

ORIGINAL ARTICLE

The influence of bromelain on platelet count and platelet activity in vitro

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Abstract

Bromelain is a general name for a family of sulfhydryl-containing, proteolytic enzymes from the pineapple plant. The aim of the present study was to investigate the influence of bromelain on platelet count, platelet aggregation and platelet activity in vitro. Blood samples were taken from the antecubital vein of 10 healthy male non-smokers. Platelet count decreased after incubation with 2.5 and 5 mg bromelain/ml from 277 ± 17 platelets/nl before to 256 ± 21 and 247 ± 19 platelets/nl after the treatment. The ADP and TRAP-6 induced platelet aggregation led to a significant decrease after the incubation with 2.5 mg (ADP: $48.6 \pm 25.7\%$; TRAP-6: $49.6 \pm 28.9\%$) or 5 mg (ADP: $5.0 \pm 4.6\%$; TRAP-6: $9.0 \pm 4.9\%$) bromelain/ml in comparison to control (ADP: $81.4 \pm 5.0\%$; TRAP-6: $77.4 \pm 10.4\%$). The percentage of unstimulated CD62P positive platelets which were investigated by flow cytometry was minimally higher after incubation with 5 mg bromelain/ml ($0.57 \pm 0.48\%$ PC) in comparison to control ($0.22 \pm 0.11\%$ PC), but after TRAP-6 stimulation the incubation with 5 mg bromelain/ml led to a remarkable decrease in comparison to the untreated control (50.4 ± 20.2 to $0.9 \pm 0.8\%$ PC). The changes of CD62P (TRAP-stimulated) and the results of platelet aggregation after incubation with bromelain in vitro may demonstrate the potential of bromelain as a substance for platelet inhibition.

Keywords: CD62P, aggregometry, platelet activity, platelet reactivity, bromelain

Introduction

Bromelain is a mixture of sulfhydryl-containing, proteolytic enzymes from the stems and immature fruits of pineapple plants (*Ananas comosus*). Its primary component is a sulfhydryl proteolytic fraction. Bromelain also contains a peroxidase, acid phosphatase, several protease inhibitors, and organically bound calcium [1, 2]. Bromelain has been used as a medical agent for centuries [3, 4]. A recent literature search revealed over 1300 scientific papers, which demonstrate a wide variety of beneficial effects from bromelain. It has been shown to be effective in the reduction of inflammation and helpful in the reduction of swelling [3, 5–7]. Furthermore, the inhibition of platelet aggregation has been demonstrated in numerous studies [8, 9]. The first conclusive evidence that bromelain prevents aggregation of platelets was reported from Heinecke et al.

in 1972 [10]. Sano et al. [11] found that bromelain counteracted the aggregation of unwashed as well as washed platelets induced by ADP or thrombin, and Livio et al. [12] investigated the influence of bromelain on the ADP-induced platelet aggregation in 10 rats. They also observed that the ADP-induced platelet aggregation in bromelain-treated animals was markedly inhibited. However, in these studies the effects of bromelain on platelet function were analysed using only optical aggregometry methods. The present study investigates in more detail the influence of bromelain on changes in platelet function not only by aggregometry, but also by flow cytometry.

The aim of this study was to answer the following two questions: First, does bromelain influence platelet count and mean platelet volume (MPV)? Secondly, does bromelain lead to a modification of receptors on the platelet surface, e.g., to a decrease of

activity receptors (CD62P) after stimulation with TRAP-6?

Materials and methods

Subjects

Blood was collected from 10 healthy male non-smokers, whose average age was 30 ± 10 (mean \pm SD) years. The mean weight was 78 ± 9 kg and the mean height was 179 ± 7 cm. The patients involved in this study had no clinical symptoms, manifestations or history of cardio- or peripheral vascular disease. The volunteers assured that they did not take any kind of drugs for at least 4 weeks before the test.

