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Mucoadhesive polymers in peroral peptide drug delivery. V. Effect of poly(acrylates) on the enzymatic degradation of peptide drugs by intestinal brush border membrane vesicles

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Abstract

The purpose of the study was to evaluate the inhibitory effect of the mucoadhesive poly(acrylates) polycarbophil and carbomer on the activity of proteolytic enzymes bound to the intestinal brush border. To that end, the degradation of a number of peptide drugs in the presence or absence of the poly(acrylates) was investigated, using rat brush border membrane vesicles (BBMV) as the protease preparation. Both carbomer and polycarbophil in concentrations of 0.25 and 0.5% (w/v) reduced rather weakly the enzymatic degradation of the peptide 9-desglycinamide, 8-arginine vasopressin (DGAVP), and only 0.5% (w/v) carbomer inhibited metkephamid degradation, but not polycarbophil. More pronounced inhibitory effects on DGAVP breakdown were found following a 30 min preincubation of the BBMV suspension with 0.5% (w/v) carbomer. However, the poly(acrylic acid) derivatives were unable to inhibit the degradation of buserelin at all. On the other hand, the polypeptide hormone insulin was remarkably stable in the BBMV preparations. In conclusion, the poly(acrylic acid) derivatives polycarbophil and carbomer show rather weak inhibitory effects on enzymes of the intestinal brush border cell membranes responsible for DGAVP and metkephamid degradation.

Keywords: Brush border membrane vesicles; Buserelin; Insulin; 9-desglycinamide, 8-arginine vasopressin (DGAVP); Metkephamid; Polycarbophil; Carbomer; Enzyme inhibition; Peroral peptide drug delivery

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1. Introduction

Successful peroral delivery of therapeutical peptide drugs has to overcome the high proteolytic activities of various enzyme systems in the gastrointestinal tract (Lee et al., 1991). Much effort has been made in the past years to study the effects of various, widely used protease inhibitors to improve peptide drug absorption (Saffran et al., 1988; Morishita et al., 1992; Zhou, 1994). Other strategies to encounter the metabolic barrier of the gut is the use of suitable carrier systems which shuttle the peptides to their absorption sites (Lowe and Temple, 1994; Constantinides, 1995). Due to different proteolytic activities at different regions of the gut, the development of sophisticated controlled release systems, e.g. for colon targeting (Rubinstein et al., 1996), also plays an important role to master this challenging issue.

It was recently found that some commonly used polymers of the poly(acrylate)-type are able to strongly reduce the activities of the intestinal proteases trypsin, α-chymotrypsin, carboxypeptidase A and cytosolic leucine aminopeptidase (Lueßen et al., 1994, 1995, 1996). A major inhibitory mechanism at neutral pH values could be lined out. At neutral pH the poly(arcylates) are mainly dissociated and, because of their poly(anionic) character, show a high binding affinity towards bivalent cations. A number of intestinal proteases requires such bivalent cations as calcium or zinc to maintain their thermodynamical stability and/or to display proteolytic activity.

The intestinal metabolic barrier towards peptide drug absorption can be divided into three different enzyme groups: (i) the luminal proteolytic enzymes originating from pancreatic secretions such as trypsin, chymotrypsins, carboxypeptidases, elastase; (ii) enzymes bound to the membrane of mucosal cells of the intestinal epithelium such as a large variety of exopeptidases; and (iii) cytosolic enzymes as lysozymes. Whereas the last group (Bohley and Seglen, 1992; Hasilik, 1992; Seglen and Bohley, 1992) is more important in the case of transcellular peptide transport which is not the most likely route for passive diffusion of such hydrophilic macromolecules, the membrane bound enzymes lining the mucosal epithelium form a highly efficient obstacle for peptide drug absorption which is also difficult to be influenced by classical enzyme inhibitors of high molecular weight. Besides a sterical protection of the enzyme by being partly embedded into or being attached to a cell membrane, the enzymes are protected from the luminal contents of the gut by the overlaying highly viscous mucus layer (Strous and Dekker, 1992). The mucus is not only a diffusion barrier for the peptide drug to be delivered, but also for protease inhibitors which require a direct contact with the enzyme (Matthes et al., 1992).

A novel class of enzyme inhibitors which are able to reduce the proteolytic activity of enzymes of the intestinal brush border without needing a direct contact, would be of high interest to improve intestinal peptide drug absorption. Poly(-acrylate) derivatives display inhibitory effects on proteases by deprivation of bivalent cations from the enzyme structure (Lueßen et al., 1995, 1996). By this mechanism a direct inhibitor–enzyme interaction is not necessary and inhibition may also be achieved over a 'far distance effect' through the mucus layer.

The aim of this study was to evaluate the potential of the mucoadhesive poly(acrylates) polycarbophil and carbomer to protect different peptide drugs from degradation by membrane bound peptidases.

2. Materials and methods

2.1. Materials

Pyroglutamyl aminopeptidase (PGAP, EC 3.4.19.3), carboxypeptidase B (CPB, EC 3.4.17.2), 2-[*N*-morpholino]ethane-sulfonic acid] (MES), (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]) (HEPES), L-leucine-*p*-nitrophenylanilide (LNA), L-pyroglutamic acid *p*-nitroanilide (PNA), *p*-nitroaniline (NA), hippuryl-L-arginine (Hipp-Arg), hippuric acid and bovine serum albumin (BSA) were obtained from Sigma Chemie, Bornem, Belgium. All other chemicals used were at least of reagent grade.

2.1.1. Mucoadhesive polymers

The poly(acrylic acid) derivatives polycarbophil (PCP, Noveon® AA-1) and carbomer (C934P, Carbopol® 934P) were generous gifts of BF Goodrich, Cleveland, Ohio, USA.

2.1.2. Peptide drugs

The peptide drugs were generously donated by the following companies: buserelin (Suprecur®, Hoechst, Frankfurt, Germany), 9-desglycinamide, 8-arginine vasopressin (DGAVP; Organon, Oss, The Netherlands), metkephamid (Ely Lilly, Indianapolis, USA) and porcine insulin (Diosynth, Oss, The Netherlands).

2.1.3. Brush border membrane vesicles (BBMV)

For the preparation of brush border membrane vesicles (BBMV) male Wistar rats, weighing approximately 250 g (SPF-status) and bred at our Center, were killed by decapitation. Decapitation was performed between 8:00 and 9:00 a.m. The BBMV were prepared from mucosal scrapings of rat whole small intestine according to Biber et al. (1981) using a Mg^{2+} precipitation method. Three rats were used for each BBMV batch. Thereafter, the vesicle suspensions were pooled to one batch, dispensed in 150 μl portions and subsequently stored at -180°C in liquid nitrogen until use.

