Send Orders for Reprints to reprints@benthamscience.net

Protein & Peptide Letters, 2020, *27*, 1-12

RESEARCH ARTICLE

Stem Bromelain Proteolytic Machinery: Study of the Effects of its Components on Fibrin (ogen) and Blood Coagulation

Mohamed Azarkan[1,*], Mariana Marta González[2], Rafaèle Calvo Esposito[1] and María Eugenia Errasti[3,*]

[1]Department of General Chemistry, Faculty Medicine, University of Brussels, B-1070 Brussels, Belgium; [2]Laboratory of Hematology, Department of Biological Sciences, Faculty of Exact Sciences, National University of La Plata, La Plata, Argentina; [3]Plant Proteins Research Center (CIProVe), Department of Biological Sciences, Faculty of Exact Sciences, National University of La Plata - CICPBA, La Plata, Argentina

Abstract: ***Background*:** Antiplatelet, anticoagulant and fibrinolytic activities of stem bromelain (EC 3.4.22.4) are well described, but more studies are still required to clearly define its usefulness as an antithrombotic agent. Besides, although some effects of bromelain are linked to its proteolytic activity, few studies were performed taking into account this relationship.

***Objective*:** We aimed at comparing the effects of stem bromelain total extract (E_T) and of its major proteolytic compounds on fibrinogen, fibrin, and blood coagulation considering the proteolytic activity.

***Methods*:** Proteolytic fractions chromatographically separated from E_T (acidic bromelains, basic bromelains, and ananains) and their irreversibly inhibited counterparts were assayed. Effects on fibrinogen were electrophoretically and spectrophotometrically evaluated. Fibrinolytic activity was measured by the fibrin plate assay. The effect on blood coagulation was evaluated by the prothrombin time (PT) and activated partial thromboplastin time (APTT) tests. Effects were compared with those of thrombin and plasmin.

***Results*:** Acidic bromelains and ananains showed thrombin-type activity and low fibrinolytic activity, with acidic bromelains being the least effective as anticoagulants and fibrinolytics; while basic bromelains, without thrombin-like activity, were the best anticoagulant and fibrinolytic proteases present in E_T. Procoagulant action was detected for E_T and its proteolytic compounds by the APTT test at low concentrations. The measured effects were dependent on proteolytic activity.

***Conclusion*:** Two sub-populations of cysteine proteases exhibiting different effects on fibrin (ogen) and blood coagulation are present in E_T. Using well characterized stem bromelain regarding its proteolytic system is a prerequisite for a better understanding of the mechanisms underlying the bromelain action.

ARTICLE HISTORY

Received: February 28, 2020
Revised: April 27, 2020
Accepted: April 30, 2020

DOI:
10.2174/0929866527666200525163622

Keywords: *Ananas comosus*, bromeliaceae, anticoagulants, fibrinolytic activity, thrombin-like activity, cysteine protease, ananain.

1. INTRODUCTION

Medicinal use of pineapple (*Ananas comosus* L. Merr) is known since 15th century, while its active component was identified as "bromelain" in the 50s [1]. The main constituent fraction of bromelain is a complex proteolytic system (also called stem bromelain; EC 3.4.22.4) containing proteases that belong to the cysteine proteases of the papain family (subfamily C1A) [2-6]. We published a paper dealing with an efficient strategy towards the purification of several catalytically active forms from pineapple stem and demonstrated that these proteolytic forms have indeed distinct biochemical/enzymological properties [7]. Additionally, other less characterized components, such as proteases inhibitors, peroxidases and glucosidases were described [2-4, 8-10]. More recently a jacalin-like lectin was also reported [11].

Pharmacological properties of bromelain such as fibrinolytic, anti-inflammatory, antiedematous, antimetastasic and anti-tumor effects, inhibition of platelet aggregation, modulation of immunity, enhanced absorption of drugs, wound healing and skin debridement have been reported [12, 13]. Bromelain is currently used as a phytotherapeutic agent to

* Address correspondence to these authors at the Department of General Chemistry, Faculty of Medicine, University of Brussels, B-1070 Brussels, Belgium; Tel/Fax: +32-2-555-6784, +32-2-555-6782;
E-mail: mazarkan@ulb.ac.be
* CIProVe, Faculty of Exact Sciences, National University of La Plata, 47 and 115, CP1900, La Plata, Argentina, Tel: +54-221-423-5333, ext. 57;
E-mails: meerrasti@biol.unlp.edu.ar / eerrasti@gmail.com

0929-8665/20 $65.00+.00

treat inflammation, oedema, and pain in cases of arthritis [14], surgical and non-surgical trauma [15-18], and acute thrombophlebitis [19] as well as debridement of deep burns [20] and as an adjunct in cancer treatment [21, 22].

