

Bromelain Proteases Reduce Human Platelet Aggregation *in Vitro*, Adhesion to Bovine Endothelial Cells and Thrombus Formation in Rat Vessels *in Vivo*

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Abstract. The thiol protease, bromelain, an extract from pine apple stem, was suggested to have antithrombotic and anticoagulant activities *in vivo*. We studied the effects of bromelain on cell size distribution of isolated human platelets *in vitro* by Coulter Counter measurements. Preincubation of platelets with bromelain (10 µg/mL) completely prevented the thrombin (0.2 U/mL) induced platelet aggregation. Papain was less active in preventing platelet aggregation. *In vitro*, bromelain (0.1 µg/mL) reduced the adhesion of bound, thrombin stimulated, fluorescent labeled platelets to bovine aorta endothelial cells. In addition, preincubation of platelets with bromelain, prior to thrombin activation, reduced the platelet adhesion to the endothelial cells to the low binding value of unstimulated platelets. On the basis of mass concentrations, the proteases papain and trypsin were as effective as bromelain. Using a laser thrombosis model, the *in vivo* effects of orally and intravenously applied bromelain on thrombus formation in rat mesenteric vessels were studied. Bromelain, orally applied at 60 mg/kg body weight, inhibited the thrombus formation in a time dependent manner, the maximum being after 2 hours in 11% of arterioles and 6% of venules. Intravenous application at 30 mg/kg was slightly more active in reducing thrombus formation in arterioles (13%) and venules (5%), suggesting that orally applied bromelain is biologically active. These results may help to explain some of the clinical effects observed after bromelain treatment in patients with thrombosis and related diseases.

Bromelain, an extract from the stem of pineapple plant, is a mixture of various basic and acidic thiol proteases and inhibitors (1, 2). As a drug, bromelain has been used for the therapy of different diseases, including thrombosis, rheumatic arthritis, inflammatory diseases such as atherosclerosis, adjuvant cancer treatment and others (for review see ref. 3).

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After oral administration, the presence of biologically active bromelain in the plasma of individuals, was demonstrated (4). *In vitro* and *in vivo* crude bromelain, as well as some purified bromelain proteases, inhibited tumor cell proliferation, invasion through matrigel and metastasis in different experimental tumor models (5,6,7). Previously bromelain was known for its antiplatelet and anticoagulating properties. The antiplatelet activities were determined *ex vivo* after oral administration of bromelain in human individuals or experimental rat models. Bromelain tablets were investigated on volunteers with high platelet aggregating activity, including patients with strokes and myocardial infarctions (8). Bromelain reduced the platelet sensitivity to ADP induced aggregation *in vitro* (9). In a rat model, oral administration inhibited the collagen induced platelet aggregation by 60% (9). However, in these studies bromelain effects on platelet functions were studied *ex vivo/in vitro*, using optical aggregometry methods. Although an interference with the arachidonic cascade on platelet activation was suggested (9), the mechanisms of bromelain antiplatelet actions are poorly understood.

Consequently, we studied in "more detail, bromelain effects on functional parameters of platelet aggregation and platelet - endothelial cell interactions *in vitro*. Furthermore, we investigated the effects of orally applied bromelain on thrombus formation *in vivo* with a laser thrombosis model, avoiding *ex vivo/in vitro* investigations.

Materials and Methods

Reagents. Human thrombin, adenosine diphosphate (ADP), papain (7.1 U/mg), trypsin (13.5-16.5 U/mg) and bovine serum albumin (BSA) were obtained from Sigma (Deisenhofen, Germany). BCECF-AM (acetoxymethyl-ester of 2',6'-biscarboxyethyl-5,6-carboxyfluorescein from Molecular Probes, Eugene, USA), was dissolved in DMSO and kept at -20°C. Crude bromelain (0.3-0.4 U/mg) was a gift from Dr. J. Houck, Seattle, USA. L-Pyr-Phe-Leu-pNA (L-pyroglyutamyl-L-phenylalanyl-L-leucine-p-nitroamide) was from Bachem (Bubendorf, Switzerland). Krebs-Ringer bicarbonate buffer (KRF3) with 5.6 mM glucose and 1% BSA) was gassed with 5% CO₂/95% O₂.