2.1.4. Buffer system

The buffer system used in all enzyme inhibition experiments was a 50 mM 2-[*N*-morpholino]ethane-sulfonic acid (MES)/KOH buffer, pH 6.7, containing 250 mM mannitol.

2.1.5. HPLC equipment

High performance liquid chromatography (HPLC) analysis was performed with a Thermo-Separations system consisting of a P 200 gradient pump, AS 100 autosampler, AS 200 UV/VIS detector and a datajet integrator. Data were calculated with the software package 'Winner on Windows' (Thermo-Separations, Breda, The Netherlands). Injection volume was 100 μl.

2.2. Methods

2.2.1. Characterization of BBMV

2.2.1.1. Protein content. The protein content of the BBMV preparations was determined according to the Lowry protein assay (Lowry et al., 1951).

2.2.1.2. Leucine aminopeptidase activity. The activity of leucine aminopeptidase (LAP) in BBMV was related to the activity of microsomal leucine aminopeptidase (mLAP) by determining the slope of the hydrolysis of the substrate LNA versus time as previously described (Lueßen et al., 1996). The LAP activity of BBMV preparations was expressed as IU_{mLAP}/mg protein.

2.2.1.3. Freeze-fracture electron microscopy. Amounts of 10 μl of BBMV preparations were mixed with 10 μl of 0.7% (w/v) polycarbophil and carbomer dispersed in a MES/KOH buffer pH 6.7, respectively (final polymer concentration of the BBMV suspension: 0.35% (w/v)). After 15 min incubation in 1.5 ml polypropylene tubes (Eppendorff, Hamburg, Germany) at 37°C, freeze-fracture electron microscopy (FFEM) of the samples was performed as described by Hofland et al. (1993).

2.2.2. Peptide degradation studies

2.2.2.1. Buserelin. Amounts of 7.7 mmol buserelin/ml were dissolved in either 50 mM MES/KOH buffer, pH 6.7, containing 250 mM mannitol (control) or in 0.25% and 0.5% (w/v) of polycarbophil and carbomer in MES/KOH buffer. After adding 10 μl of the BBMV to 100 μl of each preparation, samples of 10 μl were withdrawn from the incubation media at predetermined time points and dispersed in 500 μl stop solution (phosphoric acid, pH 2.0).

Preincubation studies were carried out at the same conditions as described above; in these cases the BBMV suspension was added to 0.5% (w/v) carbomer 30 min preceeding buserelin addition. Buserelin was measured by HPLC-UV_{210} nm. Isocratic elution was performed with 0.12 M

KH_2PO_4 buffer, pH 6.2, containing 35% (v/v) acetonitrile at a flow rate of 1 ml/min. The retention time of buserelin was 7.6 min using a Chromspher 5 C8 250 × 4.6 mm column (Chrompack, Middelburg, The Netherlands) equipped with a Chromspher 5 C8 10 × 4.6 precolumn as the stationary phase.

2.2.2.2. Desglycinamide, 8-arginine vasopressin (DGAVP). A volume of 5 μl BBMV was added to solutions of 250 μl containing 9.2 mmol DGAVP/ml of the control and polymer preparations (similar as described above for buserelin) to start the incubation. Samples of 25 μl were taken, dispersed in 1 ml of stop solution (phosphoric acid, pH 3, containing 0.01% BSA).

Preincubation studies were performed according to the above described procedure. The BBMV suspension was added to 0.5% (w/v) carbomer at 15 or 30 min prior to the start of the experiment by adding DGAVP to the incubation medium.

DGAVP was analyzed by HPLC-$UV_{210\,nm}$. Gradient elution was performed as follows: 0–15 min, linear gradient from 100% A to 70% A/30% B (flow 1.0 ml/min). Eluent A consisted of 15% (v/v) acetonitrile in a 5% tetramethyl ammonium hydroxide buffer adjusted to pH 2.8 with phosphoric acid. Eluent B contained 40% (v/v) acetonitrile in the 5% tetramethyl ammonium hydroxide buffer. The retention time of DGAVP was 9.5 min. A μ-Bondapak® C18, 10 μm, 3.9 × 300 mm column (Waters, Etten-Leur, The Netherlands) served as the stationary phase.

2.2.2.3. Insulin. Stability of insulin was studied by adding 11.5 μl of BBMV to 90 μl of 1.7 mmol insulin/ml MES/KOH buffer (pH 6.7). Samples of 10 μl were taken and dispersed in 400 μl stop solution.

Insulin was determined by HPLC-$UV_{210\,nm}$. Gradient elution was performed as follows: 0–25 min, linear gradient from 100% A to 50% A/50% B (flow 1.0 ml/min). Eluent A consisted of 22% (v/v) acetonitrile in 0.25 N phosphoric acid adjusted to pH 2.25 with triethylamine. Eluent B contained 60% (v/v) acetonitrile in the phosphoric acid/triethylamine buffer. The retention time of insulin was 15 min. For the stationary phase the same column was used as the DGAVP analysis.

2.2.2.4. Metkephamid. A volume of 15 μl of BBMV suspension was added to 150 μl of metkephamid solution (10 mmol/l) in either MES/KOH buffer pH 6.7 or different concentrations of polycarbophil and carbomer. After predetermined time points, 20 μl samples were taken and diluted in 1 ml stop solution (phosphoric acid, pH 2.0).

The disappearance of intact metkephamid and the formation of the metabolite tyrosin (Tyr) were measured by HPLC-$UV_{210\,nm}$. Gradient elution was performed as follows: 0–3 min: 100% A, isocratic (flow, 1 ml/min); 3–12 min: 25% A/75% B, linear gradient (flow, 1 ml/min). Eluent A consisted of 18% (v/v) acetonitrile in 0.05 M phosphate buffer (pH 4.0), and eluent B of 80% (v/v) acetonitrile in 0.05 M phosphate buffer. The retention times of metkephamid and Tyr were 11.75 and 3.75 min, respectively. The same stationary phase as described for the buserelin assay was used.

2.2.3. Pyroglutamyl aminopetidase activity

L-Pyroglutamic acid *p*-nitroanilide (PNA) was dissolved in a concentration of 0.2 mmol/l in different polycarbophil and carbomer preparations. The incubation was started by adding 80 IU pyroglutamyl aminopeptidase/ml. Samples of 20 μl were taken at predetermined time points and diluted in 400 μl stop solution (phosphoric acid, pH 2). Both the substrate PNA and the metabolite *p*-nitroaniline were measured by HPLC as previously described (Lueßen et al., 1996).