Written informed consent was obtained from each subject, prior to the start of the study. The procedures used in the study were approved by the Ethics Committee of the Faculty of Medicine of the Friedrich-Schiller-University Jena.

Reagents

Bromelain was provided from Ursapharm (Saarbrücken, Germany). It obtains an activity from 6.99 F.I.P./mg.

Analytical methods

Blood sampling and preparation of platelets analysis. Blood samples were taken by a venepuncture with a 20-gauge needle (Sarstedt, Nümbrecht, Germany) from an antecubital vein under controlled venous stasis (<30 s) of 40 Torr. All venepunctures were taken from the subjects in reclined position. Polypropylen vacutainers (Sarstedt) were used for collecting the blood samples. In the first vacutainer, 2.6 ml blood were added to 0.29 ml EDTA. This blood was used for the assessment of complete blood count (Act-Diff, Coulter Electronics, Krefeld, Germany). The following vacutainers for the measurements of platelet count, of mean platelet volume, the aggregometry and flow cytometry contained sodium citrate in a ratio of 1:9. To prepare platelet-rich plasma (PRP), anticoagulated blood was centrifuged with $100 \times g$ for 10 min at 22°C . After this the PRP was carefully transferred into a polystyrole plastic tube (Sarstedt). To obtain platelet-poor plasma (PPP), the remaining specimen was again centrifuged for another 15 min with $2000 \times g$ at 22°C .

All tests were performed with a bromelain-treated sample (in a respective concentration) and a control sample in one setting.

Analysis of platelet count and mean platelet volume (MPV). Platelet count and the mean platelet volume were measured in adjusted PRP with the Coulter Act-diff (Krefeld, Germany). The tests were initiated

before and after incubation with bromelain (1 mg, 2.5 mg and 5 mg/ml).

Platelet aggregometry. The platelet aggregometry was performed with the APACT aggregometer (Mölab, Hilden, Germany) according to Born [13]. At first the PRP was calibrated to 250 000–300 000 platelets/ μL with a calculated amount of PPP. PPP served as the appropriate control. The concentrations of the agonists were as follows: $100 \mu\text{M}$ TRAP-6 (Bachem, Weil am Rhein, Germany) and $0.5 \mu\text{M}$ ADP (Sigma, Deisenhofen, Germany). The aggregometry was performed before and after 5 min incubation with bromelain (1 mg, 2.5 mg and 5 mg/ml) in comparison with untreated control.

Flow cytometric analysis. For the determination of changes in platelet activity and response to agonists the direct immunofluorescence technique was used [14–16]. To investigate the influence of bromelain on this platelet functions, one PRP-sample was incubated with 5 mg bromelain (this is equivalent to an end-concentration of 1 mg bromelain/ml = 6.99 F.I.P.) for 5 min at room temperature. The second sample provided the untreated control. The following fluorescein-isothiocyanate (FITC)- and phycoerythrin (PE)-conjugated monoclonal antibodies (mabs) were purchased from Coulter-Immunotech Diagnostics (Krefeld, Germany): anti-CD62P-FITC (clone CLB-Thromb/6) and anti-CD41-PE (clone P2). Immediately after the withdrawal of blood and the preparation of PRP the platelet count in the samples was adjusted to approximately 20 000 platelets/ μL with PBS buffer (Life Technologies, Gibco BRL; Karlsruhe, Germany) including 0.5% BSA (Vitros Ortho, Clinical Diagnostics; Neckargemünd, Germany) and prewarmed to 37°C . The platelet stimulation was obtained by mixing $16 \mu\text{L}$ diluted PRP with $4 \mu\text{L}$ $7.5 \mu\text{M}$ TRAP-6 (Bachem, Heidelberg, Germany). After incubation for 10 min at 37°C , the samples were incubated for further 10 min with anti-CD41-PE ($7.5 \mu\text{L}$) and anti-CD62P-FITC ($4 \mu\text{L}$). After that, 1 ml PBS buffer with 1% BSA was added. In parallel, $20 \mu\text{L}$ of unstimulated PRP were incubated with the labelled antibodies as described above. Unspecific binding of the fluorescence-labelled antibodies was determined by adding unlabelled anti-CD62P ($4 \mu\text{L}$), respectively, anti-CD41 ($7.5 \mu\text{L}$) to PRP in a separate tube. Immediately after the immunolabelling procedure the samples were measured in a flow cytometer (Coulter® EPICS® XL-MCL™, Beckman Coulter GmbH, Krefeld, Germany); laser excitation 488 nm, detection of FITC at 525 nm and PE at 575 nm. Data acquisition and analysis were performed with System II™ software (Beckman Coulter GmbH). The activation status of the platelets measured by antibody binding were expressed by

