Placebo-controlled randomised clinical trial on the immunomodulating activities of low and high dose bromelain after oral administration – new evidence on the anti-inflammatory mode of action of bromelain

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<td>Complete List of Authors:</td>
<td>Meiser, Peter; URSAPHARM</td>
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<td>Keyword:</td>
<td>bromelain, cytokine, human, inflammation, immune system, clinical trial</td>
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Placebo-controlled randomised clinical trial on the immunomodulating activities of low and high dose bromelain after oral administration – new evidence on the anti-inflammatory mode of action of bromelain

(Running head: Immunomodulating activities of bromelain)

Silke Müller*a§, Reinhard März*a, Manfred Schmolzd*a, Bernd Drewelow*a, Klaus Eschmann*c, Peter Meiser*c§

*aInstitute for Clinical Pharmacology, Medical Faculty, University of Rostock, Schillingallee 70, 18057 Rostock, Germany

*bSCIRM, Peter-Hannweg-Str. 8, 90768 Fürth, and Ohm-University of Applied Sciences Nuremberg, Germany

*cUrsapharm Arzneimittel GmbH, Industriestr. 35, 66129 Saarbrücken, Germany

*dEDI (Experimental & Diagnostic Immunology) GmbH, Aspenhaustr. 25, 72770 Reutlingen, Germany

*These authors contributed equally to this work

§Corresponding author

Dr. Silke Müller
Tel.: +49 381 494-5784 Fax: +49 381 494-5782 email: silke.mueller@med.unirostock.de

Dr. Peter Meiser
Tel.: +49 6805 9292-105 Fax: +49 6805 9292-87 email: p.meiser@ursapharm.de

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Abstract
Bromelain is used for treatment of inflammatory diseases since decades. However, the exact mechanism of action remains poorly understood. While in vitro investigations have shown conflicting effects on the release of various cytokines, no in vivo data were available.

In this study, the effects on inflammation-related cytokines of two doses of bromelain were tested in a single dose placebo-controlled 3 x crossover randomised clinical trial. Cytokine circadian profiles were used to investigate the effects of bromelain on the human immune system by using stimulated whole-blood leukocytes.

The effects seen in these cultures demonstrated a significant shift in the circadian profiles of the Th1 cell mediator IFNγ (p<0.043) after bromelain 3000 F.I.P., and trends in those of the Th2-type cytokine IL-5 as well as the immunosuppressive cytokine IL-10. This suggests a general effect on the antigen-specific (T cell) compartment of the human immune system.

Conclusion: This is the first time that bromelain was shown to modulate the cellular responses of lymphocyte after oral use. It is postulated that the immunomodulating effect of bromelain observed in this trial is part of its known anti-inflammatory activities. Further investigations will be necessary to verify the relevance of these findings to a diseased immune system.

Keywords
bromelain, cytokine, human, inflammation, immune system, clinical trial
1. Introduction

The use of pineapple bromelain in human medicine has been known for a long time (Taussig and Batkin, 1988). One of the major indications for this enzyme are inflammatory diseases (Brien et al., 2006; Secor et al., 2005), including those of autoimmune origin (Targoni et al., 1999; Roep et al., 2002). Currently, bromelain is used for the treatment of postoperative states, sports injuries and acute inflammation (Yuan et al., 2006). It has been suggested that these effects may be the result of this protease acting either on soluble proteins (as α-2-macroglobulin, (Lauer et al., 2001) or on proteins at the cell surface level (Engwerda et al., 2001; Mynott et al., 1999; Barth et al., 2005). In vitro investigations using isolated LPS-stimulated peripheral blood mononuclear cells (PBMC) from healthy volunteers and patients revealed conflicting results after treatment of the cells with bromelain: In vitro, Barth et al. (Barth et al., 2005) found an increase in IL-6, TNFα and INFγ, while IL-4 and IL-5 were not induced. Rose et al. (Rose et al., 2005) also report a dose dependent induction of IL-6 production, while there was no effect on the production of IFNγ and IL-10. On the other hand the investigations by Huang et al (Huang et al., 2008) revealed a reduced TNFα, IL-1 beta and IL-6 production. However, only little reliable information is available on the effects bromelain triggers in vivo in humans, especially after oral ingestion.

Therefore, the aim of the study presented was to investigate the effects of bromelain on immune cell activities after oral ingestion by healthy volunteers.