2.2.4. Carboxypeptidase B activity

An amount of 1.5 mmol/l of the substrate hippuryl-L-arginine was dissolved in the control buffer and the different polymer preparations. Carboxypeptidase B, obtained as a frozen solution in 0.1 M NaCl, was diluted in ice-cold MES/KOH buffer to 15.6 U/ml. Immediately thereafter 1.6 U/ml were added to the substrate containing control or polymer preparations, and incubated at 37°C. Samples of 20 μl were taken at predetermined time points and diluted with 500 μl of a 50% (v/v) methanol/water saturated with EDTA to stop enzyme activity. Both the substrate hippuryl-L-arginine and its metabolite hippuric acid were determined by HPLC-$UV_{231\,nm}$ as described previously (Lueßen et al., in press).

3. Results

3.1. Characterization of BBMV

The protein content of the obtained BBMV preparation was 26 mg/ml, and the activity of the chosen marker enzyme (microsomal leucine aminopeptidase) 0.96 IU_{mLAP}/mg protein, corresponding to 25 $mIU_{mLAP}/\mu l$ BBMV suspension.

Freeze-fracture electron microscopy studies did not reveal substantial changes in the ultrastructural appearance of the BBMV after incubation with polycarbophil or carbomer (Fig. 1a,b). Thus, changes in the enzyme activities of BBMV were most likely not due to disruption of the vesicular structure by these polymers.

3.2. Peptide degradation studies

3.2.1. Buserelin

The two poly(acrylates) polycarbophil and carbomer showed no substantial effects on the degradation of buserelin by BBMV (Fig. 2). Compared with the control, the recovery of the peptide drug was slightly higher in presence of polycarbophil and carbomer. In the controls 65% of buserelin was degraded within 4 h, whereas approximately 50% of intact buserelin could be detected after the same incubation period in the presence of the polymers. The inhibition factors were ranging between 1.0 and 1.1, indicating no influence on the overall recovery of the peptide drug (Table 1). The inhibition factor, determined as described by Lueßen et al. (1996), was introduced as a relative measure to compare the areas under the concentration-time curve within one incubation experiment.

3.2.2. Desglycinamide, 8-arginine vasopressin (DGAVP)

Both poly(acrylic acid) derivatives were able to increase the recovery of the peptide drug DGAVP (Fig. 3a,b). The inhibition factors (IF) of the polymer preparations were ranging between 1.5 and 1.8 (Table 1), which indicates that no complete inhibition of DGAVP-degrading BBMV activity could be reached. A concentration dependent inhibitory effect of the poly(acrylates) was not observed. Preincubation of the BBMV in 0.5% (w/v) carbomer for 30 min showed a slightly higher recovery of DGAVP compared with experiments without any preincubation (Fig. 3c).

3.2.3. Insulin

Insulin appeared to be very stable in the BBMV preparations (Fig. 4). Approximately 10% was degraded over 4 h. Similar results were observed using a different BBMV preparation and another analytical method (a commercially available insulin RIA-kit; Pharmacia,Roosendaal, The Netherlands) (data not shown). Surprisingly, incubation in 0.25% (w/v) carbomer slightly increased the recovery of insulin (Fig. 4).

3.2.4. Metkephamid

Metkephamid was almost completely degraded within 240 min in the control situation (Fig. 5a,b). Polycarbophil did not show any significant inhibition on BBMV-induced metkephamid degradation (Fig. 5a). However, carbomer in a concentration of 0.5% (w/v) was able to increase the recovery of the peptide drug compared with the control (Fig. 5b), resulting in an inhibition factor of 2.1 (Table 1). The inhibitory effect was almost absent at the lower carbomer concentration of 0.25% (w/v) (IF = 1.2).

3.3. Pyroglutamyl aminopetidase activity

The activity of pyroglutamic acid aminopeptidase could not be inhibited by the poly(acrylates) polycarbophil and carbomer. In contrast, the degradation rates appeared to be increased by the polymer preparations compared with the controls. The inhibition factors were varying between 0.7 and 1.1 (Table 1).

3.4. Carboxypeptidase B activity

A concentration-dependent inhibition of carboxypeptidase B activity was observed by both polycarbophil and carbomer (Fig. 6). Carbomer was found to be more potent than polycarbophil. A concentration of 0.5% (w/v) carbomer (IF = 4.4) showed a much stronger enzyme inhibitory effect compared with 0.5% (w/v) polycarbophil

