Transport of Proteolytic Enzymes Across Caco-2 Cell Monolayers

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Received March 30, 1998; accepted June 15, 1998

Purpose. To investigate the mechanisms by which proteolytic enzymes, such as trypsin, chymotrypsin, papain, and bromelain, are able to cross the intestinal mucosal barrier after oral administration to man.
Methods. Filter-grown Caco-2 cell monolayers were incubated with proteolytic enzymes and then the transepithelial electrical resistance (TEER) and the transport of the paracellular marker fluorescein were monitored. The effects of the enzymes on the cells were investigated by light microscopy and by biochemical assays. Transport of intact proteases across the cells was verified by monitoring the proteolytic activity and MALDI-TOF mass spectroscopic identification of undegraded trypsin.
Results. Depending on time, concentration, and side of exposure to Caco-2 cell monolayers, all proteases decreased the TEER and increased the transport of fluorescein. Some morphological and metabolic changes were observed. The effects were reversible, but until 24 hours after removal of the proteases. Under the conditions of this in-vitro model, approximately 10% of the apically applied dose reached the basolateral compartment as biologically active, non-degraded molecules.
Conclusions. Proteolytic enzymes were found to exert considerable effects on the barrier function of Caco-2 monolayers, facilitating the transport of normally non-absorbable compounds. This suggests the also reported, but so far unexplained, systemic absorption of proteolytic enzymes after oral administration in vivo may occur by self-enhanced paracellular transport.

KEY WORDS: absorption; proteases; trypsin; chymotrypsin; bromelain; papain; tight junctions.

INTRODUCTION

The possibility that proteolytic enzymes, such as trypsin, chymotrypsin, papain and bromelain, can be absorbed from the gastrointestinal tract has been reported repeatedly during the past four decades (1–5). These studies were related both to the absorption of exogenous, as well as to endogenous proteolytic enzymes. In particular, enteropancreatic circulation of digestive enzymes has been discussed as a conservation mechanism (6–8). However, the phenomenon of enzyme absorption from the digestive tract has given rise to much controversy, because it contradicts the common hypothesis that proteins can only be absorbed after hydrolysis to small peptides or amino acids. The question whether orally administered proteases can become bioavailable in the systemic circulation is of particular relevance in the context of so-called systemic enzyme therapy, where relatively high doses of one or several proteolytic enzymes are administered as oral, enteric-coated formulations (9). Although the quantification of oral bioavailability of proteases is complicated by a complex disposition of these compounds in plasma, recent clinical studies employing advanced biological and immunological methods have provided unequivocal evidence for some significant absorption of such undegraded proteins after oral administration to healthy humans (10–12). What still appears to be unknown however, and largely speculative, are the mechanisms of this unusual intestinal absorption phenomenon.

Our present knowledge (13) of observed transport routes for macromolecules includes persorption (14), the M-cell route (15), and receptor-mediated transcytosis (16). As another mechanism of macromolecule absorption, however, there remains the paracellular route. The paracellular pathway relies on the passive diffusion of molecules through the junctional membrane complexes between epithelial cells, known as so-called tight junctions. While the tight junctions have originally been perceived as a permanent and rigid barrier, our knowledge of their complex molecular structure and function has increased greatly (17). Most importantly, it has been recognized, that tight junctions are capable of responding to biological signals, and hence, their resistance/permeability can be modulated by various natural or synthetic compounds (18–20). Several pharmaceutical excipients including endogenous compounds and so-called penetration enhancers, which have been explored to enhance the usually poor intestinal absorption of peptide and protein drugs, are known to affect the tight junctional resistance of the intestinal epithelium (21–22). The role of extracellular proteases in the formation and modulation of epithelial tight junctions has been discussed by Polak-Charcon (23). Based on this information, we were interested to quantitatively compare the effect of various proteolytic enzymes on the tight junctions and barrier function of the intestinal epithelium.

MATERIALS AND METHODS

Materials

Trypsin (14.2 F.I.P.-U./mg protein), chymotrypsin (298 F.I.P.-U/mg protein), papain (1.39 F.I.P.-U./mg protein) and bromelain (4.9 F.I.P.-U./mg protein) were kindly provided by Mucos Pharma GmbH, Geretsried, Germany. Fluorescein-Na and FITC-casein and horseradish peroxidase (type VI A) and the LDH assay kit were obtained from Sigma Chemicals, Deisenhofen, Germany. The WST-1 reagent was purchased from Boehringer Mannheim, Germany. All other chemicals were of analytical grade and purchased from Merck, Darmstadt, Germany.

Cell Culture and Transport Experiments

Caco-2 cells (obtained from ATCC, Rockville, U.S.A.) were grown on 12 mm polycarbonate filters (Transwell cell culture inserts, mean pore diameter 0.45 μm from Costar, Cambridge, U.S.A.). Details of the protocol adhered to in our lab can be found elsewhere (24). Cells passages 85 to 100 were used 14 to 16 days after seeding, displaying a TEER between 700 and 800 Ω*cm².

