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Improvement of the intestinal membrane permeability of low molecular weight heparin by complexation with stem bromelain

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Abstract

The aim of this study was to investigate the influence of the proteolytic enzyme bromelain on the permeation of heparin across the gastrointestinal epithelial barrier. Stability of the complex and effect of heparin on the enzymatic activity of bromelain was analysed photometrically by measuring bromelain enzymatic activity in complex with the heparin. *In vitro* permeation studies were performed with Caco-2 cell monolayer and rat small intestinal mucosa in Ussing-type chambers, respectively. Results revealed that enzymatic activity of bromelain remained uninfluenced by the immobilization of heparin on it. Transport studies across Caco-2 cell monolayer and rat small intestine showed that the permeation of heparin could be significantly increased in presence of bromelain. In the study with Caco-2 cells, the most effective molar ratio of bromelain to heparin was 2:1, leading to 6.7-fold improvement in uptake, whereas the molar ratio 1:1 showed the highest permeation enhancing effect in the study on intestinal mucosa. This study provides evidence that heparin and bromelain form stable complexes leading to a significantly improved uptake of heparin.

Keywords: Bromelain; Heparin; Permeation; Caco-2 cell monolayer

1. Introduction

Heparin is a water soluble, negatively charged polysaccharide which has been successfully used in the therapy of myocardial infarction, pulmonary embolism and deep vein thrombosis. The major disadvantage of heparin is its poor bioavailability when administered orally due to its inability to pass the gastrointestinal epithelial barrier. The epithelial cells lining of the gastrointestinal tract represent major barrier to absorption of many orally administered drugs (Jackson, 2005). For that reason heparin has been regularly dosed parenterally which is less convenient way of dosing for patients. For many years, researches have been attempting to develop an effective heparin formulation capable of passing the gastrointestinal barrier. Gastrointestinal barrier is, among others, represented by mucous gel layer covering epithelial cells and extracellular matrix. On the one hand, mucous gel layer comprises three-dimensional network of glycoproteins. On the other hand extracellular matrix comprises tight junctions, whose function is regulated by different transmembrane and intracellular proteins (Ho et al., 2000, 2004).

Recently, different compounds have been reported to enhance the intestinal permeation of heparin, including organic bases (Caramazza et al., 1991; DalPozzo et al., 1989), spermine and lysine salts (Morton et al., 1981) or [*N*-8(-2-hydroxybenzoyl)amino]caprylate (SNAC) which led in combination with heparin to a significant increase of the intestinal absorption of heparin in rats (Leone-Bay et al., 1998). All these approaches are based on the improvement of the lipophilic properties of the heparin delivery system diminishing at the same time its hydrophilic properties and favoring transport through the lipid bilayer. Proteolytic enzymes are, in contrary, capable of degrading the extracellular matrix components (Boudjennah and Pagano, 1998) such as proteins of tight junctions. They also exhibit a strong mucolytic activity by cleaving within the amino acid sequence of mucus glycoproteins (Bernkop-Schnürch et al., 2004). In previous study by our research group bromelain could be identified as permeation enhancer for low molecular size compounds like sodium-fluoresce and fluoroisothiocyanate-dextran across rat small intestine (Guggi and Bernkop-Schnürch, 2005). Bromelain is a general term for enzymes derived from fruit, stem

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Fig. 1. Schematic presentation of the complexation of bromelain with heparin. The active site of the enzyme stays free for interactions with natural substrates.

and leaves of *Ananas comosus*. Its main components are cysteine proteases and smaller amounts of other enzymes such as amylase, acid phosphatase, peroxidase, and cellulase (Rowan, 1990). Within this study stem protease has been chosen, representing a basic glycoprotein with a molecular weight of 28 kDa.

The aim of the present study was to improve the membrane permeability properties of negatively charged heparin by complexing it with the positively charged proteolytic enzyme bromelain, as illustrated in Fig. 1. Studies with heparin–bromelain complexes were performed via spectrophotometry. The effect of bromelain on the permeation of heparin was investigated with human intestinal epithelium in cell culture and with freshly excised rat small intestine.