Cell culture. Bovine aorta BKEz-7 endothelial cells were obtained from Dr. Melzig (Institut für Pharmazie, Humboldt-Universität Berlin,

Germany). Endothelial cell morphology and cell-specific marker profiles have been described (10). The cells were cultured in RPMI culture medium (Biochrom, Berlin) with 10% fetal calf serum (FCS), (Biochrom, Berlin) and 0.2% penicillin/streptomycin (Boehringer, Mannheim, Germany), splitted twice weekly and used for experiments only up to the 25th passage.

Isolation of human blood platelets. About 20 mL blood was obtained by a catheter from healthy donors of either sex between 23-47 years of age. Platelets were isolated by the method described by Sneddon *et al* (11). Blood was mixed 10:1 (v/v) with a solution of sodium citrate and glucose. The sample was centrifuged at 200 g for 15 minutes. The platelet rich plasma was mixed with an equal volume of citrate-glucose-solution and centrifuged at 1000 g for 10 minutes. The platelet pellet was resuspended in 2 mL of KRB-solution with 10 mM EDTA and centrifuged at 1000 g for 10 minutes and resuspended again in 2 mL KRB-solution. The platelet solution was stored at room temperature.

BCECF-AM labelling of platelets. 1 mL of platelet KRB-solution was incubated with 90 µl stock BCECF-AM solution (143 µg/mL) for 30 minutes at 37°C. After incubation, the platelets were centrifuged at 1000 g for 10 minutes, resuspended in 2 mL KRB-solution, centrifuged and washed 2 times. The pellet was resuspended in 0.5 mL KRB-solution.

Platelet adhesion to BKEz-7 endothelial cells. Platelet stock solution was diluted with KRB solution and adjusted to 5×10^8 cells/mL. The culture medium from confluent monolayers of endothelial cells in 96 well culture plates was aspirated and washed with 100 µL of KRB solution. The medium was again aspirated and replaced by 100 µL of KRB solution. 0.2 U/mL thrombin were added to 100 µL of the diluted platelet suspension and incubated for 15 minutes at 37°C, 5% CO₂ in humidified air. The platelet suspension was aspirated and the cell monolayers were gently washed two times with 200 µL KRB solution. After the last wash, 100 µL of lysis buffer (0.01 M Tris, 0.15 M NaCl, 1.6 mM MgCl₂, 0.2% Triton X-100, pH 7.3) were added and incubated for 15 min at room temperature. Fluorescence was measured in a fluorimeter Cytofluor 200. from Millipore, USA. Percent adhesion was calculated from the calibration curve of BCECF-labeled platelets.

Effects of proteases on platelets adhesion to endothelial cells. Bromelain, papain and trypsin effects were measured on endothelial cell-bound, thrombin (0.2 U/mL) stimulated platelets. After platelets binding to BKEz-7 cells, the KRB solution was aspirated and 100 µL of protease containing KRB solution was added and incubated for 10 minutes. Preincubation of platelets with proteases were performed by incubating the platelets (90 µL with 5×10^7 cells) with 10 µL of stock protease solutions to give final concentrations of 0.01 µg/mL and 0.1 µg/mL, for 10 minutes at room temperature. The protease was removed by centrifugation at 1000 g for 10 minutes, the platelets resuspended in 100 µL of KRB solution in the presence of 0.2 U/mL thrombin and further incubated at 37°C for 15 minutes. The plates were washed, the cells lysed and counted as described. BKEz-7 cells were treated with proteases in 100 µL KRB solution at final concentrations of 0.1 µg/mL and 0.5 µg/mL for 10 minutes at room temperature. After 2 washings with 100 µL KRB solution, thrombin activated platelets were added and counted.

Platelet aggregation. Platelet aggregation tests were performed between 2 hours and 4 hours after blood collection in a Coulter-Counter Multisizer (Coulter Electronics GmbH, Krefeld, Germany). 4×10^7 isolated platelets were incubated with and without of 0.2 U/mL thrombin, ADP (10 µg/mL) or collagen (0.5 µg/mL) for 2 minutes, transferred to 10 mL 0.9% NaCl solution and counted for 120 seconds using a 30 µm or 100 µm capillary, respectively. Preincubation of platelets with the proteases bromelain and papain was performed for 10 minutes at room temperature, the platelet solution was transferred to 10 mL saline solution, stimulated with 0.2 U/mL thrombin and measured as described.