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The transport experiments were performed by replacing the medium on both sides with Krebs-Ringer buffer (KRB) adjusted to pH 7.4. Once TEER had stabilized again—usually after 1 hour—the apical solution was replaced by the same buffer, containing the proteolytic enzymes and the hydrophilic transport marker fluorescein (0.01%). TEER was measured, and samples of 50 μl were taken at different time-points from the acceptor compartment and replaced by fresh buffer.

Reversibility studies of protease effects on TEER were performed by incubation of the Caco-2 monolayers at 37°C with proteolytic enzymes dissolved in serum-free culture medium at the previously found effective concentrations (Tab. I). After one hour of incubation the apical protease-containing solutions were replaced by fresh serum-supplemented cell culture medium and the incubation was continued. The TEER was measured at various time points during the following five days.

Light Microscopic Evaluation of Morphological Changes

Caco-2 monolayers were exposed to proteolytic enzymes at effective activities (Tab. I). At predetermined time points, the buffer was removed from both compartments and replaced by Sörensen buffer (pH 7.4) containing 2% glutaraldehyde. After 1 hour, the monolayers were post-fixed with 1% osmiumtetroxide (OsO_2) dissolved in Millonig buffer (pH 7.3), dehydrated with alcohol and placed into araldite (Polysciences, Warrington, U.S.A.). Semi-thin cuts were stained according to Richardson (25) and inspected by light (Carl Zeiss Axiophot, Oberkochen, Germany). The final magnification of the pictures shown in Fig. 6 is 1:1200.

Biochemical Evaluation of Metabolic Changes

WST-1 Assay

Confluent Caco-2 cell on filters were washed twice with KRB and finally exposed to different concentrations of proteolytic enzymes for a period of 30 minutes and 180 minutes, respectively. Triton-X-100 (0.08 up to 10 mg/ml) dissolved in KRB was used as positive control. The WST-1 reagent (diluted 1: 10 with KRB) was incubated for exactly 30 minutes at 37°C with the exposed monolayers and the absorbance was measured in a round bottom 96-well at λ = 450 nm.

Table I. Comparison of the Effective Enzyme Activities Using the Caco-2 Cell Model and the Enzyme Activity in Different Formulations Containing Proteolytic Enzymes Calculated According to the Biopharmaceutical Classification Index

Formulation \ Drug	Trypsin (mg/ml)	Chymotrypsin (mg/ml)	Papain (mg/ml)	Bromelain (mg/ml)
Effective enzyme activities[a]	2.5	5	1.25	0.5
Wobenzym® N[b]	0.24	0.01	1.18	0.46
Phlogenzym®[b]	0.48	---	---	0.92
Wobe-Mugos® E[b]	1.16	0.2	3.885	---
Mulsal® N[b]	0.36	---	1.785	0.69

[a] Effective enzyme concentrations are determined as a 50% reduction of TEER after 1 h of incubation with proteolytic enzyme in comparison to the initial values.

[b] The enzyme concentrations in commercial formulations were calculated according to the biopharmaceutical classification index (33) with a dissolution volume of 200 ml.

Lactate Dehydrogenase (LDH) Assay

Caco-2 monolayers on filters were incubated with the proteases (resp. Triton-X-100) analogously to the WST-1 assay, but for a period of 60 minutes. After that time 100 μl of the supernatant were diluted 1:6 with KRB and the absorbance was measured at λ = 490 nm with 50 μl of this dilution in a round bottom 96-well plate.

Other Analytical and Statistical Methods

Matrix Assisted Laser Desorption Ionisation—Time of Flight (MALDI-TOF) mass spectra of proteases were measured on a Micromass VG TofSpec linear mode mass spectrometer (Micromass, Idstein, Germany) with an N_2-laser (332 nm, 4ns pulse). After a typical transport experiment with trypsin the proteins were precipitated with trichloroacetic acid (TCA) and embedded at a concentration of 1 pmol/μl in a matrix of sinapinic acid (10 mg/ml) in acetonitrile/water 70:30 + 0.1% TFA.

FITC-Casein Based Protease Assay

The rate of transported proteolytic activity was determined enzymatically using FITC-casein (50–100 μg FITC/mg protein) as a substrate in a solution of 0.5% FITC-casein dissolved in water. At a temperature of 4°C a mixture of 25 μl Tris-buffer (50 mM, pH 8.0 (trypsin and chymotrypsin) and pH 6.0 (papain and bromelain)) and 25 μl of 0.5% FITC-casein solution were incubated for 1h. As a control, 10 μl Krebs-Ringer buffer or enzyme standards from 2.5 μg/ml down to 0.154 ng/ml were used. 120 μl of 5% TCA were added to precipitate during 3 hours at 4°C the non-digested FITC-casein. After centrifugation (15 min/500 g), the fluorescence of a mixture of 40 μl of the supernatant and 160 μl of a 500 mM Tris-buffer (pH 7.8) was determined using a microplate fluorescence reader with 485/20 nm excitation and 530/25 nm emission.