2. Materials and methods

2.1. Materials

Low molecular weight heparin (LMWH, 105 IU/mg, average MW 4.125 kDa) was purchased from ICN Biomedicals; bromelain (EC 3.4.22.32; 3.14 units/mg protein), heparin-acrylic beads saline suspension, *N*α-CBZ-L-lysine *p*-nitrophenyl ester and HEPES [*N*-(2-hydroxyethyl)piperizine-*N*-(2-ethanesulfonic acid)] were purchased from Sigma, Vienna, Austria.

2.2. Bromelain–heparin binding studies

2.2.1. Photometric analysis

Bromelain–heparin binding studies are based on the decrease in bromelain concentration in solution due to the addition of heparin being immobilized to acrylic beads. First, absorption maxima of increasing concentrations of bromelain (0–0.27 mg/ml) in 50 mM acetate buffer pH 5.5 were determined using spectrophotometer (Beckman DU® 650). Thereafter, to each of these bromelain solutions, 940 mg of previously washed heparin-acrylic beads were added. The samples were incubated for 1 h at 37 °C in oscillating thermomixer (Comfort, Eppendorf) with 1100 rpm. After having removed the heparin acrylic beads, absorption maxima of supernatant bromelain in each sample were determined photometrically.

2.2.2. Influence of heparin on bromelain activity at different pHs

The influence of pH on bromelain activity after having been immobilized to heparin acrylic beads was determined by measuring the activity of bromelain in sodium acetate buffer of pH 5.5 and in artificial intestinal fluid (AIF) containing 2 mM bicarbonate, 139 mM chloride, 5 mM potassium, 140 mM sodium, 4 mM calcium and magnesium and being adjusted to pH 7.4. After having being washed with sodium acetate buffer or with AIF, 940 mg of heparin acrylic beads were incubated with 1.2 ml of bromelain solution containing 0.3 IU/ml bromelain in sodium acetate buffer or in AIF. After 30 min of incubation, 0.1 ml of heparin acrylic beads suspension containing 94 mg heparin acrylic beads was pipetted from the solution, washed with sodium acetate buffer or AIF, followed by determination of activity of bromelain being bound to heparin acrylic beads as described below.

2.2.3. Assay of bromelain activity

A quantitative assay for *p*-nitrophenol being released from *N*α-CBZ-L-lysine *p*-nitrophenyl ester by bromelain was performed as follows. Briefly, a mixture of 2.6 ml of 30 mM sodium acetate buffer pH 4.6 and 0.1 ml of suspension of bromelain immobilized on acrylic heparin beads was incubated at 25 °C for 10 min. After the addition of 0.1 ml of 50 mM *N*α-CBZ-L-lysine *p*-nitrophenyl ester, heparin acrylic beads were allowed to precipitate and an increase in absorption in supernatant at 340 nm was recorded for 5 min using spectrophotometer (Beckman, DU 600, Germany). Activity was calculated using following equation:

$$\text{Units/ml enzyme} = \frac{(\Delta A_{340\,\text{nm}}/\text{min test} - \Delta A_{340\,\text{nm}}/\text{blank}) \times 2.8 \times \text{df}}{6.32 \times 0.1}$$

where 2.8 is a volume in milliliter of assay, df a dilution factor, 6.32 the millimolar extinction coefficient of *p*-nitrophenol at 340 nm and 0.1 is the volume in milliliter of enzyme used.

2.2.4. Influence of different molar ratios of heparin to bromelain on bromelain enzymatic activity

In order to determine the change in activity after addition of different molar ratios of heparin to bromelain, heparin was added to the bromelain solution in the concentrations 2.8, 1.4 and 0.7 IU/ml, respectively, corresponding to the molar ratio heparin to bromelain 2:1; 1:1 and 1:2. Resulting bromelain activities were determined and calculated as described above using 0.1 ml of bromlain–heparin solution instead of bromelain–heparin acrylic beads suspension.