antibody positive cells (isotype control was subtracted).

Statistics

Results are reported as mean ± SD and the range unless otherwise stated. Using the Shapiro–Wilk test, the data showed a normal distribution. Normally distributed data were tested by paired student’s *t*-test. The level of significance was set at *P* < 0.05. Statistical analysis was done with SPSS-11.0 software.

Results

Platelet count and mean platelet volume (MPV)

After incubation with 1 mg bromelain/ml no changes in platelet count was apparent. The use of 2.5 and 5 mg bromelain/ml led to a significant (*P* < 0.001) decrease in platelet count (Table I).

After incubation with 1 mg bromelain/ml the MPV did not change. However, MPV increased significantly (*P* < 0.001) after the incubation with 2.5 and 5 mg bromelain/ml (Table I).

Platelet aggregation

ADP-induced platelet aggregation was significantly decreased after incubation with 2.5 mg (48.6 ± 25.7%; *P* < 0.01) and 5 mg (5.0 ± 4.6%; *P* < 0.001) bromelain/ml in comparison to the control (81.4 ± 5.0%). The use of 1 mg bromelain/ml showed no effect on the ADP induced platelet aggregation (79.6 ± 5.0; Figure 1). TRAP-6-induced platelet aggregation was significantly decreased after incubation with 2.5 mg (49.6 ± 28.9%; *P* < 0.05), respectively, 5 mg (9.0 ± 4.9%; *P* < 0.001) bromelain/ml in comparison to the control (77.4 ± 10.4%). Similar to the ADP induced platelet aggregation, the incubation with 1 mg bromelain/ml showed no changes in TRAP induced platelet aggregation (74.9 ± 17.4%; Figure 1).

FSC and SSC

Evaluation of FSC showed, without stimulation, a significant increase after incubation with 5 mg bromelain/ml (11.5 ± 0.6 to 12.1 ± 0.8). TRAP-6 stimulation caused in no significant changes after the

bromelain incubation (10.4 ± 1.3 to 11.1 ± 1.1; Table II).

Evaluation of SSC showed, without stimulation, a significant decrease (9.2 ± 0.5 to 8.1 ± 0.5) and with stimulation by TRAP-6, no significant changes (9.3 ± 1.8 to 8.3 ± 0.5) after the incubation with 5 mg bromelain/ml (Table II).

% Positive cells CD62P

In comparison to the control (0.22 ± 0.11) the percentage of unstimulated CD62P positive platelets (% PC) increased by 0.57 ± 0.48 after incubation with 5 mg bromelain/ml. Using TRAP-6-stimulation the incubation with 5 mg bromelain/ml led to an remarkable decrease in comparison with the untreated control (50.40 ± 20.16 to 0.89 ± 0.77% PC, Figure 2).