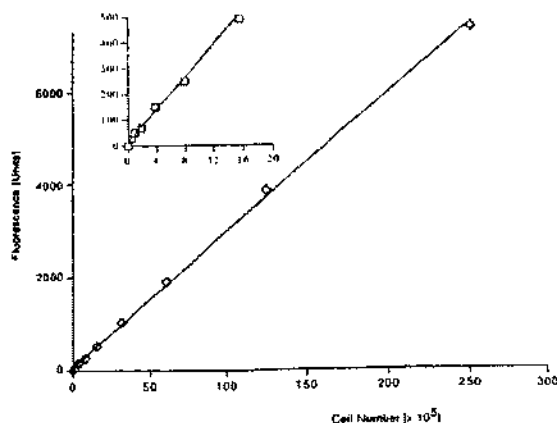


Figure 1. Calibration curve of BCECF-AM labeled human platelets. Fluorescence intensity of labeled platelets was directly related to the cell number between 3.5×10^4 - 2.5×10^7 cells. The coefficient of linear correlation regression was $r = 1.006$ for 12 data points. The insert shows an enlargement of the range 3.5×10^4 - 1.6×10^5 cells. The detection limit was 2.5×10^5 platelets. Points represent the mean of a typical experiment with three replicates of platelets isolated from one individual.

Each experiment was done in triplicates and was replicated with blood samples from at least 3 different individuals. The results shown are representative for one typical experiment. Only small differences were found between platelets isolated from different individuals.

Platelet thrombus formation in vivo. The laser microscope system and the method of laser induced thrombosis in rats were used as described (12). For drug administration, bromelain was dissolved in cold PBS at a final concentration of 25 mg/mL, centrifuged at 1000g for 5 minutes the clear supernatant was used. Male Whistar rats (120-150 g) were kept without food 2 hours prior to bromelain administration. The orally applied volume of different bromelain solutions was fixed to 0.2 mL, resulting in doses of 30, 60 and 120 mg/kg body weight. For i.v. application, about 100-500 µL of bromelain solution were injected into the rat tail vein corresponding to a dose of 30 mg/kg or 60 mg/kg body weight, respectively. Intraperitoneal application was performed with 120 mg/kg body weight. At different time intervals thereafter, i.e. 1 hour, 2 hours or 3 hours the rats were anesthetized and thrombus formation was induced by laser shots (12). For evaluation, the thrombus formation index (TFI) was determined. It represents the average shot number necessary to form a thrombus with at least 10 platelets (about the size of one leukocyte). When no thrombus was formed after 5 shots a number of 6 was assumed. Therefore, a TFI=6 means, that no thrombus could be observed at all. The inhibition of thrombus formation by bromelain was calculated using the formula:

$$\% \text{ inhibition} = \frac{\text{mTFI (bromelain treated)} - \text{mTFI (control)}}{\text{mTFI (control)}} \times 100$$

Results

Platelet labeling with BCECF-AM and adhesion to BKEz-7 endothelial cells. To quantify platelet adhesion to endothelial cells, the effector cells were labeled with the fluorescent dye BCECF-AM. Loading of the platelets with the probe did not

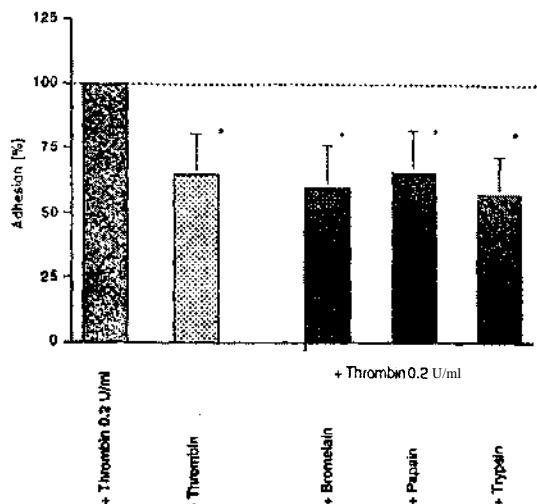


Figure 2. Bromelain effects on the adhesion of bound, thrombin stimulated platelets to bovine endothelial cells. Platelets were incubated in the absence or presence of 0.2 U/ml thrombin. Proteases were added, incubated for 10 min, cells were washed and lysed as described. The % adhesion value of thrombin stimulated platelets was expressed as 100% binding. Each bar represents the mean \pm SEM of seven independent experiments. One tailed student t test, * $p < 0.05$.