Although the potential therapeutic effect of bromelain for the treatment of cardiovascular diseases has been indicated, there have been few and no substantive human and animal trials intended to elucidate this therapeutic effect [23]. Results from *in vivo* and *in vitro* studies showed that bromelain inhibits platelet aggregation and adhesion, decreases the thrombus formation, increases serum levels of fibrinolytic activity and plasmin as well as decreases that of fibrinogen and activates plasminogen to yield plasmin [13, 23]. However, results about its action on the blood coagulation are not conclusive. According to some animal studies, bromelain acts as an anticoagulant agent since lengthens the prothrombin time (PT), antithrombin time (ATT), and activated partial thromboplastin time (APTT) [13, 24]. Nevertheless, bromelain showed a tendency toward hypercoagulability in a recent *in vivo* study, which was paradoxical regarding the *in vitro* and *ex vivo* results [25]. On the other hand, coagulation time and PT increased slightly after oral administration of bromelain to patients with oedema and inflammation, though they were not considered to have practical significance [26]. In a study with healthy persons, PT was not modified and APTT showed a moderate increase within normal range [13], while bromelain lengthened PT and ATT during a clinical pharmacological study [27]. Recently, a clinical case of patient with burns who presented prolonged values of PT and APTT after treatment with a bromelain-based debridement agent was published [28].

Results reported by our group were controversial since bromelain showed both procoagulant and anticoagulant effects when human plasma was incubated with a commercial stem bromelain extract. Shortened APTT were registered at low concentrations while the elongated values were reached at high concentrations of the bromelain, both effects being dependent on the proteolytic activity [29].

An aspect that affects the correct interpretation and comparison of results from several studies is the lack of information about activity and proteolytic composition of the tested bromelain samples [23, 30]. This fact becomes more important if it is considered that each of the proteases present in bromelain extract showed different substrate specificity, stability, and enzymatic activity, which could lead to different biological effects and consequently to different therapeutic actions [7, 30].

According to the above, there is still much to know about the bromelain action on the haemostasis, not only for the management of possible adverse effects but also for therapeutic applications. Considering our previous results [7, 29] from the perspective of the proteolytic composition complexity of bromelain, the aim of this study was to compare the *in vitro* effects of the stem bromelain total extract and its major proteolytic compounds, characterized as acidic bromelains, basic bromelains and ananains, on fibrinogen, fibrin, PT, and APTT. Additionally, the present study was conducted by comparing catalytically competent species and their irreversibly inhibited counterparts in order to shed light on the implication of the proteolytic activity on the evaluated effects.

2. MATERIALS AND METHODS

2.1. Chemicals and Materials

Stem bromelain (B4882), dithiothreitol (DTT) (43815), S-methyl methanethiosulfonate (MMTS) (208795), Nα-benzoyl-DL-arginine p-nitroanilide hydrochloride (DL-BAPNA) (B4875), casein from bovine milk (C5890), Coomassie brilliant blue G-250 (B0770), L-cysteine (168149), bovine fibrinogen (F8630), bovine thrombin (T7513), human plasmin (P1867), and Tris (T1503) were purchased from Sigma-Aldrich. SP-Sepharose Fast Flow (17072901) and Q-Sepharose Fast Flow (17051001) were from Amersham Biosciences, part of GE Healthcare. Iodoacetamide (407710) was purchased from Merck. Methoxy polyethylene glycol ortho-pyridine disulfide (mPEG-OPSS, MW 5000Da) (PLS-293) was provided by Shearwater Polymers. Acrylamide (1610101), bis-acrylamide (1610201), tricine (1610713), molecular weight standards (1610304) were purchased from Bio-Rad. Sodium dodecyl sulfate (409504) was purchased from J.T.Baker. Kits for determination of PT (C1705001) and APTT (C1705002) were obtained from Wiener lab Group. All the other chemicals were of analytical grade.