2.3. Diffusion studies

In order to evaluate the extent of heparin release from the complex with bromelain, diffusion experiments were performed using Franz diffusion cells displaying an acceptor chamber volume of 2 ml and a diffusion area of 1 cm^2. Further, 10 IU of heparin and 0.7 mg of bromelain, corresponding to the molar ratio of 1:1, were dissolved in 50 mM sodium acetate buffer pH 5.5. As a control heparin solution of final concentration of 10 IU/ml in 50 mM sodium acetate buffer pH 5.5 was used. The solutions were applied on a pre-hydrated dialysis tubing membrane (Sigma, MWCO 12 kDa). The lower chamber was filled with 2 ml of pure 50 mM sodium acetate buffer pH 5.5 used as

acceptor medium. Experiments were performed at room temperature over 8 h. At predetermined time points samples of 100 μl were withdrawn from acceptor chamber. Sink conditions were maintained throughout the study. Cumulative corrections were made for previously removed samples.

2.4. Permeation studies in vitro

2.4.1. Transport studies across Caco-2 monolayer

Caco-2 cells were kindly donated by Prof. Pfaller, Institute for Physiology and Balneology, Innsbruck. The cells of passage nos. 28–29 were seeded on 12 mm polycarbonate filter inserts (Transwell cell culture chambers, mean pore diameter 0.45 μm purchased from Costar, Cambridge, USA) and maintained at 37 °C in Dulbecco's modified Eagle medium supplemented with 20% fetal calf serum and streptomycin in an atmosphere of 5% CO_2 and 90% relative humidity. Cells were allowed to grow and differentiate to confluent monolayers for 21 days displaying a TEER between 800 and 900 Ω.

In order to determine an effect of bromelain onto apical to basolateral permeability of heparin, heparin and bromelain were applied to the apical side in molar ratios 1:2, 1:1 and 2:1 corresponding to bromelain activity of 2.25, 1.25 and 0.625 FIP/ml. Final activity of heparin on the apical side was 5 IU/ml. Bromelain and heparin were dissolved in medium containing 250 mM NaCl, 2.6 mM $MgSO_4$, 10 mM KCl, 40 mM glucose, 50 mM $NaHCO_3$ and 40 mM HEPES as a buffer, maintaining pH 7.4 during the whole experiment and incubated at 37 °C. As a control, heparin dissolved in medium without bromelain was used. At predetermined time points samples were withdrawn from the basolateral chamber. Sink conditions were maintained during the whole experiment. Cumulative corrections were made for previously removed samples. The quantity of permeated heparin was determined indirectly by measuring anti-Xa activity utilizing COAMATIC® LMWH (CoaChrom, Diagnostica, Vienna, Austria) test kit and detection by Fluostar Galaxy spectrometer (BMG Labtechnologies, Offenburg, Germany) at 405 nm.

2.4.2. Transepithelial electrical resistance measurements

The transepithelial electrical resistance (TEER) was measured to determine an effect of bromelain on monolayer integrity. A decrease in TEER indicates that passive permeability to ions has been increased. Reversibility of the effect of bromelain on TEER was measured within 5 days after replacing the permeation medium with serum containing culture medium.

2.4.3. Permeation studies on freshly excised mucosa

The studies were performed in Ussing-type chamber measuring 1 cm^3 (1 ml) volume in both donor- and acceptor-chambers and permeation area of 0.64 cm^2. As a permeation barrier, rat small intestine was used (duodenum). Immediately after sacrificing rat, its duodenum was excised and mounted in the Ussing-type chambers and displaced in incubation medium as described above. The pH of the medium was adjusted to pH 6.8.

In order to evaluate an influence of bromelain concentration on permeation of heparin, bromelain was added in three different molar ratios to heparin—2:1, 1:1 and 1:2. The heparin concentration was thereby constant, with 10 IU/ml. After 10–15 min of pre-incubation of small intestine with pure incubation medium, the incubation media in donor compartments were substituted by solutions containing 10 IU/ml heparin and bromelain in following concentrations: 0.35, 0.7 and 1.4 mg/ml. Samples of 100 μl were withdrawn from the acceptor compartment every 30 min over a period of 150 min. The removed quantities were replaced by fresh artificial intestinal fluid equilibrated at 37 °C. All experiments were performed at least five times in an atmosphere of 95% O_2 and 5% CO_2 at 37 °C. Anti-Xa activity of permeated heparin was determined as described above. Cumulative corrections were made for previously removed samples.