Horseradish Peroxidase (HRP) Assay

A solution of 20 mM 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) dissolved in phosphate buffer (10 mM Na_2HPO_4 and 60 mM KH_2PO_4, pH 6.0) was freshly prepared and stored at 4°C. 200 μl of the sample to be assessed for HRP activity, 200 μl KRB or 200 μl HRP standard solution (from 0.001 up to 1 mg/ml HRP) were mixed with 20 μl of the ABTS solution and 20 μl of a freshly prepared 10 mM H_2O_2 solution. Absorption was measured after 1 and after 6 minutes in a microtiter plate UV/VIS reader at 405 nm.

Statistics

Unless marked otherwise, the data shown in figures and tables represents the mean ± standard deviation of three experiments with different cell passages, each performed in 3–4 repetitions. The level of statistical significance was evaluated using Student's t-test (Statgraphics Plus V6.0, STSC Inc., Rockville, Maryland, USA).

RESULTS

Time and Concentration Dependent Effects of Proteolytic Enzymes on TEER and Fluorescein Transport

The proteolytic enzymes decreased TEER and increased the transport of the paracellular marker substrate fluorescein-Na, depending on the time and concentration of exposure. Figure 1 shows a typical example of the data involving trypsin. At low enzyme concentrations, the TEER was first transiently increased, but finally decreased during the course of the experiments. Higher enzyme concentrations decreased the TEER faster than lower enzyme concentrations, but after a certain period of time the TEER finally dropped to approximately 20% of the initial value, comparable to the baseline resistance of the transwell filter membrane. The TEER-reducing potential of the various proteases can be compared by determining the enzyme concentrations which reduced the TEER by 50% within one hour of incubation. These concentrations as determined under the conditions of this in-vitro model are referred to as effective enzyme activities (Tab. I).

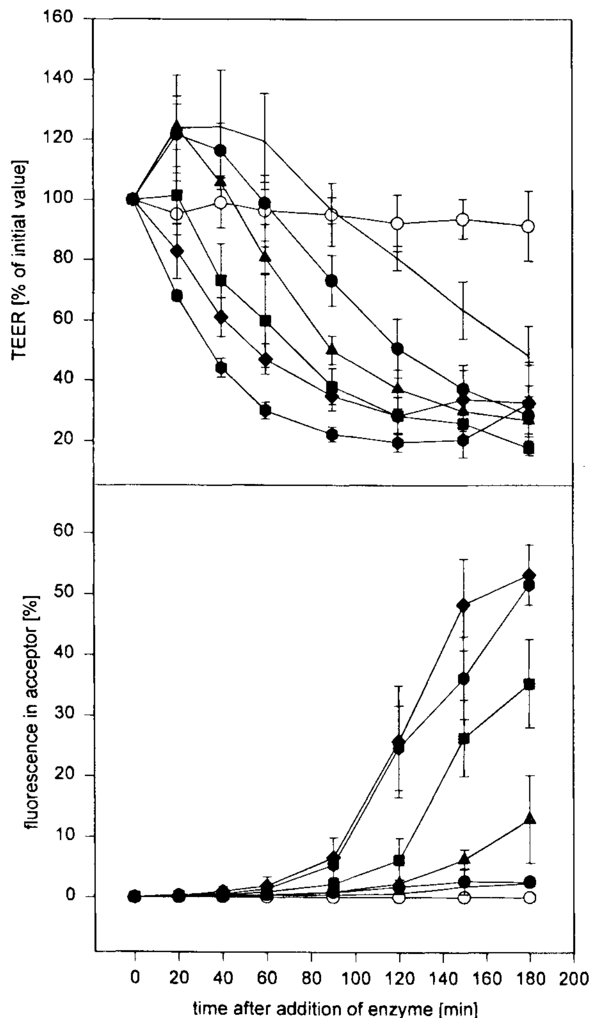

Fig. 1. Time course of TEER (upper panel) and fluorescein transport (lower panel) across Caco-2 cell monolayers under influence of trypsin at various concentrations. —○— control without trypsin; —— 2.22 F.I.P.-U./well trypsin in KRB, —•— 4.44 F.I.P.-U./well trypsin in KRB, —▲— 8.88 F.I.P.-U./well trypsin in KRB, —■— 17.75 F.I.P.-U./well trypsin in KRB, —♦— 35.5 F.I.P.-U./well trypsin in KRB, —⬣— 71 F.I.P.-U./well trypsin in KRB.

Transport of Proteolytic and Non-Proteolytic Enzymes Across Caco-2 Monolayers

By measuring the proteolytic activity in the basolateral receiver compartment at the end of a 3-hour experiment and comparing it to the apically applied dose, between 10 to 15% of the applied dose of all proteases were found to have crossed the epithelium (Fig. 2). In contrast, the transport of the non-proteolytic enzyme horseradish peroxidase (HRP), which is of similar size (approx. 40 kDa) was negligibly small (0.07%) and had no effect on TEER during the course of the experiment. In order to prove that the proteases detected in the basolateral compartment were intact and did not represent some still proteolytically active fragments, we compared the MALDI-TOF mass spectra of a sample taken from the apical donor compartment at the beginning of a transport experiment (A) with a sample taken 3 hours later (B) from the basolateral receiver compartment. As shown in Fig. 3, the two mass spectra for trypsin showed the same high-MW peaks at approximately 23 kD (z = 1) and 11.5 kD (z = 2), proving that both samples did contain substantial amounts of undegraded trypsin.