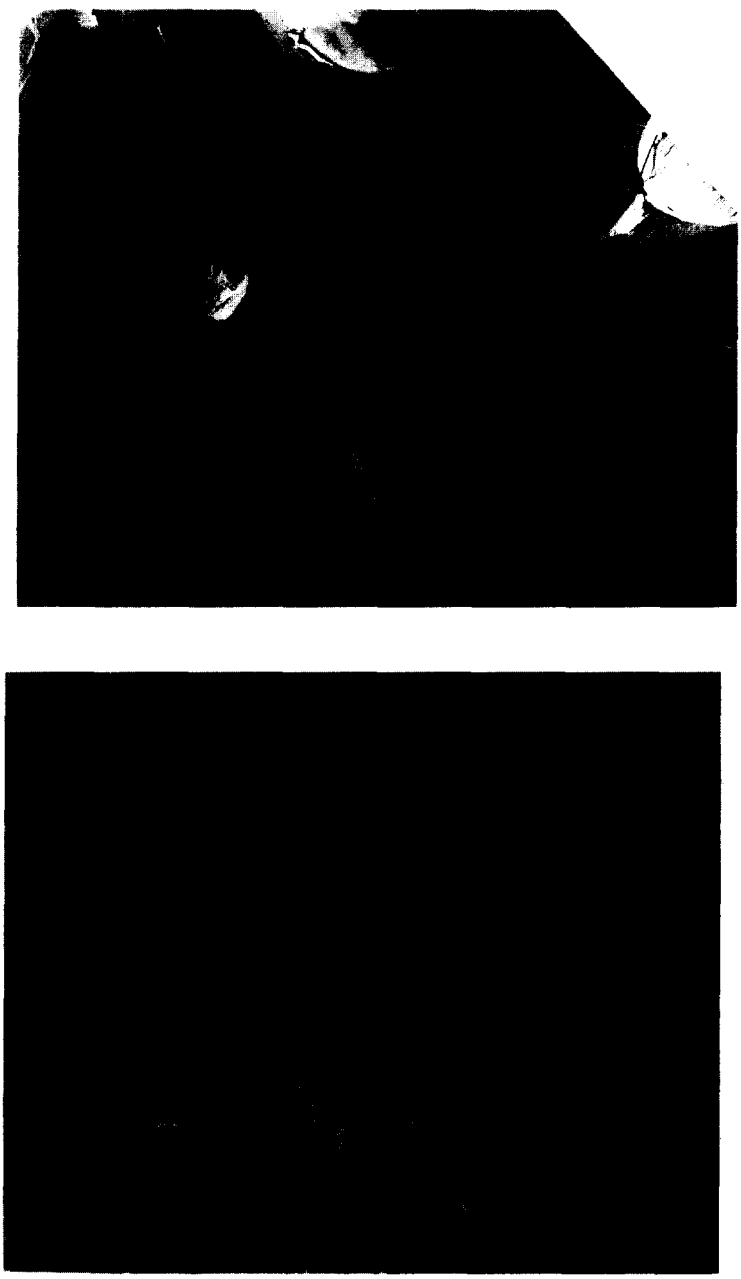

Fig. 1. Electronmicrographs of BBMV: (a) BBMV suspension mixed with MES/KOH buffer in a 1:1 ratio (control); and (b) BBMV suspension mixed with 0.7% (w/v) polycarbophil in a ratio of 1:1.

(IF = 2.2), whereas the differences at polymer concentrations of 0.25% (w/v) were not so pronounced (Table 1).

4. Discussion

From all peptide drugs studied, DGAVP degra-

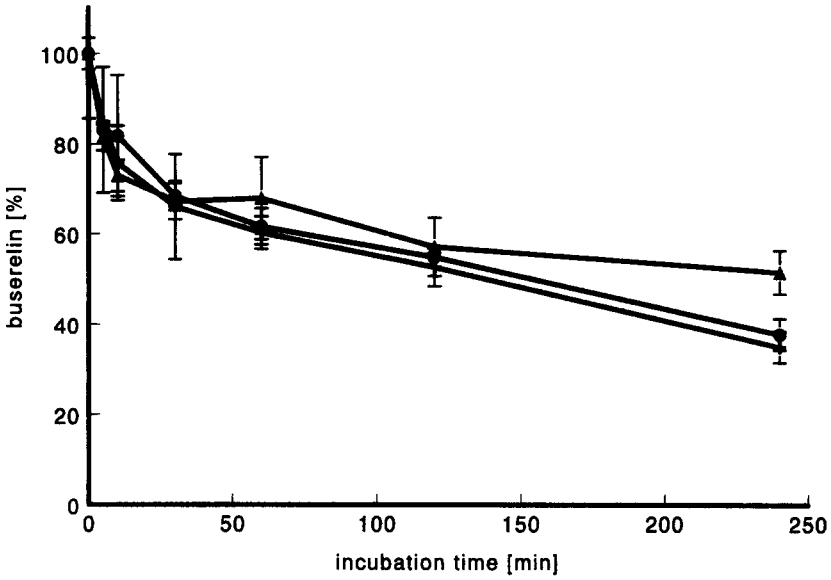

Fig. 2. Degradation of buserelin by BBMV in presence of polycarbophil and carbomer: +, control; ▲, 0.5% (w/v) polycarbophil; ●, 0.5% (w/v) carbomer (mean ± S.D., $N = 3$).

dation could be partly inhibited by the poly(-acrylic acid) derivatives polycarbophil and carbomer in concentrations of 0.25 and 0.5% (w/v). In the case of metkephamid only carbomer at a concentration of 0.5% (w/v) was able to display any inhibitory effect, whereas buserelin degradation was not inhibited by both poly(acrylates) in concentrations up to 0.5% (w/v).

DGAVP is a vasopressin fragment which was originally developed for the treatment of memory disorders (Bruins et al., 1990). It is highly stabilized against metabolic degradation and cleavage occurs preferentially at the carboxy-terminal L-arginine by carboxypeptidase B or prolyl-endopeptidase like activities (Verhoef et al., 1986). Although the high structural similarity between carboxypeptidase A and B (Guasch et al., 1992; Chan and Pfuetzner, 1993), the inhibitory effect of the poly(acrylates) on carboxypeptidase B was not so strong pronounced compared with carboxypeptidase A (Lueßen et al., 1996), which may explain the weak inhibitory effect on DGAVP degradation by the polymers. An explanation for this phenomenon may be that the binding affinity of carboxypeptidase B to its co-factor Zn^{2+} is higher compared with carboxypeptidase A.

Metkephamid is an enkephalin analogue with the following amino acid sequence: Tyr-D-Ala-Gly-Phe-*N*-Me-Met-NH_2. This peptide shows high stability against proteolytic degradation and is mainly degraded by aminopeptidase activity (Langguth et al., 1994a). The high resistancy against pancreatic endo- and exopeptidases makes metkephamid an interesting model peptide to study enzyme activities of the intestinal brush border (Langguth et al., 1994b). Of the poly(acrylate) preparations studied, only 0.5% (w/v) carbomer dispersion was able to display an inhibitory effect on metkephamid-degrading BBMV enzyme activities. Assuming that deprivation of Zn^{2+} out of the metkephamid-degrading aminopeptidase structure by carbomer serves as a mechanism for the observed inhibitory effect (Lueßen et al., 1995, 1996), it may be suggested that the higher binding affinity of carbomer for bivalent cations as Zn^{2+} and Ca^{2+} is responsible for the differences in efficacy between carbomer and polycarbophil.

Insulin was relatively stable against degradation by BBMV. This may be due to the conformational structure of this polypeptide drug, where

Table 1
Inhibition factors of different peptide drugs in the presence of polycarbophil or carbomer

Peptide drug/substrate	Polymer	Polymer conc. (w/v)	IF[a] (experimental)
Buserelin	Control		1.0
	Polycarbophil	0.25%	1.1
		0.5%	1.1
	Carbomer	0.25%	1.1
		0.5%	1.0
		0.5%[c]	1.0
DGAVP	Control	—	1.0
	Polycarbophil	0.25%	1.8
		0.5%	1.6
	Carbomer	0.25%	1.5
		0.5%	1.7
		0.5%[b]	2.0
		0.5%[c]	2.4
Metkephamid	Control		1.0
	Polycarbophil	0.25%	1.3
		0.5%	1.7
	Carbomer	0.25%	1.2
		0.5%	2.1
PGNA	Control	—	1.0
	Polycarbophil	0.1%	0.8
		0.25%	0.7
		0.5%	1.1
	Carbomer	0.1%	0.7
		0.25%	0.7
		0.5%	0.7
Hipp-Arg	Control	—	1.0
	Polycarbophil	0.25%	1.7
		0.5%	2.2
	Carbomer	0.25%	2.0
		0.5%	4.4