2.4.4. Calculation of apparent permeability coefficient

The apparent permeability coefficients (P_{app}) for different bromelain concentrations were calculated according to the following equation:

$$P_{app} = \frac{Q}{Act}$$

where P_{app} is the apparent permeability coefficient (cm/s), Q the total amount permeated within the incubation time (μg), A the diffusion area of the Ussing chamber/filter insert (cm^2), c the initial concentration of the heparin within the donor/apical compartment (μg/cm^3) and t is the total time of the experiment (s). Transport enhancement ratios (R) were calculated from P_{app} values using the following equation:

$$R = \frac{P_{app}(\text{sample})}{P_{app}(\text{control})}$$

2.5. Statistical data analysis

Statistical data analysis was performed using the Student's t-test with $p < 0.05$ as the minimal level of significance unless indicated otherwise.

3. Results

3.1. Binding studies

3.1.1. Photometric analysis

After being incubated with bromelain solution for 1 h, heparin acrylic beads were removed from the solution by centrifugation. The amount of bromelain bound to 940 mg of heparin-acrylic beads in sodium acetate buffer at pH 5.5 was plotted against the equilibrium concentration (Fig. 2). When bromelain concentration was below the binding capacity of the heparin acrylic beads a linear relationship was found. The average percentage of bromelain bound to heparin acrylic beads amounted 97.7 ± 1.2%.

3.1.2. Influence of heparin on bromelain activity at different pHs

An effect of heparin immobilized on acrylic beads on bromelain activity was determined photometrically by monitoring the enzyme catalysed hydrolysis of the substrate at 340 nm. The reaction rates of substrate hydrolysis in sodium acetate buffer

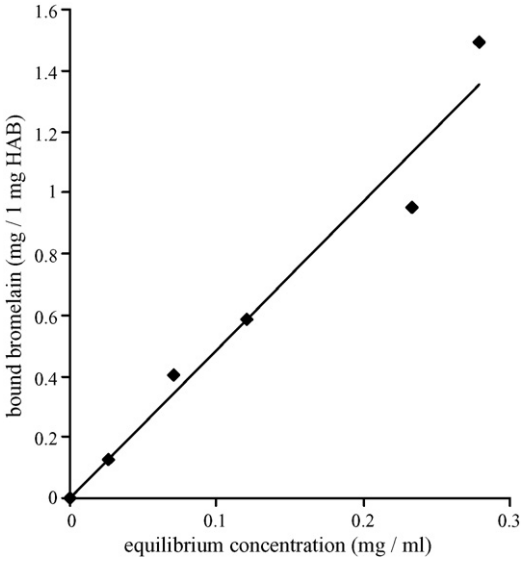

Fig. 2. Binding affinity of bromelain to heparin acrylic beads in 50 mM sodium acetate buffer at pH 5.5.

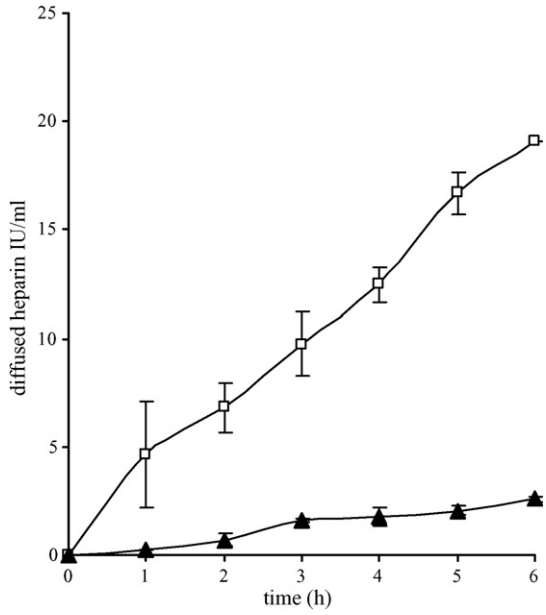

Fig. 3. Diffusion studies of heparin and bromelain complex (▲) across 12 kDa membrane in comparison to control containing only heparin (□).

and in AIF, respectively, did not differ significantly from the reaction rate of the free bromelain (data not shown) in both media. Results demonstrate that the enzymatic activity of bromelain immobilized on heparin acrylic beads remains uninfluenced by the pH values of the surrounding medium.