Reversibility of Protease Effects on Monolayer Permeability

The reversibility of enzyme effects was studied using culture medium instead of KRB to provide the cells with the FCS necessary for the restoration of the monolayer integrity. However, trypsin dissolved in the FCS containing medium, failed to decrease the TEER after 60 minutes, probably as a consequence of inactivation by antitrypsin and other serpins (serine proteinase inhibitors) usually present in serum (Fig.

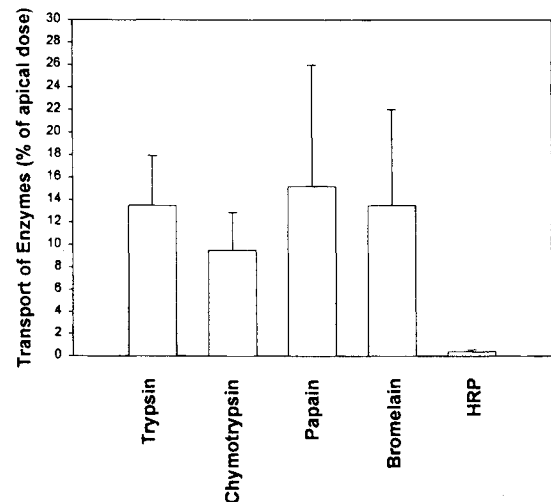

Fig. 2. Cumulative transport of proteolytic enzymes and horseradish peroxidase across confluent Caco-2 monolayers after 180 minutes of incubation. Enzymes were used of their effective concentrations respectively. 17.75 F.I.P.-U./well trypsin in KRB, 745 F.I.P.-U./well chymotrypsin in KRB, 0.87 F.I.P.-U./well papain in KRB, 1.23 F.I.P.-U./well bromelain in KRB, 1 mg/ml HRP in KRB.

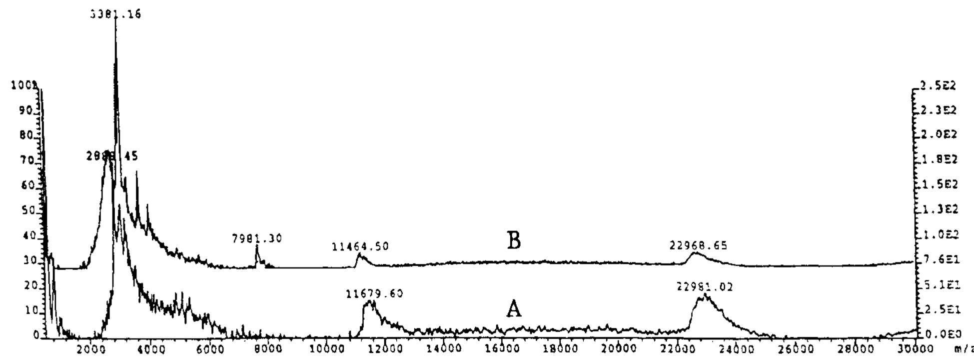

Fig. 3. MALDI-TOF mass spectra of trypsin: A: as added to the apical compartment at the beginning of a transport experiment, B: taken from the basolateral compartment at the end of a 3 hours transport experiment.

4). The other proteases effectively decreased the TEER, as experienced previously in KRB. The strongest TEER decreasing effect under these conditions was observed for papain, followed by bromelain, and, on account of corresponding antiproteinases in higher rates, only slight effects were observed for chymotrypsin. After removal of the proteases and continued incubation of the monolayers in the presence of serum-containing culture medium, the TEER did not recover immediately, but recovered within 5 days with values as high or higher than the initial values for trypsin, chymotrypsin (after 24 hours), bromelain (within 2 days), and papain.

pH- and Side-Dependence of Protease Effects

The pH-optimum of the plant proteolytic enzymes, bromelain and papain (pH 6.0), differs markedly from the optimum of animal proteases, trypsin and chymotrypsin (pH 8.0). The pH-dependence of enzyme effects was studied using KRB adjusted to pH 6.5, pH 7.4, and pH 8.0. But apart from a slightly increased initial TEER, (compared to the control) which was observed for the pH 8.0 buffer, the pH of the buffer had no significant effect on the TEER decreasing activity of the proteolytic enzymes (data not shown). The decrease of TEER for the Caco-2 cell monolayers 40 minutes after addition of proteolytic enzymes to either the apical or basolateral compartment is shown in Fig. 5. Apical addition of the serine proteases trypsin and chymotrypsin reduced the TEER only slightly, but the effects were much more pronounced when these enzymes were applied to the basolateral side. In contrast, the cysteine proteases bromelain and papain significantly decreased the TEER to approximately 50%, regardless of whether they were applied to the compartment apically or basolaterally. Comparing only their apical efficiency, the plant proteolytic enzymes appeared to be more effective in opening the tight junctions than the animal proteases, while the latter were more potent in opening tight junctions when they had access to the basolateral side of epithelial cells.