[a] Inhibition factors (IF), calculated by the formula $AUC_{polymer}/AUC_{control}$ for areas under the percentage of substrate-time curve (for buserelin, DGAVP and metkephamid). For areas under the percentage of metabolite-time curve (in the case of pyroglutamyl aminopeptidase) following coefficient was determined: $AUC_{control}/AUC_{polymer}$.
[b] 15 min preincubation;
[c] 30 min preincubation.

the terminal ends of the A- and B-chains can be sterically protected from exopeptidase degradation. The observed stability of insulin may also be explained by the formation of insulin associates and agglomerates (Cleland et al., 1993). The fact that insulin is highly resistant against degradation by BBMV makes insulin an interesting peptide drug for the design of peroral delivery systems based on polycarbophil or carbomer, since the major insulin degrading proteases, α-chymotrypsin and trypsin (Schilling and Mitra, 1991), are strongly inhibited by these poly(acrylates). This is also a possible explanation for the increased recovery of insulin in the presence of 0.25% carbomer (Fig. 4). There is always some luminal proteolytic activity in the BBMV preparations left, despite the cleaning procedures during their preparation. Another aspect is the possibility

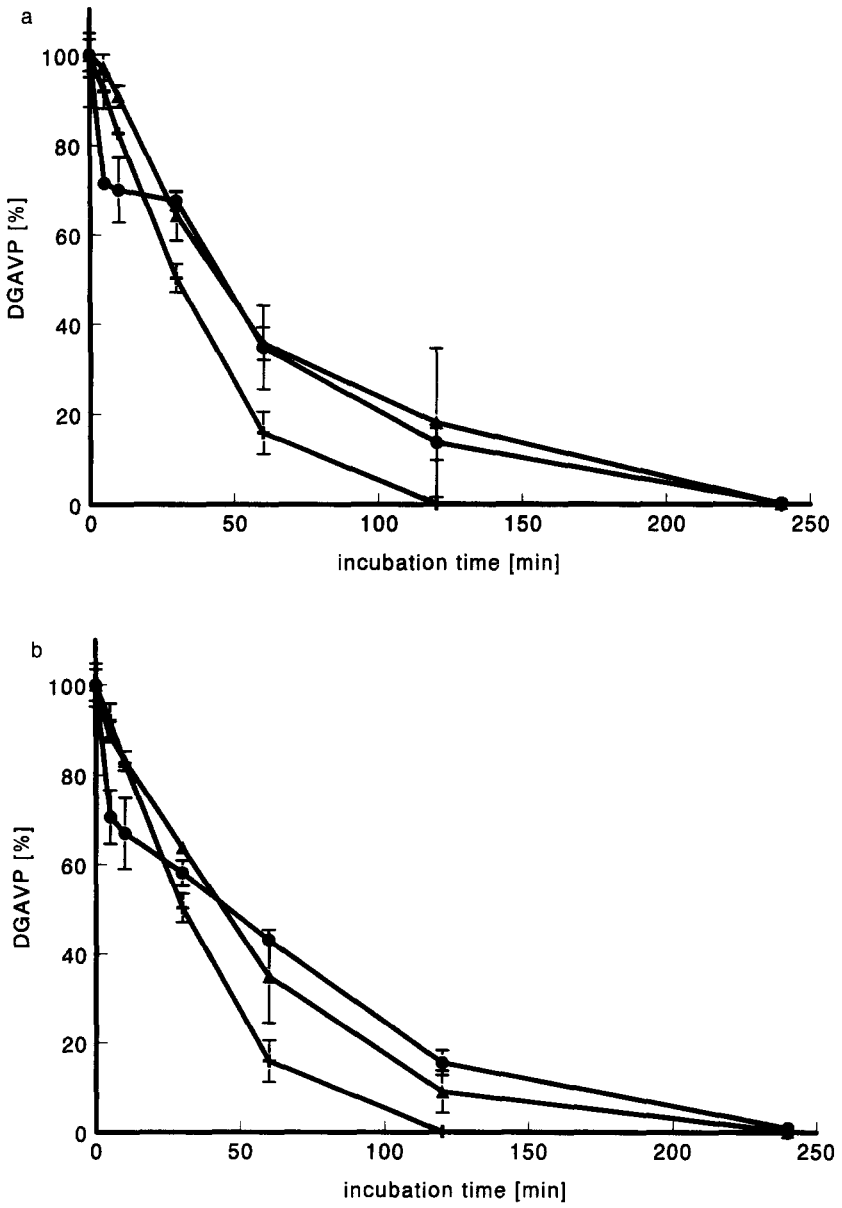

Fig. 3. (a) Degradation of DGAVP by BBMV in presence of polycarbophil: +, control; ▲, 0.25% (w/v) polycarbophil; ●, 0.5% (w/v) polycarbophil (mean ± S.D., $N = 3$). (b) Degradation of DGAVP by BBMV in presence of carbomer: +, control; ▲, 0.25% (w/v) carbomer; ●, 0.5% (w/v) carbomer (mean ± S.D., $N = 3$). (c) Degradation of DGAVP by BBMV. Effect of preincubation of BBMV in 0.5% (w/v) carbomer: +, control; ▲, 0.5% (w/v) carbomer without preincubation of BBMV; ●, 0.5% (w/v) carbomer with 15 min preincubation; ■, 0.5% (w/v) carbomer with 30 min preincubation of BBMV (mean ± S.D., $N = 3$).

of binding the Zn^{2+} ions, which are supporting the association of insulin monomers (Cleland et al., 1993). Because of their Zn^{2+}-binding properties, poly(acrylates) as polycarbophil and carbomer may stabilize insulin by avoiding the formation of irreversible insulin agglomerates. In this respect the recently developed insulin derivatives of Ely Lilly are of interest, which do not