3.1.3. Influence of different molar ratios of heparin to bromelain on bromelain enzymatic activity

After the addition of different concentration of heparin to bromelain solution there were not any changes in its activity observed (data not shown), indicating that interactions with heparin do not affect the active site of the enzyme that is necessary for the proteolytic activity.

3.2. Diffusion studies

Since LMWH displays a molecular weight of 4.125 kDa, it is small enough to pass the 12 kDa MWCO membrane. The influence of bromelain on heparin diffusion through a membrane of MWCO 12 kDa is shown in Fig. 3. The rate of diffusion for heparin alone showed to be 3 ± 0.5-fold higher than for a complex. The reason for diminished permeation can be explained by the complex formation of which molecular weight is higher than a molecular weight of heparin molecule, in this case 4.125 kDa. Since diffusion studies were performed over 8 h providing equilibrium, alternations in the diffusion velocity of bromelain and heparin can be excluded.

3.3. Permeation studies

3.3.1. Effect of bromelain on TEER

The effect of bromelain on an integrity of Caco-2 monolayer was monitored as a function of time at three different concentrations of bromelain (Fig. 4). It was shown that when bromelain concentration was 0.7 FIP/well, TEER decreased slightly within first 60 min of the experiment and up to 50% of the initial TEER by the end of 150 min transport experiment. After removal of bromelain and heparin and replacing the transport medium with a serum-containing culture medium, TEER was completely recovered within 4.5 days. In comparison to these results concentration of 1.25 FIP/well showed final decrement of TEER up to 30%, being recovered after 4.5 days of incubation with serum-containing culture medium. At the bromelain concentration of 2.5 FIP, drastic decrement of TEER was observed, reaching the same value as the resistance of the Transwell filter membrane and indicating a complete disruption of the cell integrity. In this case, even after 5 days of incubation with culture medium TEER could not be completely recovered reaching only 40% of the initial value.

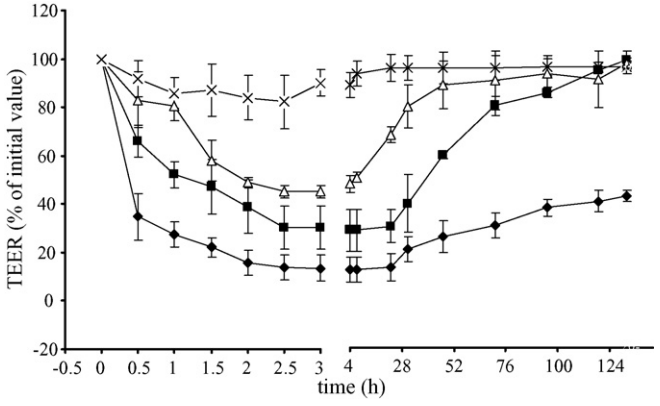

Fig. 4. Effect of different bromelain concentrations on transepithelial electrical resistance (TEER) of Caco-2 cell monolayer. Data represent the percentage of initial TEER values over the experimental time. 2.5 FIP/ml (♦); 1.25 FIP/ml (■); 0.625 FIP/ml (△) in HEPES buffer pH 7.4 compared to control (×). Reversibility experiment started as indicated after 150 min of incubation. Each point represents the mean ± S.D. of at least three experiments.