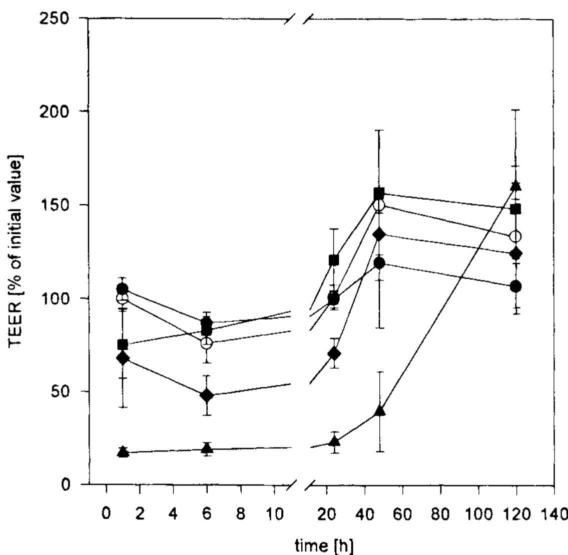

Fig. 4. Recovery of TEER 1 hour exposure to, and subsequent removal of proteolytic enzymes. —○— Control without proteolytic enzymes, —●— 17.75 F.I.P.-U./well trypsin, —■— 745 F.I.P.-U./well chymotrypsin, —▲— 0.87 F.I.P.-U./well papain, —⬢— 1.23 F.I.P.-U./well bromelain.

Biochemical and Morphological Alterations of Caco-2 Cell Monolayers Under Influence of Proteolytic Enzymes

WST-1 Assay

The cytotoxic effects of molecules can be determined by measuring the metabolism of tetrazolium salts in the respiratory

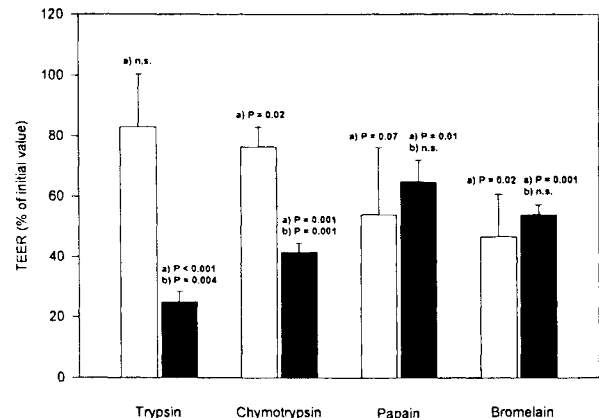

Fig. 5. Decrease of TEER of Caco-2 monolayers 40 minutes after addition of proteolytic enzymes (17.75 F.I.P.-U./well trypsin; 745 F.I.P.-U./well chymotrypsin; 0.87 F.I.P.-U./well papain; 1.23 F.I.P.-U./well bromelain) to the apical or serosal compartment (□ apical Addition, ■ basolateral Addition) Statistical significance: a) compared to the control, b) compared to the apical addition.

chain of mitochondria in viable cells. Using small concentrations of Triton-X-100 the activity of the STR increased, which is a known phenomenon for cytotoxic compounds acting at sub-toxic concentrations. A decreased STR activity was observed using 0.3 mg/ml, and complete inactivation of this mitochondrial enzyme was observed using ≥ 0.625 mg/ml Triton-X-100 (Tab. II). Incubation with proteolytic enzymes for a period of 30 minutes caused an increase of STR activity for low papain and bromelain concentrations. Larger amounts of these enzymes slightly decreased the STR activity, indicating cell damage. Trypsin did not show any STR-decreasing effects, and chymotrypsin decreased the STR activity only with higher enzyme concentrations. Extending the incubation time to 180 minutes yielded less pronounced metabolic changes than after 30 minutes for all enzymes.

LDH-Assay

Lactate dehydrogenase (LDH) is released from cells when their membrane is damaged and can be detected in the extracellular medium. LDH activity in the supernatant linearly increased with Triton-X-100 concentrations after 30 minutes of incubation. After 60 minutes of incubation with the proteolytic enzymes, trypsin and bromelain showed effects comparable to 10 mg/ml of Triton-X-100. Papain and chymotrypsin appeared to be less cytotoxic in this assay, but still showed a significant deviation from the negative control (Tab. II).

Table II. Effect of Triton-X-100 and Proteolytic Enzymes on the Activity of Lactate-Dehydrogenase (LDH) and of Succinate-Tetrazolium-Reductase (STR) (n = 4)