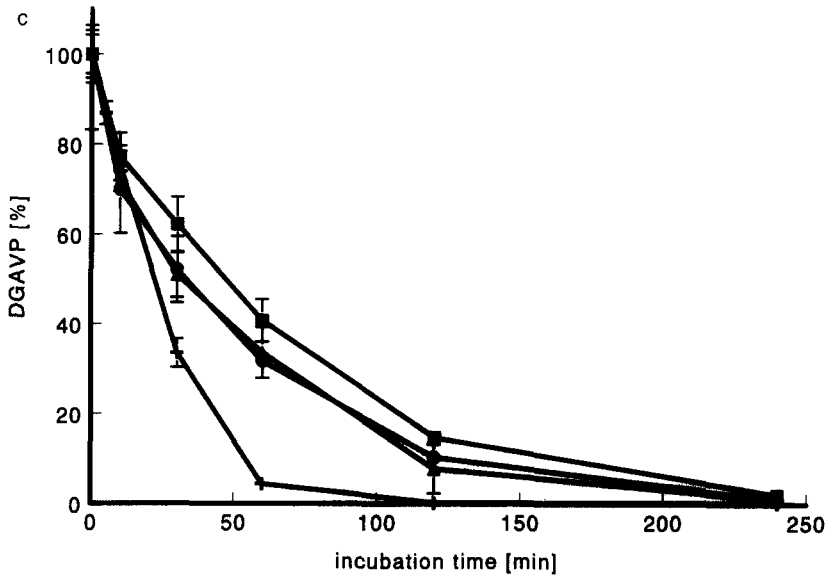

Fig. 3C.

show association and aggregation (Ter Braak et al., 1993).

Buserelin degradation is mainly initiated by α-chymotrypsin, neutral endopeptidases or pyroglutamic acid aminopeptidase. In the intestinal brush border and in the systemic circulation pyroglutamic aminopeptidase is the major enzyme responsible for the inactivation of this peptide drug (Sandow, 1989). Pyroglutamic aminopeptidase belongs to the group of cysteine peptidases. This

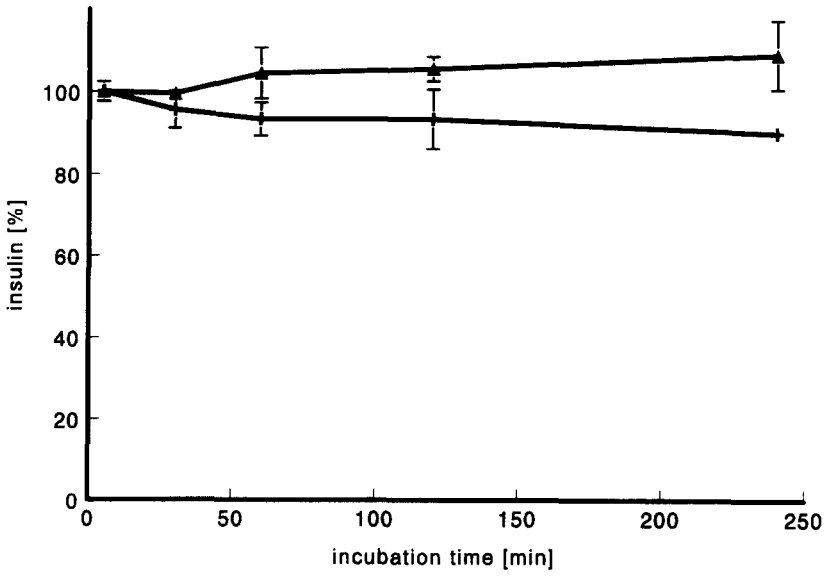

Fig. 4. Incubation of insulin in a BBMV containing preparation with and without carbomer: +, control; ▲, 0.25% (w/v) carbomer (mean ± S.D., $N = 3$).

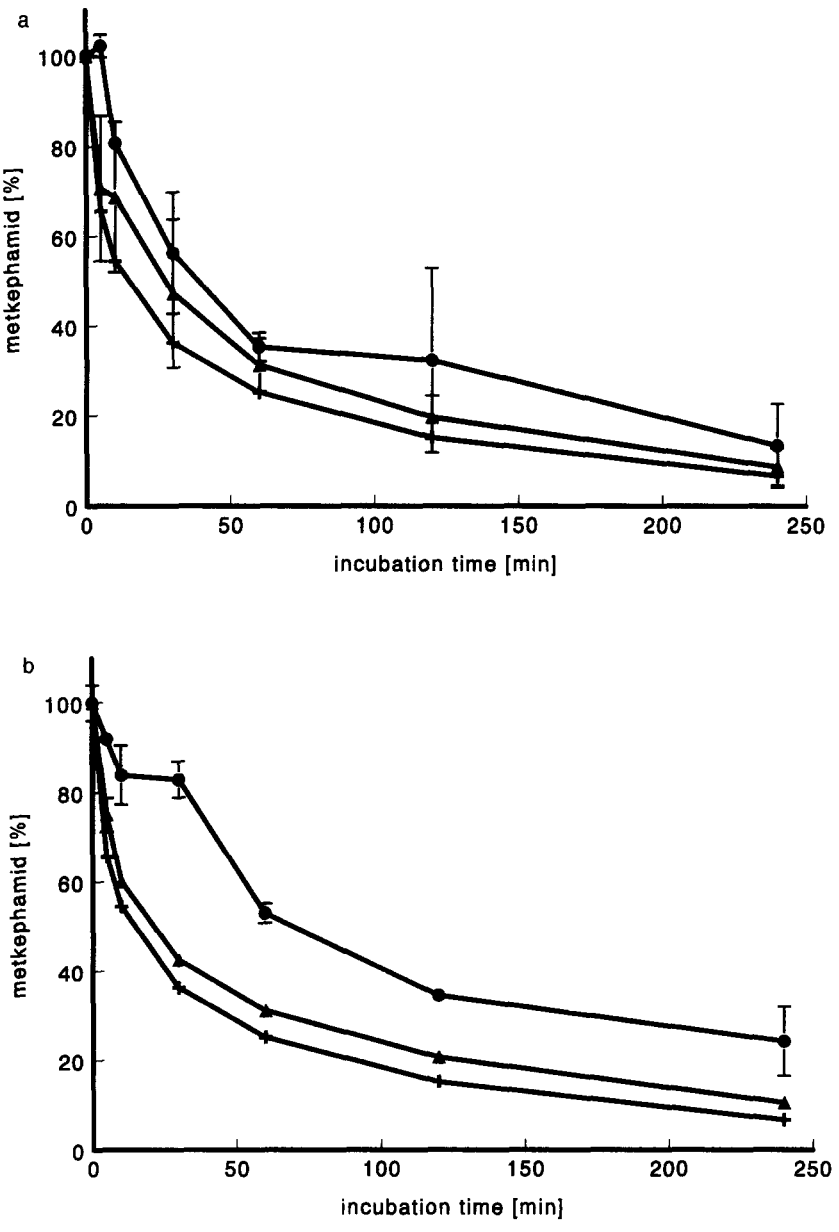

Fig. 5. (a) Degradation of metkephamid by BBMV in presence of polycarbophil: +, control; ▲, 0.25% (w/v) polycarbophil; ●, 0.5% (w/v) polycarbophil (mean ± S.D., $N = 3$). (b) Degradation of metkephamid by BBMV in presence of carbomer: +, control; ▲, 0.25% (w/v) carbomer; ●, 0.5% (w/v) carbomer (mean ± S.D., $N = 3$).

enzyme does not contain any bivalent cations within its structure (Szewczuk and Mulczyk, 1969; Armentrout and Doolittle, 1969), and therefore can not be inhibited by poly(acrylic acid) derivatives (Lueßen et al., 1996). Thus it is not surprising that a 30 min preincubation of the BBMV suspensions in the carbomer preparation did not show any inhibitory effect.