The following cytokines and chemokines relevant to judge inflammatory processes were selected as relevant endpoints. IL-2 and IFNγ both pertain to the Th1-type of immune response (Schroder et al., 2004), while IL-5 and IL-13 rather mark the Th2-driven reactions (O’Byrne, 2006). These two T helper cell types are seen mutual counter-regulators (Santoni et al., 2008). Under the conditions chosen in these experiments, IL-6 and IL-10 are mainly produced by the cells of the native immune system (as a product of zymosan stimulation) and
also can be seen as mediators that have opposite effects in the activation of immune cells (IL-6 being mostly of pro-inflammatory activity (Kishimoto, 2006), while one of the major functions of IL-10 is its inhibitory effect on cytokine synthesis (Mosser and Zhang, 2008). IL-8 and MCP-1 belong to the chemokines and can be seen the most prominent representatives of the both major sub-classes, the α- (or CXC) and the β-chemokines (CC), (Viola and Luster, 2008).

2. Methods

2.1. Study design and Volunteers

The trial was performed as a monocentric, partially placebo-controlled, double-blind, randomised, 3 x cross-over study at the Center for Pharmacology and Toxicology, Medical Faculty, University of Rostock. It was performed according to the German Drug Law (Arzneimittelgesetz), the GCP guideline CPMP/ICH/135/95, as well as the guideline 2001/20/EG, and in compliance with the Declaration of Helsinki (EudraCT-Nr 2005-002302-28). After approval of the study by the ethics committee of the University of Rostock 15 healthy male, no-smoking volunteers with an age of 18-50 years and a body mass index >17.5 kg/m² to <30 mg/m² were included into the study. Volunteers were determined to be healthy on the basis of medical history, physical examination, routine clinical chemistry laboratory (including negative virus screen on hepatitis B, C and HIV and a negative drug screening) and electrocardiogram. Volunteers were excluded from participation if they had known allergy to pineapple ingredients, used any drugs at present or up to 4 weeks prior to study start, had an acute infection at present or up to 2 weeks before the start of the study, underwent surgery or had larger injuries until 12 weeks before the study, had an vaccination within 4 weeks before the start of the study or had any chronic or inflammatory disease. Furthermore any self-medication with drugs or food supplements, excessive exercise, intake of pineapple or papaya (fruit and juice) and alcohol were prohibited throughout the study period.
The study period consisted of three identical one week periods, in which the study medication was provided in a randomized manner. The trial preparations (in the following scheme called “X1, X2, …”) were applied orally once a week with 1 week of wash-out in between. The order of intake of the trial preparations bromelain 3000 F.I.P. units, bromelain 1000 F.I.P. units and placebo were determined by a randomization scheme. TruCulture blood sampling (TC) was done according to the scheme in table 1.

On the day of trial drug intake the first blood sample was drawn before breakfast, for each blood sample a new puncture was performed. No venous catheter was inserted and no blood sample was drawn through a venous catheter. Blood donors who missed more than 1 time point or developed an infection were excluded from this evaluation.

### 2.2. Trial medication

The trial drug medication was manufactured at Ursapharm Arzneimittel GmbH, Saarbrücken, Germany. These were placebo tablets (lot No.: 004035), as well as two different concentrations of bromelain tablets: 500 F.I.P. (Fédération Internationale Pharmaceutique) units (lot No.: 018035) or 1000 F.I.P. units (lot No.: 005025). While those containing 500 F.I.P. units were the commercial product Bromelain-POS®, as marketed by Ursapharm Arzneimittel GmbH, the medication containing the higher dose, as well as the placebo tablets, were produced separately as trial medication.

The daily dose of 1000 F.I.P. units was achieved by applying 2 tablets of Bromelain-POS® (open label), for the dose of 3000 F.I.P. units 3 tablets with 1000 F.I.P. units were given. Thus, trial participants, physicians involved into the study and staff assessing the primary outcome of the study were blinded with regard to the therapies bromelain 3000 F.I.P. units and placebo.

### 2.3. TruCulture® blood collection and whole-blood culture system

The TruCulture® blood collection and whole-blood culture device was operated as described in the manufacturer’s instruction manual (EDI GmbH). In brief, the TruCulture® syringe-
tubes, containing a proprietary nutrient solution together with a combination of stimulants, were sent to the trial site at -40 °C on dry ice and stored at -20 °C at the trial site until use. The blood leukocytes in this trial were stimulated by (a) zymosan, a β-glucan from yeast (Sigma-Aldrich, Deisenhofen, Germany), used at a final concentration of 300 µg/mL; (b) *Staphylococcus* enterotoxin B (SEB, from the Bernhard Nocht Institute, Hamburg, Germany; final conc. 200 ng/mL); as well as (c) anti-CD28 antibodies (Beckmann-Coulter, Frankfurt, Germany, final conc. 1 µg/mL). This combination of stimuli was used to activate simultaneously the phagocytes (granulocytes and monocytes) by zymosan, as well as the T-cells by SEB + anti-CD28, giving a very physiological pattern of activation. The TruCulture® syringe front end is a screw cap into which a male-type connector with a rubber septum is inserted. Together with a special butterfly needle system (21G) attached to a female-type connector that punctures this rubber septum during the blood draw (Sarstedt, Nümbrecht, Germany), this ensures a closed state of these syringe-tubes, before as well as after the blood draw, thereby preventing the contamination of the cultures.

