Transport of Proteolytic Enzymes Across Caco-2 Cell Monolayers

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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 spectrometric 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 transepithyosis (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-Chacon (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. 1). 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. 1). 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% osmium tetroxide (OsO₄) 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.

**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 N₂-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 Na₂HPO₄ and 60 mM KH₂PO₄, 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 H₂O₂ 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).

<table>
<thead>
<tr>
<th>Drug</th>
<th>Trypsin (mg/ml)</th>
<th>Chymotrypsin (mg/ml)</th>
<th>Papain (mg/ml)</th>
<th>Bromelain (mg/ml)</th>
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</thead>
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<tr>
<td>Effective enzyme activities&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.5</td>
<td>5</td>
<td>1.25</td>
<td>0.5</td>
</tr>
<tr>
<td>Wobenzym® N&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>0.01</td>
<td>1.18</td>
<td>0.46</td>
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<tr>
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<td>---</td>
<td>---</td>
<td>0.92</td>
</tr>
<tr>
<td>Wobe-Mugo® E&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>0.2</td>
<td>3.885</td>
<td>---</td>
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<tr>
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<td>0.36</td>
<td>---</td>
<td>1.785</td>
<td>0.69</td>
</tr>
</tbody>
</table>

<sup>a</sup> Effective enzyme concentrations are determined as a 50% reduction of TEER after 1 hour of incubation with proteolytic enzyme in comparison to the initial values.

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