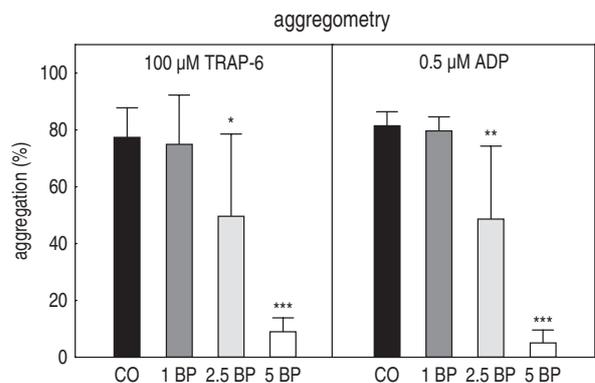


Figure 1. ADP- and TRAP-6-induced platelet aggregation after incubation with bromelain (in different concentrations: 1 BP= 1 mg bromelain/ml; 2.5 BP= 2.5 mg bromelain/ml; 5 BP= 5 mg bromelain/ml) in comparison to control (CO); Values are means ± SD; ****P* < 0.001; ***P* < 0.01; **P* < 0.05 pre to post.

Table II. FSS and SSC after incubation with 5 mg bromelain/ml in comparison to control.

	Unstimulated		TRAP-6 stimulated	
	Control	5 mg Bromelain	Control	5 mg Bromelain
FSS	11.5 ± 0.6	12.1 ± 0.8**	10.4 ± 1.3	11.1 ± 1.1
SSC	9.2 ± 0.5	8.1 ± 0.5***	9.3 ± 1.8	8.3 ± 0.5

****P* < 0.001; ***P* < 0.01 pre to post. Values are means ± SD.

Table I. Platelet count and MPV before and after incubation with bromelain (in different concentrations) in comparison to control.

	1 mg Bromelain		2.5 mg Bromelain		5 mg Bromelain	
	Pre	Post	Pre	Post	Pre	Post
Platelet count (1/nl)	278 ± 22	273 ± 21	276 ± 19	256 ± 21***	278 ± 19	247 ± 19***
MPV (fl)	6.7 ± 0.9	6.8 ± 0.8	6.7 ± 0.9	7.2 ± 0.8***	6.7 ± 0.8	7.5 ± 0.8***

****P* < 0.001 pre to post. Values are means ± SD.

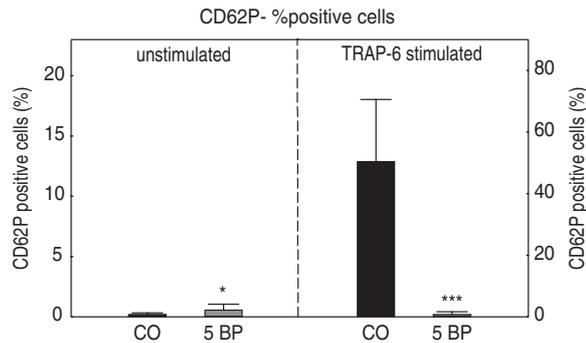


Figure 2. CD62P% positive cells after incubation with 5 mg bromelain/ml (5BP) in comparison to control (CO); Values are means \pm SD; *** $P < 0.001$; * $P < 0.05$ pre to post.

Discussion

The aim of this study was to investigate the influence of bromelain on platelet count and function. Incubation with 2.5 and 5 mg bromelain/ml led to a significant decrease in platelet count and additionally to a significant increase in mean platelet volume. Similar changes were also investigated by measuring the forward scatter characteristics, which underpinned the changes in platelet volume. The results of FSC showed a significant increase in the unstimulated samples after the bromelain application in comparison to the untreated control. Based on these facts that MPV and FSC increased after bromelain treatment, we may speculate that small platelets are more affected, which can possibly lead to the lower count of platelets after bromelain incubation. To examine the granularity of the platelets, the side scatter characteristics were analysed. In the unstimulated sample a significant decrease after the bromelain incubation could be found but not in the stimulated trial. Whether bromelain definitely leads to changes in the granularity of platelets needs further study.