The observed inhibitory activities of the poly(-acrylates) on metkephamid and DGAVP degrada-

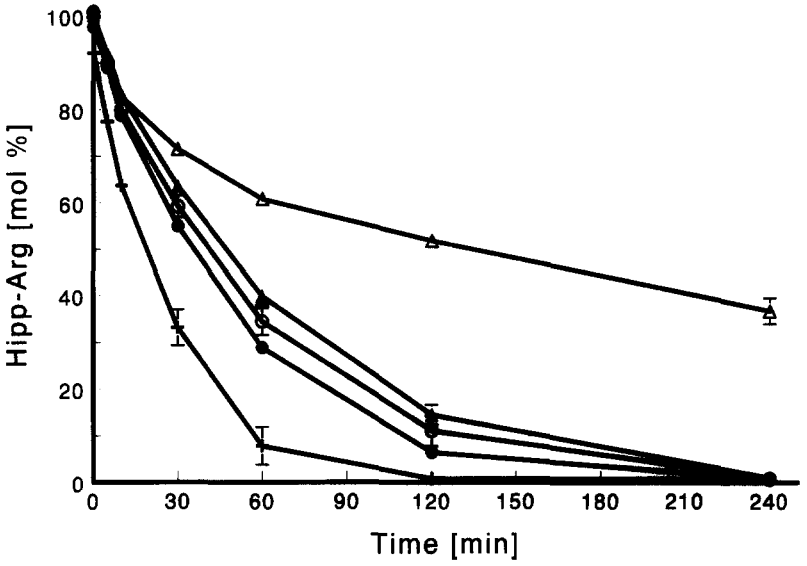

Fig. 6. Degradation of hippuryl-L-arginine (Hipp-Arg) by carboxypeptidase B in presence of polycarbophil and carbomer: +, control; ●, 0.25% (w/v) polycarbophil; ▲, 0.5% (w/v) polycarbophil; O, 0.25% (w/v) carbomer; △, 0.5% (w/v) carbomer (mean ± S.D., $N = 3$).

tion by BBMV were not as pronounced as previously shown for trypsin, α-chymotrypsin, carboxypeptidase A and cytosolic aminopeptidase (Lueßen et al., 1995; Lueßen et al., 1996). This may be explained by different reasons. One reason may be the bivalent cation-containing peptidase itself. The binding affinity of the poly(acrylate) should be much higher than the binding affinity of the enzyme towards bivalent cations. Thus, if the dissociation constant of the enzyme-X^{2+} (X = bivalent cations, such as Zn^{2+} or Ca^{2+}) complex is very small, the capability of the X^{2+}-binding polymer to deprive the bivalent cation from the enzyme structure gets more aggravated. Another reason for the relatively weak inhibitory effect of the poly(acrylates) on the brush border bound enzymes may be the environment of the peptidases. Because of being attached to or embedded in the lipid bilayer of the cell membrane, the X^{2+}-bearing part of the enzyme may be sterically protected, resulting in an apparent lower dissociation constant of the X^{2+}-enzyme complex. Both reasons may occur simultaneously for the BBMV, which make it very difficult to inhibit membrane-bound proteolytic activities by X^{2+}-binding polymers, such as polycarbophil and carbomer. The inhibitory effect of the poly(acrylates) can be improved if the peptidase is preincubated in the polymer environment for approximately 30 min (as found for DGAVP; Fig. 3c), before the peptide drug comes into contact with the enzyme. This will require the design of dosage forms which release the peptide drug in a delayed burst after the poly(acrylate) has been swollen and became capable to bind bivalent cations. This principle is indeed effective, as has been demonstrated recently (Akiyama et al., 1996a,b).

5. Conclusions

The poly(acrylates) polycarbophil and carbomer showed a weak inhibitory effect on metkephamid- and DGAVP-degrading brush border bound proteolytic activities, whereas buserelin degradation was not affected by the poly(acrylates) at all. The polypeptide insulin displayed a high resistance against degradation by brush-border membrane vesicles. It is suggested that the weak inhibitory potency of the poly(acrylates) is due to the high affinity of the X^{2+}-containing peptidase towards its bivalent cation which may

be even enhanced by being embedded in or attached to the epithelial cell membrane in comparison with the free enzyme. To improve the inhibitory effect of the poly(acrylates), it is interesting to design polymers which display a higher binding affinity for bivalent cations such as zinc or calcium. Only then, the 'far distant inhibitory effect', as mentioned in Section 1, will be powerful enough to protect peptide drugs from degradation by membrane-bound proteases.

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References

Akiyama, Y., Lueßen, H.L., de Boer, A.G., Verhoef, J.C. and Junginger, H.E., Novel peroral dosage forms with protease inhibitory activities. I. Design of capsules with fast gel-forming and fast drug-releasing properties. *Int. J. Pharm.*, 136 (1996a) 155–163.

Akiyama, Y., Lueßen, H.L., de Boer, A.G., Verhoef, J.C. and Junginger, H.E., Novel peroral dosage forms with protease inhibitory activities. II. Design of fast dissolving poly(acrylate) and controlled drug-releasing capsule formulations with trypsin inhibiting properties. *Int. J. Pharm.*, (1996a) in press.

Armentrout, R.W. and Doolittle, R.F., Pyrrolidonecarboxyl peptidase: stabilization and purification. *Arch. Biochem. Biophys.*, 132 (1969) 80–90.

Biber, J., Stieger, B., Haase, W. and Murer, H., A high yield preparation for rat kidney brush border membranes—different behaviour of lysosomal markers. *Biochim. Biophys. Acta*, 647 (1981) 169–176.

Bohley, P. and Seglen, P.O., Proteases and proteolysis in the lysosome. *Experientia*, 48 (1992) 151–157.

Bruins, J., Kumar, A. and Schneider-Helmert, D., Influence of desglycinamide-(Arg^8)-vasopressin on memory in healthy subjects. *Neuropsychobiology*, 23 (1990) 82–88.