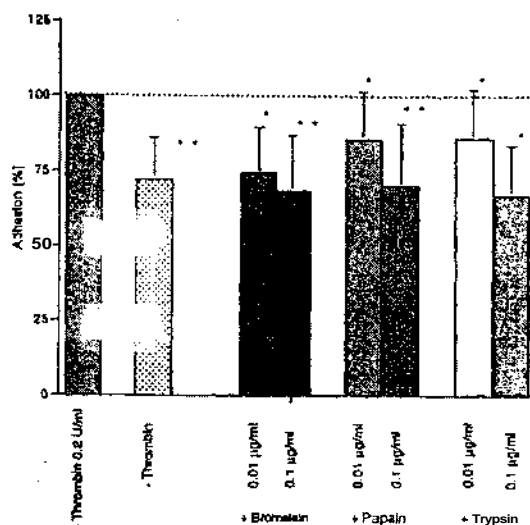


Figure 3. Effects of protease preincubation on platelet adhesion to BKEz-7 cells. Isolated platelets were preincubated with the proteases for 10 minutes, washed and incubated in the absence or presence of 0.2 U/ml thrombin. The % adhesion value of thrombin stimulated platelets, in the absence of proteases, is expressed as 100% binding. Each bar represents the mean \pm SEM of independent experiments. Student's t test * $p < 0.07$, ** $p < 0.05$.

alter the aggregation state. Furthermore, at the BCECF-sensitive wavelengths, platelets did not exhibit any significant autofluorescence. As shown in Figure 1, using a 12 point standard calibration curve, a linear relationship between fluorescence intensity and platelet number in the range between 3.5×10^4 - 2.5×10^7 cells ($r = 1.000$) was found. As few as 2.5×10^5 platelets could be counted. To measure the adhesion of BCECF-loaded platelets to confluent BKEz-7 cells endothelial cell monolayers, incubation of 5×10^7 platelets over a time period of 15 minutes was found to be optimal. Under these conditions, about 7.25, 4.5 and 7.5×10^5 platelets were bound (data of three independent experiments).

Bromelain effects of platelet adhesion to endothelial cells. The addition of 0.2 U/ml thrombin to platelets increased their adhesion to endothelial cells about 1.35 fold, relative to untreated platelets (Figure 2). Under these conditions, about 1.1×10^6 platelets were bound, which corresponded to 2.2% adhesion. The incubation of bound, thrombin stimulated platelets with bromelain (0.1 $\mu\text{g}/\text{mL}$) reduced the platelet binding to about 40 %, which nearly corresponded to platelet binding in the absence of thrombin. Trypsin and papain, applied at 0.1 $\mu\text{g}/\text{mL}$, were equally effective and reduced the platelet binding to 41% and 35%, respectively. Preincubation of platelets with bromelain, followed by incubation with thrombin, significantly reduced the binding of platelets to the BKEz-7 endothelial cells (Figure 3). At the lower protease concentration (0.01 $\mu\text{g}/\text{mL}$), bromelain was somewhat more

Table I. Effects of thrombin and bromelain on number and size of platelets and platelet aggregates.

	Number of platelets ¹⁾	Median diameter (um)	<90 % diameter ²⁾ (um)
- Thrombin	53,600	4.57	5.32
+ Thrombin (0.2 U/ml)	131,000	5.06	6.51
+ Thrombin (0.2 U/ml) + Bromelain (10 $\mu\text{g}/\text{ml}$)	41,600	4.61	5.64

1) diameter range of platelets and platelet aggregates: 4.23 - 7.32 μm

2) diameter of platelets and platelet aggregates comprising up to 90 % of the total population

effective in reducing platelet adhesion (25%), compared with papain and trypsin (19% and 13%, respectively). At the higher protease concentration of 0.1 $\mu\text{g}/\text{mL}$, bromelain, papain and trypsin were equally effective (31%, 30% and 33% reduction). Again, these values of platelet adhesion corresponded to the value of platelet binding to endothelial cells in the absence of thrombin. In a set of experiments, BKEz-7 cells were preincubated with bromelain at 0.1 $\mu\text{g}/\text{mL}$ and 0.5 $\mu\text{g}/\text{mL}$, and the adhesion values of unstimulated and thrombin stimulated platelets were determined. No visible morphological effects on the confluent endothelial cell monolayer could be detected. Under these conditions, only

