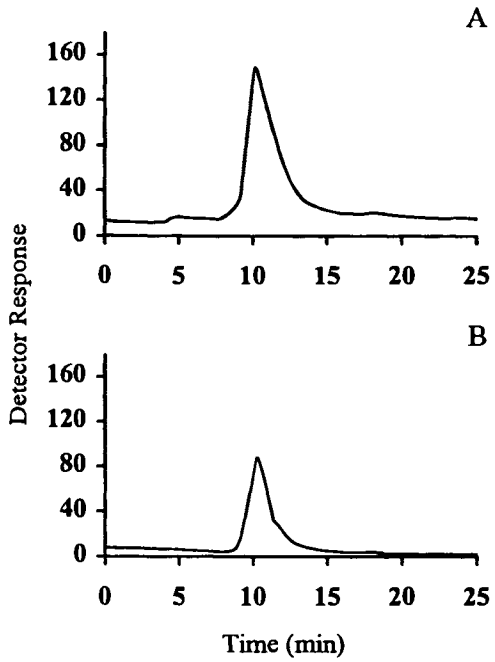

Fig. 5. Gel filtration of trypsin (50 μg/ml) stored on ice (A), and trypsin (50 μg/ml) incubated at 60°C for 45 min (B) in 50 mM phosphate buffer pH 7.0.

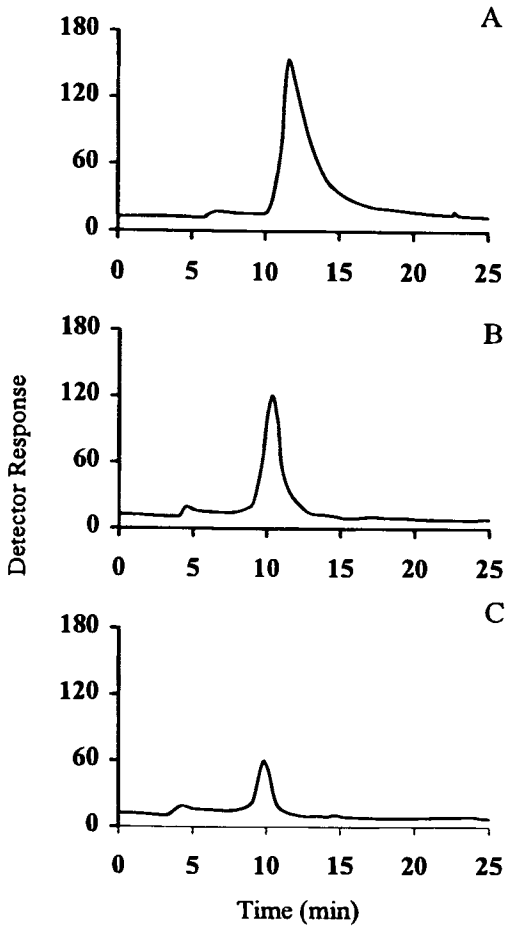

Fig. 6. Gel filtration of centrifugal filtrates of trypsin (50 μg/ml) incubated in the absence of carbomer (A), trypsin incubated in the presence of 0.35% (w/v) carbomer (B) and 0.35% (w/v) carbomer only (C) in 50 mM phosphate buffer pH 7.0 at 37°C for 30 min.