Substance	Concentration	LDH-assay absorbance	WST-assay absorbance
Control		1.254 ± 0.03	0.508 ± 0.08
Triton-X-100	0.3 mg/ml		0.133 ± 0.05
	0.625 mg/ml		−0.016 ± 0.02
	10 mg/ml	0.320 ± 0.08	
Trypsin	155 μg/ml (30 min)		0.519 ± 0.02
	2.5 mg/ml (30 min)		0.536 ± 0.05
	2.5 mg/ml (60 min)	0.432 ± 0.21	
	2.5 mg/ml (180 min)		0.536 ± 0.10
Chymotrypsin	312 μg/ml (30 min)		0.546 ± 0.02
	5 mg/ml (30 min)		0.366 ± 0.05
	5 mg/ml (60 min)	0.947 ± 0.37	
	5 mg/ml (180 min)		0.416 ± 0.10
Papain	71.9 μg/ml (30 min)		0.621 ± 0.02
	1.25 mg/ml (30 min)		0.415 ± 0.01
	1.25 mg/ml (60 min)	0.761 ± 0.09	
	1.25 mg/ml (180 min)		0.565 ± 0.02
Bromelain	32.65 μg/ml (30 min)		0.605 ± 0.05
	0.5 mg/ml (30 min)		0.542 ± 0.02
	0.5 mg/ml (60 min)	0.393 ± 0.13	
	0.5 mg/ml (180 min)		0.592 ± 0.10

Note: Caco-2 cells were incubated with proteolytic enzymes, or Triton X-100, for a period of 60 minutes (LDH-assay) and 30 or 180 min (WST-assay). *LDH-assay*: The supernatant was taken and the LDH content was measured. High absorbance is measured in viable cells, while low absorbances indicate damage of the cell membrane. *WST-assay*: The enzymes were removed and replaced by the tetrazolium salt WST-1. After 30 and 180 minutes of incubation the absorbance was measured. High absorbance is measured in viable cells, while low absorbances indicate cell damage.

Light Microscopical Evaluation of Morphological Alterations

The control experiment shows a continuous cell monolayer, strictly attached to the polycarbonate filter membrane. In comparison with the control experiment, trypsin on the left-side of Fig. 6 caused the normally elongated nuclei at the basolateral side to become more round and a migration of the nuclei to the center of the cells. These effects were observed first after 40 minutes and were still more pronounced after prolonged incubation time periods. After 150 minutes of trypsin incubation, the cells assumed a round shape and partially detached from the polycarbonate filter. This detachment was almost complete after 3 hours. Compared to trypsin treatment, the rounding and migration of the nuclei was observed already somewhat earlier under the influence of papain in the left column. Although papain is apparently faster in loosening the contacts between the tight junctions of neighbouring cells, the Caco-2 monolayer was never completely detached from the filter substrate as opposed to the trypsin treatment. The observed morphological changes corroborate the differences between the serine proteases in contrast to the cysteine proteases.

DISCUSSION

A time and concentration dependent decrease of the TEER under the influence of proteolytic enzymes followed by an increase of marker transport was observed, indicating a loosening of the tightness of intercellular junctions, i. e. the opening of the paracellular route across the epithelium for normally non-absorbable compounds (21,22). Even the transport of FITC-dextrans with molecular weights in the range of 4.4 to 580 kDa was observed after addition of proteolytical enzymes to the donor compartment (13). Such facilitated transport of macromolecules was not observed using other intestinal permeability enhancing substrates such as sodium caprate (26). Sodium caprate has been reported to change the morphology of the cytoskeleton and alter the structure of the tight junctions, but to have no effects on the apical cell membrane (27). The absorption enhancing effect of sodium caprate is reportedly regulated by changes of the intracellular calcium concentrations (28). But the dramatic enhancement of penetration mediated by proteolytic enzymes, which also affects the transport of macromolecules, suggests that it probably involves other and/or additional mechanisms.

In contrast to absorption enhancement with EGTA as reported in the literature (29), the restoration of the membrane integrity after protease treatment was not possible within minutes. However, after a prolonged recuperation and in the presence of FCS, the initial TEER values were reached again. This indicates that the proteases influences the structure of the tight junctions in a way that fast restoration is impossible. The loss of epithelial barrier function is paralleled by a loss of cellular polarization and differentiation, but the cells are obviously not irreversibly damaged and are still capable to redifferentiate after a certain period of time. Possibly, the occurrence of such morphological changes is due to some peculiarities of the Caco-2 cell monolayer. In this *in-vitro* model there is no apical mucus gel layer, and the cells are not grown on a natural basal membrane. In a recent *in-vivo* study using the rat and the guinea pig as models (30), the absorption of trypsin was measured from the jejunum into blood by ELISA, but the authors did not observe any morphological changes or damages on the jejunal mucosa. Ultrastructural investigations demonstrated that