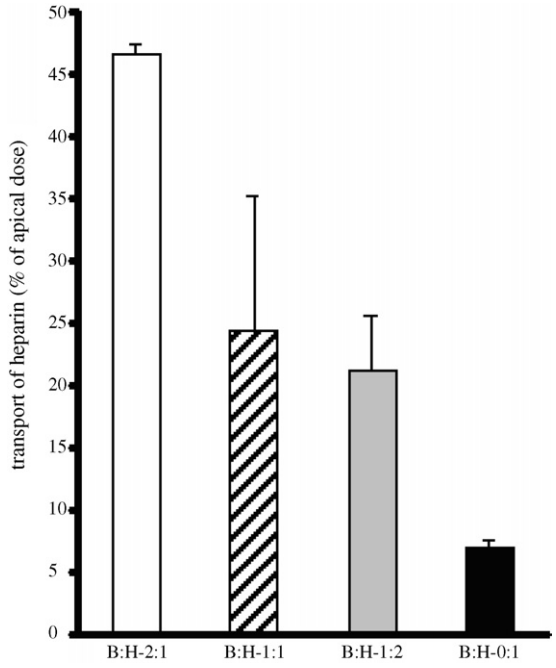

Fig. 5. Cumulative transport of heparin across Caco-2 monolayer from the apical to the basolateral side after 150 min of incubation in the presence of bromelain in molar ratios to heparin 2:1 (blank bars); 1:1 (hatched bars); 1:2 (grey bars) in comparison to control (black bars). Initial concentration of heparin in apical chamber was 5 IU/ml. Data represent the mean ± S.D. of at least five experiments.

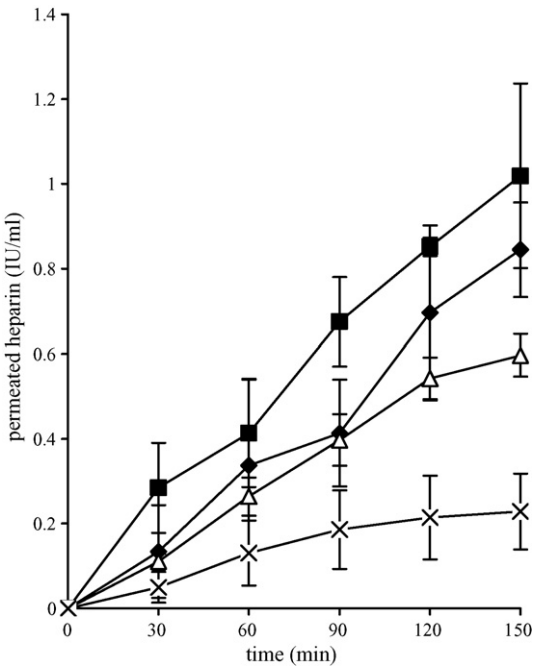

Fig. 6. Comparison of the permeation of heparin in presence of bromelain in increasing concentrations *in vitro*. Donor chambers contained 10 IU/ml heparin and bromelain in molar ratios bromelain to heparin 2:1 (♦), 1:1 (■) and 1:2 (△). Control contained 10 IU/ml heparin (×). Data represent the transport of heparin through intestinal mucosa of mean ± S.D. of at least five experiments.

3.3.2. Transport of heparin across Caco-2 monolayer

Caco-2 cell model represent plausible conditions for mimicking the transport from intestinal lumen to blood vessels. Within 150 min of incubation of cells with HEPES buffer containing bromelain and heparin, the transport of heparin across Caco-2 cell monolayer was significantly increased as shown in Fig. 5. Comparing heparin concentrations in the basolateral compartment at the end of the experiment it was found that the highest transport rate had been provided by the molar ratio bromelain to heparin 2:1, given in percentage of the initial concentration in apical chamber. In this case, 47% of the initial heparin concentration was transported across the cell line indicating a complete disruption of the cell monolayer supported by the measurement of TEER across cell monolayers as given above. Comparatively lower enhancement of the permeation was shown by bromelain in molar ratios to heparin 1:1 and 1:2. These setups showed a clear time- and concentration-dependent effect indicating preserved integrity of the cell layer. The addition of bromelain in molar ratio to heparin 1:1 induced transport of 24% of heparin from the apical chamber. In contrary, 21% of heparin was transported across the layer utilizing half of the bromelain molar concentration. At molar ratio bromelain to heparin 2:1 the apparent permeability coefficients P_{app} was increased by 6.7-fold compared to control (Table 1), whereas at ratios 1:1 and 1:2, P_{app} was increased by 3.5 and 3.0, respectively.