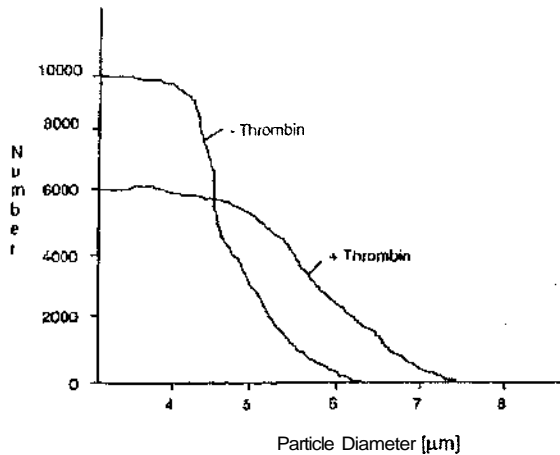


Figure 4. Platelet size distribution of unstimulated and 0.2 U/mL thrombin stimulated cells estimated by Coulter Counter Multizer measurements. Isolated platelets were counted in the range from 3.5 µm-8.7 µm using a 100 µm capillary.

protease treatment at 0.5 µg/mL moderately reduced the adhesion of thrombin stimulated platelets to endothelial cells (28%, 22% and 27% reduction for bromelain, papain and trypsin).

Platelet aggregation. On a cellular basis, the size distribution of isolated human platelets were determined by Coulter Counter measurements in the range between 0.6 µm - 18 µm. Using a 30 µm capillary, the mean diameter of platelets was 2.46 µm with a median value of 2.43 µm, suggesting a normal Gauß size distribution. The < 90% value of platelets was 2.94 µm. In order to measure the effect of thrombin on platelet aggregation, a 100 µm capillary with a working range between 2 - 60 µm was used. Under these conditions, the platelets diameter was 4.3 µm, with an < 90% value of 5.26 µm. The mean diameter of thrombin (0.2 U/mL) induced platelet aggregates slightly increased to 4.86 µm, with a < 90% value of 6.02 µm (Figure 4). In comparison, significantly lower effects on platelets aggregation were found with ADP (10 µg/mL) and collagen (0.5 µg/mL, data not shown). Preincubation of platelets with bromelain at 0.1, 1.0 and 10 µg/mL, followed by the addition of thrombin, reduced the platelet aggregation in a protease concentration dependent manner. In the range between 4.04 - 7.27 µm, bromelain (10 µg/mL) inhibited the thrombin induced platelet aggregation from a mean diameter of 5.06 µm in the presence of thrombin alone to 4.61 µm, which nearly corresponded to the value in the absence of thrombin (Table 1). More significantly, the number of platelets in the diameter range between 4.23 - 7.32 µm was reduced from 131 000 in the presence of thrombin to 41 600 after bromelain pretreatment, whereas, 53 600 platelets were measured for unstimulated cells. The < 90%