After filling the TruCulture® tubes with blood they were immediately transferred into a desktop dry-block incubator (VLM Vogler, Bielefeld, Germany), where they were incubated for 24h at 37 °C.

At the end of this incubation the supernatants were separated by insertion of a valve separator and the tubes were frozen at -20 °C until measuring the cytokine contents.

### 2.4. Mediator quantification

Cytokine and chemokine concentrations in the supernatants were determined by sandwich-type enzyme-linked immune-sorbent assays (ELISAs). These were performed strictly according to the procedures suggested by the manufacturer using the matched antibody pairs for IL-2, IL-5, IL-6, IL-8, IL-10, IL-13, IFNγ, and MCP-1, but also the cytokine standards from R&D Systems.

### 2.5. Statistical methods

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Only few data about circadian rhythms of the cytokines have been published (Petrovsky et al., 1998) and no data on the effects of bromelain or its variability. The sample size of n = 12 was therefore a matter of agreement in this study that addressed complete new questions. The statistical analysis was performed at SCIRM, Fürth, and Ohm-University of Applied Sciences, Nuremberg.

Statistical analysis: A thorough exploratory analysis of the data revealed that the considerable variability in the data of each cytokine was due to extreme differences between individual subjects (up to a factor of 70), while the within-subject variability was very small and mainly caused by the circadian rhythm (publication in preparation). Thus it was decided to z-transform the cytokine data individually for each subject for each cytokine. The resulting data were then adjusted to zero at 8 a.m. of the medication day. For the analysis, a linear mixed model with "group" and "time" as fixed effects and an interaction term "group*time" was used to compare the effects of the medication and the changes during daytime and to identify significant changes over the day. All p-values given below are Bonferroni-adjusted for multiple comparisons.

3. Results
12 volunteers with a mean age of 27.1 ± 4.3 years, a mean height of 183.9 ± 5.8 cm, a mean weight of 77.3 ± 10.3 kg and a mean body mass index of 22.8 ± 2.8 kg/m2 finished the study and were available for evaluation. Three volunteers were withdrawn from the study because of acute respiratory infection during the study period. For all cytokines investigated in the study, significant changes in the circadian profile have been established, with IFN-γ, IL-8, IL and IL-13 showing a profound diurnal increase (p < 0.001; p = 0.008, and p < 0.001, respectively), and IL-10 as well as MCP-1 display a diurnal decrease (for both: p < 0.001). IL-6 has a morning and an afternoon peak (p = 0.001) and IL-5 a steady increase towards the evening hours (p < 0.001) (Schmolz et al., paper submitted).
3.1. **IL-2, IL-6, IL-8, IL-13, MCP-1**

The release of the cytokines IL-2, IL-6 and IL-13, as well as of the two chemokines, IL-8 and MCP-1, tested in this clinical trial did not show statistically significant drug effects in this trial (data not shown).

3.2. **IL-10 diurnal profile modulation after bromelain 3000**

The daily profile of the release of IL-10 after treatment with bromelain 3000 F.I.P. showed a statistically significant difference to bromelain 1000 F.I.P. (p = 0.028) and a borderline significance vs. placebo (p = 0.069) (Fig. 1.). After placebo and Bromelain 1000, IL-10 decreased during morning hours and returned to baseline in the evening, while after Bromelain 3000, this decrease in the morning hours was only marginal. Thus Bromelain 3000 prolonged IL-10 production as compared to both other treatments. For the rest of the day, all groups showed similar courses.

3.3. **IFNγ diurnal profile modulation after bromelain**

For IFNγ, the placebo group demonstrated a peak in cell activity around noon, with a slow decrease towards the evening hours until evening (see Fig. 2). Both bromelain treatments did exhibit differences compared to placebo, but significance was found only with the higher dose (p = 0.043), which was apparently caused by a faster rise in cell activity in the morning and an increased secretion upon stimulation towards the evening. This latter activity seems to be prolonged even until the next morning.