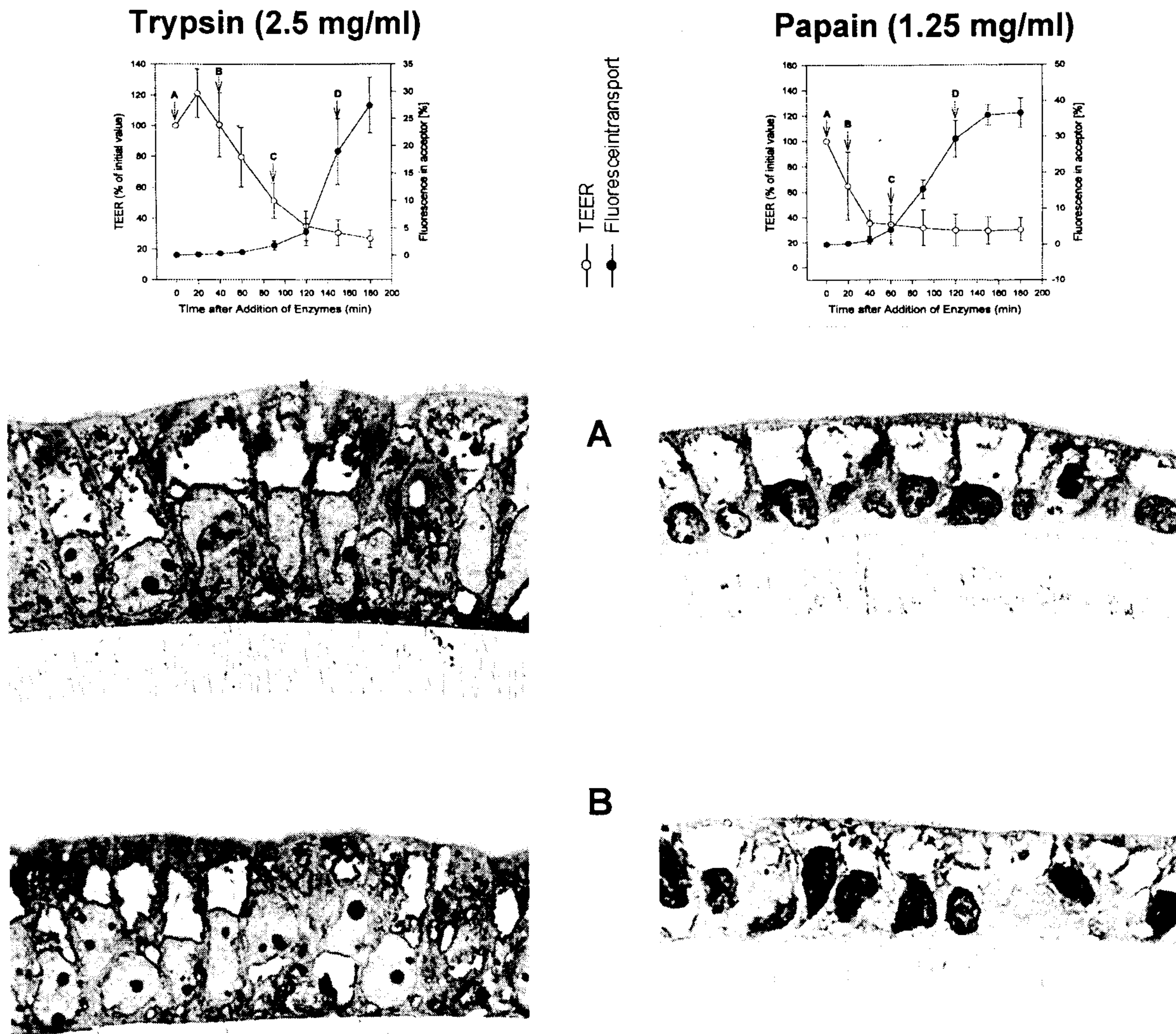

Fig. 6. Changes of TEER (open circles) and transport of fluorescein (black circles) across Caco-2 monolayers in comparison to morphological changes after addition of proteolytic enzymes (17.75 F.I.P.-U./well trypsin (left row) and 0.87 F.I.P.-U./well papain (right row)).

the absorption of trypsin into the blood was real, and not due to leaks through major destruction of areas of the mucosa. It could not be distinguished by the authors whether tight junctions between epithelial cells were involved.

Recognizing the essential role of proteins for the structural integrity and function of tight junctions, it is of interest that protease treatment has two different effects on cell monolayers. The proteases act as efficient inducers of tight junction formation over a very wide range of concentrations (23,31) for a small incubation time, but after a rigorous proteolytic treatment, the cells are unable to restore the tight junction assembly (23) unless they were allowed to recover for a relatively long period of time depending on the extent of proteolytic treatment. Moreover, restoration of tight junctions was prevented when cellular protein synthesis was inhibited (e.g. by adding cycloheximide) (23,32). This data is in some agreement with the changes of cellular metabolism as revealed by the biochemical assays in this study. While the LDH-assay indicates some leakage of the plasma membrane through proteases, the results of the WST-1 assay point in another direction: after either short incubation at low enzyme concentrations, or after a long incubation at higher enzyme concentration, the activity of the succinate-tetrazolium-reductase is increased compared to control cells. The light microscopy studies on the cell monolayers suggest that under the influence of proteases, the proteins involved are obviously affected in the structure of polarity which induces tight junctions and the cell shape changes from the differentiated columnar to the non-differentiated rounded shape.