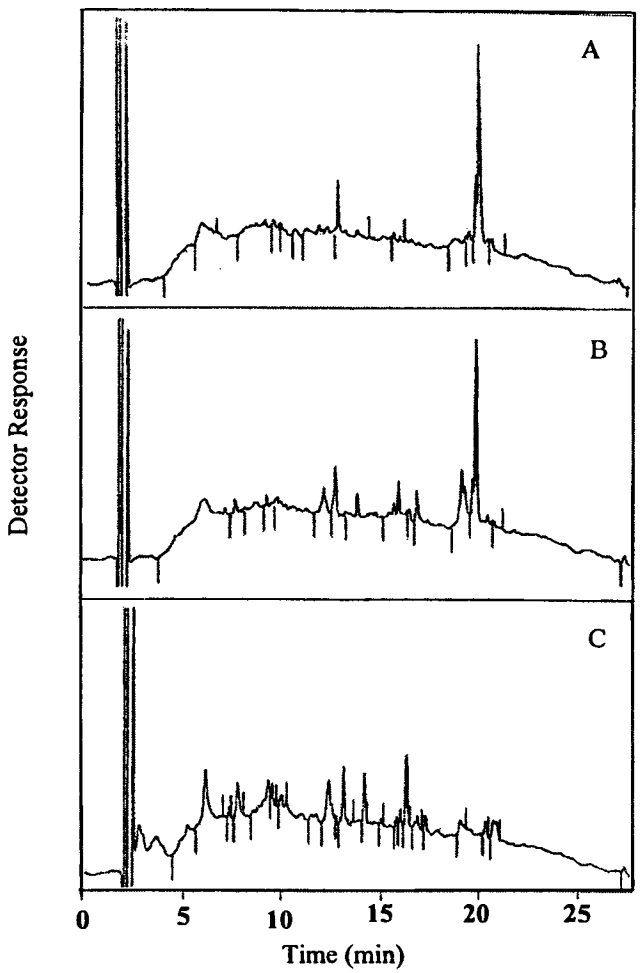

Fig. 7. Reverse-phase HPLC of centrifugal filtrates of trypsin (50 μg/ml) maintained at 4°C (A), trypsin incubated in the absence of carbomer (B), and trypsin incubated in the presence of 0.35% (w/v) carbomer (C), in 50 mM phosphate buffer pH 7.0 at 37°C for 30 min.

Reverse Phase HPLC

HPLC analysis of the centrifugal filtrates of trypsin in 50 mM sodium phosphate buffer pH 7.0 in the presence and absence of carbomer is shown in Fig. 7. The chromatographs of trypsin in phosphate buffer only (Fig. 7(A) and (B)) show that the trypsin filtrate after heating at 37°C for 30 min has a greater number of peaks when compared to trypsin that was maintained at 4°C. A dominant peak at 20 min was observed in both filtrates. Trypsin filtrates incubated at 37°C for 30 min in the presence of carbomer (C) had a greater number of peaks in comparison to trypsin incubated in the absence of carbomer. The dominant peak at 20 min was not present in the filtrate.

DISCUSSION

The results demonstrate that the carbomer (Carbopol 934P) reduces the proteolytic activity of trypsin *in vitro*. BAEE and LHRH were used as substrates to measure the activity of trypsin. The degree of proteolytic inhibition by carbomer was similar for both substrates, with hydrolysis rates 34 and 28% of the controls, respectively (Figs. 1 and 3). Under the conditions of this study the rate of metabolite formation for both substrates was constant both in the presence and absence of carbomer. The constant rate of metabolite formation in the presence of carbomer suggests that enhanced enzyme autolysis does not play a role in the reduced proteolytic activity of trypsin and this is supported by the gel filtration results (Figs. 5 and 6). This is in contrast to the postulate of others (24).

Carbomer could reduce the proteolytic activity by interacting with either the substrate or the enzyme or both. Centrifugal filtration was used to assess the binding of trypsin, BAEE and LHRH to Carbopol 934P. No LHRH and only 10% of the BAEE was bound to the carbomer suggesting that the enzyme binds to the carbomer. Protein analysis of the trypsin filtrate showed that trypsin binds strongly to the carbomer (Table I). The trypsin filtrates were analysed by gel filtration (Fig. 6) and reverse phase HPLC (Fig. 7) to confirm protein estimations and to determine the autolytic and conformational stability of trypsin.

Autolysis is associated with the loss of activity of trypsin in solution incubated at optimum pH: trypsin molecules can cleave each other at lysyl and arginyl bonds. Three autolytic cleavage sites have been identified in trypsin, Lys145-Ser146, Arg117-Val118, and Lys61-Ser62 (conventional chymotrypsin numbering is used). Only cleavage at Lys61-Ser62 resulted in

lower tryptic activity (25). Maroux and Desnuelle (25) suggested that no part of the trypsin molecule will be released after cleavage at these sites as they are interconnected by disulfide bonds. However, Varallyay *et al.* (26) showed that if either the Arg117-Val118, or Lys61-Ser62 sites are cleaved, the multiple cleavage sites within the Arg117-Val118, Lys61-Ser62 segment become subject to autolytic attack. Multiple cleavage in this segment results in the release of trypsin fragments and loss in tryptic activity. This 57 residue segment contains 10 potential cleavage sites and two members of the catalytic triad. It is believed that the loss of activity is due to the multiple cleavage of this segment resulting in either the disruption of the trypsin structure or the displacement of the members of the catalytic triad. The displaced fragments of autolysis will not significantly change the molecular weight of the protein in relation to the native trypsin.