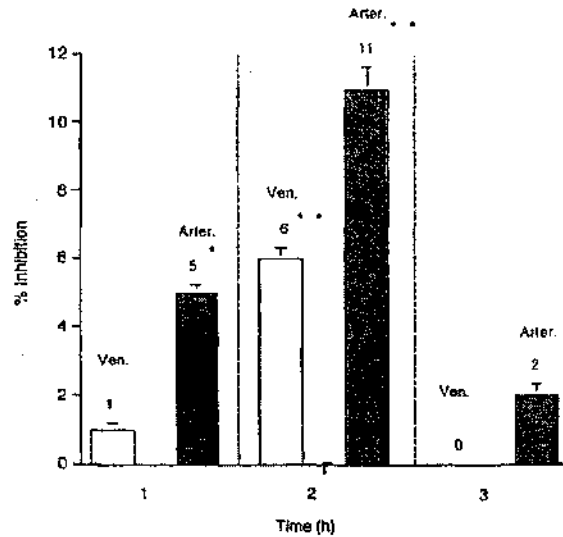


Figure 5. Time dependency of in vivo bromelain effects on the inhibition of laser beam induced thrombus formation in Wistar rat vessels. Bromelain was orally applied at a single dose of 60 mg/kg body weight by syringe with a blunt cannula. After time intervals of 1, 2 and 3 hours, thrombus formation was induced by laser shots and the number necessary for the formation of thrombi in mesenteric arterioles (blank column) and venoles (white column) counted. The percentage of inhibition was calculated by relating these counts to those of untreated animals. The bars represent the mean \pm SD of independent experiments. * $p < 0.07$, ** $p < 0.02$

value was 5.64 µm for bromelain pretreated platelets, 6.51 µm for thrombin stimulated platelets and 5.32 µm for untreated platelets. Compared with bromelain, papain at concentrations of 1.0 µg/mL and 10.0 µg/mL was less active in preventing platelet aggregation by thrombin (from 4.67 µm plus thrombin to 4.60 µm for 10 µg/mL papain pretreated platelets, data not shown).

In vivo effects of bromelain on thrombus formation in rat mesenteric vessels. The formation of thrombi in mesenteric arterioles and venoles of male Wistar rats was induced by a laser beam. The mean thrombus formation index (mTFI) in arterioles and venoles was 1.54 and 1.26, respectively. The antithrombotic effect of orally applied bromelain at a single dose of 60 mg/kg body weight was measured in a set of three independent experiments. The mTFI value of thrombus formation was determined 1, 2 and 3 hours after protease treatment. In general higher antithrombotic bromelain effects were found in arterioles than in venoles. Bromelain inhibited thrombus formation in a time dependent manner, with a maximum antithrombotic effect after 2 hours with 11% and 6% ($p < 0.02$) inhibition of thrombus formation corresponding to mTFI values of 2.03 in arterioles and 1.53 in venoles, respectively (Figure 5). Significantly lower ($p < 0.07$) bromelain effects were measured after 1 hour with 5%

inhibition in arterioles and 1% in venoles and after 3 hours with 2% inhibition in arterioles and no effects in venoles. The high oral bromelain dose of 120 mg/kg body weight did not further increase the antithrombotic protease effects. Compared with orally applied bromelain, intravenous application at 30 mg/kg body weight resulted in 13% (arterioles) and 8% (venoles) inhibition of thrombus formation. The intraperitoneal administration of bromelain (120 mg/kg body weight) is not suitable because toxic effects were observed.

Discussion

Here we demonstrated, that bromelain *in vitro* prevents thrombin induced human platelet aggregation and platelet adhesion to bovine endothelial cells. Furthermore, oral and intravenous bromelain application reduced thrombus formation in rat mesenteric vessels.

Bromelain effects on isolated platelet size distribution were studied by Coulter Counter measurements. The measured mean size of isolated, unstimulated platelets of 2.46 μm (30 μm capillary) agreed with data on the basis of different optical methods (17). Thrombin at optimal concentrations of 0.2 U/mL induced platelet aggregation to a mean diameter of 4.86 μm (100 μm capillary) ADP and collagen were less active. This small increase in thrombin induced platelet size suggested, that the system (isolated platelets in buffer) measured the formation of small rather than large platelet aggregates, as routinely produced in the aggregometer cuvette with platelet rich plasma (18). However, preincubation of platelets with bromelain, followed by thrombin, completely abolished the thrombin induced aggregation, as measured in the narrow size range between 4-7 μm . The measured platelet size distribution corresponded to that of unstimulated platelets. On these platelet aggregation parameters, bromelain was more active than papain. For comparison, using optical aggregometry (Born test) with platelet rich plasma and collagen, no bromelain effects at the used low concentrations were detectable. However, significant higher bromelain concentrations up to 1 mg/mL were reported to be effective (19).