Compared to the usually minimal transport of proteins and other macromolecules across tight epithelia, the transport of the proteases trypsin, chymotrypsin, papain, and bromelain observed with the Caco-2 cell model is very high. Whereas, the transport rates for the non-proteolytic enzyme horseradish peroxidase did not exceed 1 percent of the apically added dose and the transport rates for the proteases were about 10 percent. These exceptionally high values, as observed for the proteolytic

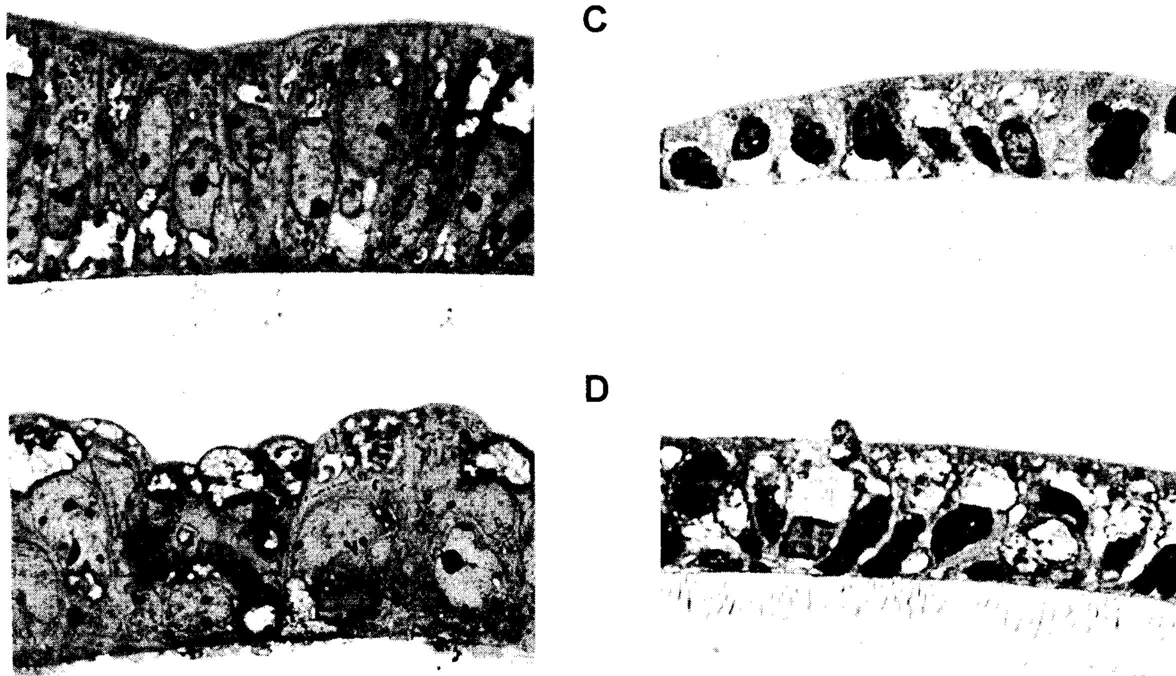

Fig. 6. Continued.

enzymes studied here, are explained by a mechanism of self-enhanced paracellular transport. The MALDI-TOF mass spectra of trypsin added to the apical and transported to the basolateral side, unambiguously proved the transport of some intact and non-degraded enzyme across the Caco-2 cell monolayer. Notably, the transport into the blood plasma of undegraded bromelain and trypsin has also been reported after oral administration to healthy humans (10–12).

Addition of the proteases to the basolateral side of the monolayers also resulted in a decrease of the TEER and an increase of fluorescein transport. Thus, proteases are active on the tight junctions from both sides of the cell monolayer. However, the effect of the two animal serine proteases trypsin and chymotrypsin on the TEER was very steep when the enzymes had access to the basolateral sides, but much less pronounced when their access was restricted to the apical side of the tight junctional complexes. In contrast, the two plant thiol proteases, papain and bromelain, acted independently in regards to the site of administration. This suggests that there are some differences in the mechanisms of action between these two classes of proteolytic enzymes, which remain to be further elucidated in more detail. Nevertheless, it may be speculated that in combinations of apically applied thiol and serine proteases, the first group of enzymes facilitates the access of the latter to the more sensitive basolateral side of the tight junctions, which explains the observed synergism of such enzymes in combinations.

By using the approach of Amidon et al. (33) for the so-called biopharmaceutical classification index, the enzyme activities of commercially available oral formulations (13) can be converted in concentrations by assuming a dissolution volume of 200 ml. The calculated data for four different formulations are shown in Table I. At least by an order of magnitude, these data reflecting the situation in-vivo are comparable with the effective enzyme concentrations found in the Caco-2 cell model. Moreover, most of the commercial preparations contain combinations of several enzymes, which were found to have synergistic effects. In some of the clinical studies up to eight tablets have been administered simultaneously.

In conclusion, proteolytic enzymes were found to have dramatic effects on the permeability of the intestinal epithelium, which facilitated the transport of normally non-absorbable compounds. Our data, obtained with the Caco-2 *in-vitro* model, suggests the transport of orally administered enzymes *in-vivo* across the gut wall may occur by self-enhanced paracellular transport.

ACKNOWLEDGMENTS

This work was supported by grants from Mucos Pharma GmbH, Geretsried, Germany. The MALDI-TOF analysis of trypsin samples were performed by Mrs. H. Brill, Department of Organic Chemistry (Prof. J. Engels), Johann-Wolfgang Goethe University, Frankfurt/M., Germany.

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