The gel filtration system had insufficient resolution to identify the autolysed and intact trypsin forms. However we were able to use gel filtration to monitor autolysis by relating peak area of the trypsin species to the amount of tyrosine reactive groups by the modified method of Lowry (21). This was demonstrated by gel filtration (Fig. 5) and protein estimations of trypsin which had undergone accelerated autolysis by heating at 60°C for 45 min. For the autolysed trypsin sample there was a drop in the peak area with no change in the protein content. This suggests that the drop in the trypsin peak area is due to less absorbing material, as a result of the displacement of low molecular weight fragments which could not be identified by the chromatograhpic system. The chromatograph of the carbomer-treated-trypsin showed a drop in the area of the trypsin peak compared to the untreated trypsin (Fig. 6). The drop in peak area to 28% of the control, corresponded to the lower protein content of the carbomer-treated-trypsin filtrate (Table I). These results suggest that accelerated autolysis is not involved in the inactivation of the trypsin enzyme. We conclude that trypsin is immobilised on to the carbomer thereby reducing the amount of free trypsin available to hydrolyse the substrate.

Reverse phase HPLC of trypsin filtrates identified a number of different structural conformations of trypsin present in the different treatments (Fig. 7). Trypsin that was incubated at 37°C for 30 min had a greater number of peaks than the trypsin maintained and filtered at 4°C. This could be due to limited autolysis of trypsin at the elevated temperature. A greater number of peaks was observed for the carbomer-treated-trypsin filtrate that was incubated at 37°C. The largest peak (20 min), presumably native trypsin, was not present in the carbomer-treated-trypsin filtrate, again confirming that trypsin is bound to the carbomer. The greater number of peaks present in the carbomer-treated-trypsin filtrate suggests that there is a greater number of conformational forms of trypsin. This is supported by the lower specific activity of the unbound carbomer-treated-trypsin, being 34% of the untreated trypsin (Table I).

Studies investigating protein polymer surface interactions have demonstrated that these interactions induce protein conformational changes (27,28). A number of kinetic models have been proposed for the adsorption, desorption of proteins. The most recent kinetic model was based on the interactions between the interleukin-2 and silicone rubber tubing (18). The model assumes that the active protein can bind to a surface in a reversible fashion, so loosely bound protein can desorb and remain active but some of the bound protein may undergo structural rearrangements to develop more energetically favorable contacts with the surface. These structurally altered proteins may remain permanently bound or desorb. We suggest that the lower specific activity of the trypsin filtered after incubation with carbomer maybe the result of this protein–polymer interaction. However this observation could be explained if the carbomer has a lower affinity for the limited autolysed trypsin expected to be present after incubation at 37°C for 30 min in phosphate buffer pH 7.0.

Typically, Michaelis-Menton kinetic parameters (K_m, K_{cat}, K_i) are useful for determining the type of inhibition (non- and competitive inhibition) and relative potency of inhibitors. However, these parameters cannot be determined for this type of inhibition as the interaction between carbomer and trypsin is unlikely to be specific to a small number of loci on the trypsin protein, and the interaction will result in significant disruption of the three dimensional structure of trypsin. For this type of inhibition the binding capacity and affinity of carbomer for trypsin are more useful parameters to determine the potency of the carbomer to inhibit trypsin. The data showed a good correlation to the Langmuir model with a correlation coefficient of 0.997 for a linear fit. The binding capacity of carbomer for trypsin was 30 mg/g with an affinity constant of 0.028 ml/μg (Fig. 4).

The non-specific adsorption of proteins to interfaces have generally been attributed to electrostatic and hydrophobic interactions (29), and the amount of protein adsorbed has been shown to be dependent on pH, ionic strength and temperature (28,30). It remains for these variables to be investigated in the context of the luminal conditions before predicting the effectiveness of carbomer to inhibit the luminal proteolytic enzymes *in vivo*. Furthermore, the inhibition of luminal enzymes *in vivo* by carbomer may involve other mechanisms, including pH change (14) and autolysis (24). Binding of substrate (e.g., BSA) can also occur which may inhibit metabolism but also absorption (unpublished results).

We conclude that the inhibition of trypsin by carbomer is the result of enzyme–polymer interaction so reducing the free concentration of trypsin and in part denaturing the enzyme. This is another mechanism by which carbomer can reduce the proteolytic activity of luminal enzymes.

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