The results of the ADP- and TRAP-6-induced platelet aggregation of this *in vitro* study show clear dose-dependent effects of bromelain on platelet function. The measurement was done with an aggregometer according to Born [13]. The concentration of 5 mg bromelain/ml (end concentration 1 mg bromelain/ml = 6.99 F.I.P.) led to a significant inhibition of the ADP- and TRAP-6-induced platelet aggregation in comparison to the untreated control by 94 and 88%. After PRP incubation with 2.5 mg bromelain/ml (end concentration 450 μ g bromelain/ml = 3.14 F.I.P.) a significant, but smaller, inhibition of the ADP- and TRAP-6-induced platelet aggregation by 40 and 36% could be observed in comparison with the untreated control. The application with 1 mg bromelain/ml (end concentration 182 μ g bromelain/ml = 1.27 F.I.P.) caused no significant change of the ADP- and TRAP-6-induced platelet aggregation in comparison with the untreated control. These results correspond with those mentioned

in the literature. These studies carried out by different work groups show unanimously a dose-dependent inhibition of platelet aggregation after a bromelain application. In 1972, Heinicke et al. [10] showed that bromelain, administered orally to 20 volunteers (two Ananase tablets of 30 mg bromelain), reduced ADP-induced platelet aggregation significantly. In another study using five male rat pairs, Livio et al. [12] investigated the effect of bromelain on platelet aggregation. It was shown that in the rats treated with 30 mg bromelain/kg body weight, 16 μ M ADP-induced platelet aggregation was reduced by 58% in contrast to the control rats. Sano et al. [11] analysed *in vitro* the changes in the sensitivity of unwashed and washed platelets of the stimulants ADP and thrombin after the treatment with 50 mg bromelain. The sensitivity of the unwashed and washed platelets against ADP and thrombin after bromelain treatment at concentrations of 10 and 100 μ g/ml decreased significantly. With these results the authors showed that bromelain counteracts the ADP- and thrombin-induced platelet aggregation in both unwashed and washed platelets. This suggests that the inhibitory effect is based on the reactions of proteolytical enzymes with the platelet membrane. Metzger et al. [17] investigated the platelet aggregation and adhesion on endothelial cells. These cells, treated with bromelain before thrombin activation, showed a complete inhibition of platelet aggregation. The authors assume a modulation of the thrombin receptor. Metzger et al. found that bromelain-preincubated platelets did not respond to the stimulatory action of thrombin. Using two different stimulants for inducing platelet aggregation, the effect of bromelain on the different mechanisms of platelet activation could be more closely examined. The TRAP-6 peptides connect to the corresponding sequences of the thrombin receptors PAR 1 [18–20], which provides a signal transduction to the inside of the cell through a conformation change [21]. The consequence of this signal transduction is a degranulation of the dense granules and the α -granules, which induces platelet aggregation. The ADP, however, leads to a change of the cytoskeleton, thus causing a release of the receptor GP IIb/IIIa, which then allows a fibrinogen connection and sets in motion a reversible primary aggregation [22, 23]. Both, the ADP- and TRAP-6-induced platelet aggregation showed a similar dose-dependent inhibition after the bromelain treatment, which suggests that changes of the fibrinogen receptor and not of the PAR 1 or P2 Y1 receptor are responsible for the effects. At this point further studies on the fibrinogen receptor are required, e.g., with the help of the antibody PAC-1, which connects only at the activated fibrinogen receptor [24–26].

The analysis of CD62P% positive cells showed, in the unstimulated sample, a significant increase from 0.22% (control) to 0.57% positive cells after the

bromelain application. This low increase can be found in the lowest measure range and, therefore, has to be interpreted carefully. The TRAP-6-stimulated sample shows a very significant decrease of CD62P% positive cells (from 50.4 to 0.9%) after bromelain incubation. These results confirm that bromelain has an inhibitory effect on platelet reactivity. The mechanism by which bromelain may inhibit degranulation remains unclear. It could also be possible that the expression of P-selectin is influenced by proteolytic reactions of bromelain [27, 28].

The changes of CD62P (TRAP-stimulated) and the results of platelet aggregation after incubation with bromelain *in vitro* may demonstrate the potential of bromelain as a substance for platelet inhibition.

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