To quantify bromelain effects on platelet-endothelial cell interactions, we established an *in vitro* adhesion assay using BCECF-labeled platelets and cultured bovine aorta endothelial cells as targets. So far, in adhesion studies, platelets were labeled with different radioactive chemicals, like ^3H -adenine (11). BCECF-labeling was used in one study for measuring cytosolic pH and Ca^{2+} fluxes in human platelets (16). We found that platelet labeling did not effect platelet size distribution and different platelet functions, like depolarization or degranulation (16). The advantage of platelet labeling with BCECF-AM, besides being a nonradioactive and a highly sensitive method (at least 2×10^5 cells could be counted) is that it correlates to platelet viability. In agreement with data on platelet binding to human umbilical vein endothelial cells (HUVEC), BKEz-7

endothelial cells express antiadhesive properties against unstimulated platelets, Thrombin stimulation of platelets increased their binding to values in the range of 1.5-5%. Taking into account the different experimental conditions used, thrombin stimulated platelet binding to HUVEC was found to be 6.4%, and to cultured bovine aorta endothelial cells 8.8% (11). Preincubation of platelets with bromelain, prior to thrombin stimulation, reduced platelet binding to the low value of unstimulated platelet binding to endothelial cells. Papain and trypsin were equally effective. Furthermore, bromelain reduced the number of endothelial cell bound, thrombin stimulated platelets, but was less effective on endothelial cells, working only at higher concentrations. This may suggest a bromelain effect on surface molecules like GMP-140 or CD31 on platelets, which have been discussed to be involved in platelet-endothelial cell interactions (13).

Using the laser thrombosis model, orally applied bromelain induced inhibition of thrombus formation in rat mesenteric cells, was measured directly *in vivo* thus avoiding *ex vivo/in vitro* studies. Our finding, that bromelain was more effective in arterioles than in venoles is consistent with previous data with different antithrombotic and anticoagulant compounds (12). The lower blood flow rate in venoles than in arterioles has been thought to be at least partially responsible for this observation (13). Bromelain showed a concentration and time dependent effect on the reduction of thrombus formation, with a maximum 2 hours after administration. This value was consistent with the plasma half life time for orally applied bromelain at doses of 10 - 50 mg/kg body weight in rabbits, estimated to be 1.5-2.5 hours (14). Whereas, *i.v.* administration of 30 mg/kg body weight bromelain was only slightly more effective in preventing thrombus formation in rat vessels. In a pharmacological study on humans it was demonstrated, that oral administration of high bromelain doses of 3 g/day resulted in a low bromelain plasma concentration of 5 ng/mL, with proteolytic activity (4). Comparable low bromelain plasma concentrations were found in experimental animal models (15). This suggested, that in our rat model, the applied *i.v.* dose generated saturating levels and that the orally applied bromelain was proteolytically active. Bromelain effects after oral administration were studied *ex vivo/in vitro* in human individuals and experimental rat models. Here, the sensitivity of *in vivo* bromelain effects were examined *in vitro* by ADP (8) and collagen (9) induced platelet aggregation. In the rat model bromelain, at a dose of 0.5 - 1.0 mg/kg body weight over 3 days reduced the collagen induced platelet aggregation by about 60%. However, these studies neglect blood fluidity parameters and the important platelet - endothelial cell interactions.

The nature of active bromelain components and the mechanism of the antiplatelet bromelain action are still poorly known. Crude bromelain was found to be a mixture of different basic and acidic proteases, which are members of the papain superfamily (20), and of other non-characterized

components (1,2). On the basis of the applied mass concentrations of 0.01-0.1 µg/mL, crude bromelain with a proteolytic activity of 0.4 U/mg was more active than papain (7.1 U/mg). Therefore, the combined action of different bromelain components, including different proteases, may be responsible for the high activity of crude bromelain. In earlier papers it was suggested, that the proteolytic activity may be related to the antiplatelet aggregation activity (21). In our functional studies we found, that bromelain preincubated platelets did not respond to the stimulatory action of thrombin. This suggests an interaction of bromelain with thrombin receptors on the cell surface of platelets or with signal transduction factors, is necessary for platelet activation (22). So far, bromelain induced structure modulations of different cell surface molecules, including adhesion molecules on peripheral blood lymphocytes and cultured tumor cells have been studied (5, 23, 24). The purified bromelain protease F9 selectively modulated the CD44 surface molecules of lymphocytes, thus reducing the CD44 mediated lymphocyte binding to human umbilical vein endothelial cells (25).

The results of the present paper may provide some further indications for the usefulness of bromelain in the management of different thrombotic diseases.

Acknowledgements

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