Chan, W.W.-C. and Pfuetzner, R.A., General occurence of binding synergism in zinc proteases and its possible significance. *Eur. J. Biochem.*, 218 (1993) 529–534.

Cleland, J.L., Powell, M.F. and Shire, S.J., The development of stable protein formulations: a close look at protein aggregation, deamidation, and oxidation. *Crit. Rev. Therapeutic Drug Carrier Syst.*, 10 (1993) 307–377.

Constantinides, P.P., Lipid microemulsions for improving drug dissolution and oral absorption: physical and biopharmaceutical aspects. *Pharm. Res.*, 12 (1995) 1561–1572.

Guasch, A., Coll, M., Avilés, F.X. and Huber, R., Three-dimensional structure of porcine pancreatic procarboxypeptidase A—a comparison of the A and B zymogens and their determinants for inhibition and activation. *J. Mol. Biol.*, 224 (1992) 141–157.

Hasilik, A., The early and late processing of lysosomal enzymes: proteolysis and compartimentation. *Experientia*, 48 (1992) 130–150.

Hofland, H.E.J., Bouwstra, J.A., Spies, F., Talsma, H. and Junginger, H.E., Non-ionic surfactant vesicles: a study of vesicle formation, characterization, and stability. *J. Colloid Interface Sci.*, 161 (1993) 366–376. *

Langguth, P., Bohner, V., Biber, J. and Merkle, H.P., Metabolism and transport of the pentapeptide by brush-border membrane vesicles of rat intestine. *J. Pharm. Pharmacol.*, 46 (1994a) 34–40.

Langguth, P., Merkle, H.P. and Amidon, G.L., Oral absorption of peptides: the effect of absorption site and enzyme inhibition on the systemic availability of metkephamid. *Pharm. Res.*, 11 (1994b) 528–535.

Lee, V.H.L., Traver, R.D. and Taub, M.E., Enzymatic barriers to peptide and protein drug delivery. In Lee, V.H.L. (Ed.), *Peptide and Protein Drug Delivery*, Marcel Dekker, New York, 1991, pp. 303–358.

Lowe, P.J. and Temple, C.S., Calcitonin and insulin in isobutylcyanoacrylate nanocapsules: protection against proteases and effect on intestinal absorption in rats. *J. Pharm. Pharmacol.*, 46 (1994) 547–552.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J., Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, 193 (1951) 265–275.

Lueßen, H.L., Borchard, G., Verhoef, J.C., Lehr, C.-M., de Boer, A.G. and Junginger, H.E., Mucoadhesive polymers in peroral peptide drug delivery. II. Carbomer and polycarbophil are potent inhibitors of the intestinal proteolytic enzyme trypsin. *Pharm. Res.*, 12 (1995) 1293–1298.

Lueßen, H.L., de Leeuw, B.J., Pérard, D., Lehr, C.-M., de Boer, A.G., Verhoef, J.C. and Junginger, H.E., Mucoadhesive excipients in peroral peptide drug delivery. I. Influence of mucoadhesive excipients on the proteolytic activity of intestinal enzymes. *Eur. J. Pharm. Sci.*, 4 (1996) 117–128.

Lueßen, H.L., Lehr C.-M., Rentel, C.-O., Noach, A.B.J., de Boer, A.G., Verhoef, J.C. and Junginger, H.E., Bioadhesive polymers for the peroral delivery of peptide drugs. *J. Controlled Rel.*, 29 (1994) 329–338.

Lueßen, H.L., Rentel, C.-O., Kotzé, A., Lehr, C.-M., de Boer, A.G., Verhoef, J.C. and Junginger, H.E., Mucoadhesive polymers in peroral peptide drug delivery. IV. Polycarbophil and chitosan are potent enhancers of peptide transport across intestinal mucosae in vitro. *J. Controlled Rel.*, (in press).

Matthes, I., Nimmerfall, F. and Sucker, H., Mucus models for investigation of intestinal absorption. Part 2: Mechanisms of drug interaction with intestinal mucus. *Pharmazie*, 47 (1992) 609–613.

Morishita, I., Morishita, M., Takayama, K., Machida, Y. and Nagai, T., Hypoglycemic effect of novel oral microspheres of insulin with protease inhibitor in normal and diabetic rats. *Int. J. Pharm.*, 78 (1992) 9–16.

Rubinstein, A., Tirosh, B., Baluom, M., Nassar, T., David, A., Radai, R., Gliko-Kabir, I. and Friedmann, M., The rationale for peptide drug delivery to the colon and the potential of polymeric carriers as effective tools. *J. Controlled Rel.*, (1996) in press.

Saffran, M., Breda, C., Kumar, G.S. and Neckers, D.C., Vasopressin: a model for the study of effects of additives on the oral and rectal administration of peptide drugs. *J. Pharm. Sci.*, 77 (1988) 33–38.

Sandow, J., Physiologische und pharmakologische Wirkungen von Buserelin: präklinische Untersuchungen. In Schindler, A.E. and Schweppe K.W. (Eds.), *Endometriose-Neue Therapiemöglichkeiten durch Buserelin*. Walter de Gryter, Berlin, 1989, pp. 23–42.

Schilling, R.J. and Mitra, A., Degradation of insulin by trypsin and α-chymotrypsin. *Pharm. Res.*, 8 (1991) 721–727.

Seglen, P.O. and Bohley, P., Autophagy and other vacuolar protein degradation mechanisms. *Experientia*, 48 (1992) 158–171.

Strous, G.J. and Dekker, J., Mucin-type glycoproteins. *Crit. Rev. Biochem. Mol.*, 27 (1992) 57–92.

Szewczuk, A. and Mulczyk, M., Pyrrolidonyl peptidase in bacteria. The enzyme from Bacillus subtillis. *Eur. J. Biochem.*, 8 (1969) 63–67.

Ter Braak, E.W.T.M., Bianci, R. and Erkelens, D.W., Faster, shorter and more profound action of Lys (B28) Pro (B29) human insulin analogue compared to regular insulin irrespective of the injection site. *Diabetes*, 42 (1993) 207A.

Verhoef, J., van den Wildenberg, H.M. and van Nispen, J.W., [^{3}H]9-desglycinamide, 8-arginine vasopressin: metabolism and in-vivo fate. *J. Endocrinol.*, 110 (1986) 557–562.

Zhou, X.H., Overcoming enzymatic and absorption barriers to non-parenterally administered protein and peptide drugs. *J. Controlled Rel.*, 29 (1994) 239–252.