3.3.3. Permeation of heparin across rat small intestinal mucosa

The evaluation of permeation enhancing effect of bromelain from *A. comosus* was performed in Ussing-type chamber using rat small intestine as a permeation membrane. The permeation of heparin was tested in presence of three different bromelain concentrations. Results show that presence of bromelain exerts a significant enhancing effect regardless of its concentration. Permeated amount of heparin was plotted against time (Fig. 6). The highest increment in permeation rate showed the experimental setup with a molar ratio bromelain to heparin of 1:1. After 150 min, for the ratio 1:1, the average concentration of heparin found in acceptor chamber was 1.81 IU/ml, while the concentration found in acceptor chamber of the control was 0.228 IU/ml, resulting in enhancement ratio of 4.5. The apparent permeability coefficients and corresponding enhancement ratios are shown in

Table 1
Apparent permeability (P_{app}) for heparin transport across Caco-2 monolayers in presence of four different molar ratio of bromelain to heparin

Ratio bromelain:heparin	Apparent permeability coefficient, P_{app} ($\times 10^{-5}$ cm/s) (mean ± S.D., $n = 5$)	Enhancement ratio, P_{app} sample/P_{app} control
2:1	4.5 ± 0.09	6.7
1:1	2.4 ± 0.1	3.5
1:2	2.0 ± 0.4	3.0
Control	0.6 ± 0.07	1

Table 2
Apparent permeability (P_{app}) for heparin permeation through small rat intestine in presence of four different molar ratio of bromelain to heparin

Ratio bromelain:heparin	Apparent permeability coefficient, P_{app} ($\times 10^{-5}$ cm/s) (mean ± S.D., $n = 5$)	Enhancement ratio, P_{app} sample/P_{app} control
1:1	1.7 ± 0.3	4.5
2:1	1.4 ± 0.2	3.7
1:2	1.0 ± 0.08	2.6
Control	0.4 ± 0.15	1

Table 2. Although, molar ratios bromelain to heparin 2:1 and 1:2 provided a significantly lower enhancing effect than 1:1, they were nevertheless more effective than control. On the one hand, low permeation rate of heparin in the presence of two-fold higher concentration of bromelain might be explained by possible competition of bromelain with heparin–bromelain complex for the paracellular route of uptake. On the other hand, the lowest permeation enhancing effect of the molar ratio bromelain to heparin 1:2 can be reasoned with the insufficient quantity of bromelain that is necessary to facilitate the transport.

4. Discussion

The results of this study provide the proof of concept that permeation of heparin through the intestinal membrane can be enhanced by its complexation with proteolytic enzyme bromelain. According to Capila and Linhardt (2002) interactions of heparin with proteins are mainly based on ionic interactions. Clusters of positively charged basic amino acids on proteins form ion pairs with spatially defined negatively charged sulfo- and carboxyl groups on the heparin chain. Further on, hydrogen bonds were shown to play an important role in binding to heparin (Capila and Linhardt, 2002). The same authors reported about the existence of heparin-binding domains on the proteins containing the amino acids asparagine and glutamine, being capable of hydrogen bonding. They have also shown that the amino acid arginine binds very tightly to a sulfo group of heparin. Moreover, the primary structure of bromelain, investigated by Ritonja et al. (1989), reveals arginine, asparagine and glutamine as its constructive amino acids.

Data obtained by photometric analysis show that increasing the concentration of bromelain in the solution does not automatically increase the binding rate, indicating dissociation constant and interactions between bromelain and heparin. The intestinal epithelium shows a fairly stable surface pH compartment (microclimate pH) with a pH that is >6.25 and <6.75 (McEwan et al., 1988). Since the complex showed stability at pH 5.5 and 7.4, stability of the complex should also be provided on the intestinal membrane.