3.4. **Possible IL-5 diurnal profile modulation after bromelain 3000**

Bromelain at 3000 F.I.P. units also seems to affect the production upon stimulation of IL-5, also a T-cell cytokine, although produced by Th2 lymphocytes. However, due to high variation, significance was not established (p=0.146). The reason why this increase in IL-5 secretion did not lead to a
significant result seems to be the particularly high data scatter in the placebo group at time point 11 h (see Fig. 3).

3.5. Tolerability of the study medication

The study medication was well tolerated. During the study no serious adverse events and no study medication related adverse drug effects were observed. The three volunteers that were withdrawn from the study developed an acute respiratory infection, which was an exclusion criterion because it would have affected the cytokine release. The respiratory infection episodes were not regarded as being related to the medication.

4. Discussion

Bromelain is a crude extract from the pineapple plant that contains, besides low molecular weight compounds and other proteins, various closely related cysteine proteases. It is medicinally used for decades in the treatment of various diseases, such as injuries, like postoperative states, sinusitis and inflammatory diseases (Braun et al., 2005; Inchingolo et al., 2010; Maurer, 2001; Yuan et al., 2006). Especially the treatment of inflammatory diseases remains an important research area since the available therapeutic drugs are often not sufficiently efficient or side-effects are intolerable, all the more in cases of long-time therapy (Trelle et al., 2011). Therefore this study aimed – for the first time – to characterize the effects of orally taken bromelain on immuno-modulation with regard to the cytokine secreting capacities of peripheral blood leukocytes.

One of the biggest obstacles in the characterisation of pharmacological effects on cells of the human immune system is the well-known inter-individual variability in leukocyte activities (Yaqoob et al., 1999; Wurfel et al., 2005). By means of the highly standardised test system we used (TruCulture®), the variability of the cell cultivation procedure could also be reduced to a minimum (Schmolz et al., paper submitted). An additional source of increasing the data scatter of especially investigations looking at short term kinetics (over 24 h), is the circadian
changes in the reactivity of the cells of the human immune system. There are only a few reports on such rhythmic changes in immune cell functions so far (Petrovsky et al., 1998). We controlled this factor by tight measurement intervals during the day and comparing the same individuals after application of the two study drugs and placebo in randomised order. Finally, we analysed the remaining variability, which was still considerable. It turned out that the intra-individual levels were of remarkable stability over the whole study period of 4 weeks for each volunteer, and that the inter-individual variability is a consequence of the fact that obviously low, middle and high “producers” are existing, who differed up to a factor of 70, depending on the cytokine analysed.

Based on this finding of individual corridors of cytokine production levels within each subject, z-standardisation of each individual’s cytokine data was the means to transform the original data into a dataset with a mean of zero and a standard deviation of 1 to make them comparable. Additionally, the data were adjusted to have their mean at 8 a.m. Thus, the circadian profile is expressed in terms of deviation from the 8 a.m. value.

Results obtained within this study showed that all cytokines exhibited significant diurnal changes with p-values < 0.02. (Schmolz et al., paper submitted). Although there are investigations on circadian rhythms regarding leukocyte activities, it is difficult to compare their data to ours, as they were using different cell culture systems and/or other activators (Petrovsky and Harrison, 1998). However, when investigating effects of drugs on cytokine production, circadian changes have to be taken into account; this is one of the important general results of the study.

The goal of the current trial was to describe any type of systemic effect that can be related to the administration of bromelain on the cells of the human immune system, irrespective of the underlying mechanism. The trial revealed a significant modulation in the release of IFNγ, seen as an increase in the afternoon hours (p<0.043 vs. placebo) after bromelain 3000 F.I.P.

For IL-10, the effect of group was significant (p = 0.019), but the Bonferroni-adjusted p-value
vs. placebo was not significant (p = 0.069). With respect to IL-5 (p=0.146), the profiles differed obviously between bromelain 3000 F.I.P. and the other groups in the morning hours, but significance was not established (p = 0.07 for the group effect and p = 0.146 vs placebo). Larger group sizes are necessary to find out if this was a random or a systematic difference. Only at the higher dose of bromelain (3000 F.I.P.) significant changes of the diurnal profiles were seen; they seem to affect both types of T helper cells, Th1 (IFNγ), as well as Th2 (IL-5). This suggests a general effect of this enzyme preparation on T lymphocytes, regardless of the different sub-populations of these cells existing in the human body. The third mediator, IL-10, is rather known for its suppressive effects on cytokine synthesis (Mosser and Zhang, 2008) and – according to the type of leukocyte activation that was chosen in these cultures – can be considered to be secreted by monocytes rather than by Th2 lymphocytes. As this effect was most pronounced in the morning hours (while that on IFNγ had an additional late component in the evening hours), one might assume that at least at its onset the bromelain-mediated modulation of immune cell activities touches a broad spectrum of leukocyte sub-types, while the prolonged effect might affect mainly the T cells. The effects observed during bromelain treatment in this trial were detected in cultures that involved a physiologic pro-inflammatory activation of immune cells, very similar to what occurs during acute or chronic inflammation in vivo. It cannot be speculated, how these effects of bromelain would modulate the course of any type of inflammatory disease, at least because the treatment would usually involve repeated application of the medication. Despite that, two major findings can already be derived from these results:

(a) Even after a single oral dose of bromelain significant changes in the response of physiologically stimulated immune cells were found.

This demonstrates that the enzyme is able to either reach the blood circulation in an intact, biologically active form (as was suggested earlier by others (Castell, 1996; Desser et al., 2001), or it acts indirectly, e.g. on the class of protease-activated
receptors (PARs), which was shown for bromelain recently (Reddy and Lerner, 2010), thereby possibly stimulating intestinal epithelial cells which, in turn, could then change the activities of close-by cells of the mucosal immune system of the gut. In the latter case, one further step in this reaction cascade would have to be postulated, which could be a re-circulation of these cells, or a local secretion of systemically active mediators that consequently modulate the activities of leukocytes in the blood circulation (giving then rise to the changes seen in the whole-blood cultures of this clinical trial).

(b) The fact that all of the effects reaching significant p-levels or showing clear trends were observed after applying 3000 F.I.P. units can be regarded an indicator of a concentration-dependency of these bromelain activities.

Conclusions
The immunomodulating effect of bromelain 3000 F.I.P. demonstrated in this study is an initial step towards the understanding of this natural compound, as it could be demonstrated that effects on the immune system are present after a single application. Further studies should analyse repeated applications and effects in inflammatory conditions to close the gap between in vitro results and empirical use.

In a remarkable set of animal experiments, Baez and coworkers (Baez et al., 2007) have demonstrated, in vivo, convincing antitumor effects of the substance. It is a serious and valuable task to elaborate the mechanisms behind these results in order to lay the grounds of their transfer to human cancer treatment and other immune system related diseases, where bromelain has a more or less empirical role as adjunct therapy.

Taken together, the benefits of the medicinal product bromelain are - and might become even more - a valuable constituent of a modern (supportive) treatment of inflammatory diseases.

The results of these experiments support the idea of bromelain having a “multiple-target
activity profile”, similar to what is claimed for several other herbal preparations. This is postulated to have advantages over the inhibition of single targets, especially in the treatment of complex diseases, such as chronic inflammation.

Further investigations will be necessary to evaluate the pharmacodynamics of repeated bromelain application and the spectrum of effects under disease situations.

Acknowledgements

The trial was funded by URSAPHARM Arzneimittel GmbH.

Klaus Eschmann and Peter Meiser are employees of URSAPHARM Arzneimittel GmbH.

Manfred Schmolz is the CEO of EDI GmbH, a CRO, which developed the TruCulture® blood collection and whole-blood culture system. We thank Ms Grit Haase and Ms Josefine Bookholdt for their support in taking blood samples and care of the volunteers.

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Table 1: Drug application and sampling scheme

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X1: drug 1; X2: drug 2; TC: TrueCulture blood sampling
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Figure captions:

Figure 1: Means with 95 % confidence intervals in the circadian profile of leukocyte activity of n = 12 healthy volunteers regarding the experimentally stimulated release of IL-10 for placebo, bromelain 1000 and 3000 F.I.P. units. After bromelain 3000 F.I.P, the levels of IL-10 are statistically significantly elevated compared to bromelain 1000 F.I.P. (p = 0.028), but not to placebo (p = 0.069) due to the inhibition of the decrease between 10 a.m. and 11 a.m. (all p-values are Bonferroni-adjusted).

Figure 2: Means with 95 % confidence intervals in the circadian profile of leukocyte activity of n = 12 healthy volunteers; test of the experimentally stimulated release of IFN-γ for placebo, bromelain 1000 and 3000 F.I.P. units. After bromelain 3000 F.I.P. units, IFN-γ levels were increased especially in the afternoon hours (p = 0.043; Bonferroni-adjusted).

Figure 3: Means with 95 % confidence intervals in the circadian profile of leukocyte activity of n = 12 healthy volunteers regarding the experimentally stimulated release of IL-5 for placebo, 1000 and 3000 F.I.P. units bromelain, respectively. The differences between bromelain 3000 F.I.P and placebo were statistically not significant (p = 0.146).