Low intestinal permeability of heparin has been the objective of many researches. Despite of its low molecular mass and its low charge heparin cannot permeate through the mucosal epithelia lining intestinal tract. Drugs can pass the gastrointestinal barrier by two parallel routes: transcellular and paracellular (Artursson and Magnusson, 1990). The absorption of hydrophilic drugs is limited to the paracellular pathway (Ma et al., 1991). Moreover, the permeation of hydrophilic compounds through paracellular pathway is restricted by tight junctions (Lee and Yamamoto, 1990). Bock et al. (1998) reported that proteolytic enzymes open the tight junctions between the cells, altering at the same time the structure of tight junctions proteins. Beside tight junctions, mucus layer covering epithelial cells represents another barrier. Mucolytic protein bromelain has shown to cleave between amino acids sequence of mucous protein (Bernkop-Schnürch et al., 2004).

Using Caco-2 cell line the complex barrier of the mucosal membrane can be reduced to just one cell line. Decrement of TEER under the influence of proteolytic enzymes indicates loosening of the tightness of intercellular junctions, i.e., opening of the paracellular route across the epithelium for normally non-absorbable compounds (Bock et al., 1998). However, recovery of TEER of the membrane after treatment with bromelain for 150 min has not occurred immediately after removing the bromelain from the apical side. The recuperation of membrane integrity and restoration of initial TEER values was possible after a prolonged time and in the presence of fetal calf serum. These results suggest that bromelain influences the structure of the tight junctions leading to the loss of epithelial barrier function as a result of the loss of cellular polarization and differentiation (Bock et al., 1998). The mechanism of action of proteolytic enzyme has still not been completely understood. Nevertheless, it is known that proteolytic enzymes exert two different effects on tight junctions. On the one hand, they can act as efficient inducers of tight junction formation and on the other hand, can inhibit the restorations of tight junctions when used in high concentrations over a long period of time (Polak-Charcon, 1992). Although Caco-2 cell layer is morphologically and biochemically similar to the normal intestinal cell layer, it lacks factors like mucus and other cell population. This might be the reason for incoherence of the results of permeation studies performed on small intestine with results of experiments performed on Caco-2 cell line. The answer is partially to be found in the histological differences between small rat intestine and Caco-2 cell monolayer. Small intestine displays more complicated structure that obviously influences the permeation enhancing potential of the bromelain. According to our working hypothesis, bromelain complexated with heparin based on ionic interactions should provide an enhanced permeation of heparin across the intestinal membranes. Permeation studies on both Caco-2 monolayer and freshly excised mucosa were performed with heparin in final concentration of 10 IU/ml. Results of this study however, revealed that on the Caco-2 monolayer the heparin/bromelain complex leads to such an extensive degradation of tight junctions that cells get already separated from each other (data not shown). Consequently, the concentration of heparin and in parallel the concentration of bromelain were lowered

to 5 IU/ml in studies on the Caco-2 monolayer, guaranteeing the cohesiveness of the tissue.

Previous studies have shown that drug delivery system based on soybean phosphatidylcholine and medium chain monoacylglycerol induced an approximately 10-fold increase in the transport rate of LMWH across Caco-2 cell layer (Lohikangas et al., 1994). Further on, sodium caprate as paracellular permeation enhancer increased transport of mannitol by 15-fold. In both cases, the hydrophilic substance was complexated with lipids. In contrast to these lipids of which safety remains questionable, bromelain is known to be a toxicologically harmless substance since it is approved by the Food and Drug Administration as a nutritional supplement for various diseases. Moreover, bromelain is used as anti-inflammatory agent and for treatment of edema. It favors fibrinolysis, prolongation of blood clotting time and prothrombin time, as well as inhibition of thrombocytes aggregation. These additional effects of bromelain could intensify the action of heparin, leading to a very potent combination.

5. Conclusion

Within this study, it was shown that heparin complexates with bromelain without influencing its enzymatic activity of bromelain. The stability of the complex at different pH levels was confirmed. Further on, it was demonstrated that bromelain increases the paracellular transport of heparin across both Caco-2 monolayer and rat small intestinal mucosa. Since bromelain exerts a mild and reversible effect on tight junctions, using bromelain as permeation enhancer could represent a promising novel strategy for mucosal delivery of heparin in future.

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