2.2. Purification of Stem Bromelain Proteases

Stem bromelain proteases were purified as described previously [7]. This purification strategy achieves an efficient chromatographic separation of the catalytically active proteases from the irreversibly oxidized counterparts by the S-pegylation with mPEG-OPSS, which reacts only with proteases having free and accessible thiol functions (catalytically active forms), because thiol-pegylated species elute from ion-exchange supports faster than the un-pegylated species [7, 31, 32]. Two home-made columns (12.5 x 1.5 cm i.d) were used: SP-Sepharose Fast Flow column pre-equilibrated with 100 mM sodium acetate buffer, pH 5.0, and Q-Sepharose Fast Flow column pre-equilibrated with 50 mM Tris-HCl buffer, pH 7.4. The chromatographic fractions were analyzed by measuring the absorbance at 280 nm and the amidase activity.

Briefly, stem bromelain powder was suspended in 100 mM sodium acetate buffer, pH 5.0, and centrifugated. The supernatant constituting the total soluble protein fraction (E_T) was applied onto the SP-Sepharose Fast Flow column and eluted with a linear concentration gradient from 100 mM to 800 mM sodium acetate buffer, pH 5.0. The chromatographic fractions constituting the different proteases were pooled according to their amidase activity profile, concentrated by ultrafiltration and exhaustively dialyzed against water at 4°C. Each pool was then activated with DTT and loaded on the SP-Sepharose Fast Flow column (for pools with the basic forms) or the Q-Sepharose Fast Flow column (for pools with the acidic forms). The columns were washed with the respective pre-equilibrating buffers to remove the excess of DTT. The bound proteins were eluted with 1 M

sodium acetate buffer at pH 5.0 or 1 M NaCl in 50 mM Tris-HCl buffer at pH 7.4, respectively, and directly collected in solid mPEG-OPSS. Each reaction mixture was exhaustively dialyzed against water at 4°C. The dialyzed solutions containing the mixture of S-pegylated and underivatized (irreversibly oxidized) protease forms were individually applied to the SP-Sepharose Fast Flow (for basic forms) or Q-Sepharose Fast Flow (for acidic forms) columns. The columns were washed by the respective pre-equilibrating buffers to eliminate the unreacted mPEG-OPSS. Bounded proteins were eluted with a linear gradient from 100 to 800 mM sodium acetate buffer at pH 5.0 (SP-Sepharose Fast Flow) or from 0 to 1 M NaCl in 50 mM Tris-HCl buffer at pH 7.4 (Q-Sepharose Fast Flow). The chromatographic fractions containing the pegylated forms were pooled and exhaustively dialyzed against water at 4°C. MMTS was used to selectively and reversibly block the cysteine proteases, preventing their autolysis and/or irreversible oxidation of their catalytic cysteine residues [7]. S-methylthio-derivatives of the different stem bromelain protease forms were regenerated from their S-pegylthio-conjugates by reaction with MMTS in 100 mM sodium acetate buffer, pH 5.0, containing DTT. The resulting mixture was dialyzed against the suitable pre-equilibrating buffer and applied onto SP-Sepharose Fast Flow (for basic forms) or Q-Sepharose Fast Flow (for acidic forms) columns. The columns were washed with the respective pre-equilibrating buffers to eliminate the released mPEG moieties and the bound proteins were eluted with a linear gradient of the respective buffers. Proteolytic activity of the fractions was analysed and their purity was checked by SDS-PAGE. Proteolytic fractions chromatographically separated (Fs) from E_T were lyophilized and stored at -20°C until use.

E_T was activated with DTT and treated with MMTS to prepare its S-methylthio-derivative, which was lyophilized and stored at -20°C until use.

2.3. Irreversible Inhibition of the Stem Bromelain Proteases

E_T and Fs were activated with DTT and subsequently irreversibly inhibited with a large excess of iodoacetamide (10 to 20 mM) until the inhibition was completely achieved. After removing the iodoacetamide excess by exhaustive dialysis, residual proteolytic activity was checked fluorometrically using appropriate substrates [7]. The irreversibly inhibited samples were lyophilized and stored at -20°C until use.

2.4. Samples Conditioning

Stock solutions (10.0 to 15.0 mg/ml) of the S-methylthio-derivates of E_T, Fs and the irreversibly inhibited samples were prepared by dissolving the lyophilized samples in sterile distilled water, aliquoted and stored at -20°C for one month. Immediately before each assay, different dilutions were prepared from the stock solutions and activation of Fs and E_T was performed by incubation with L-cysteine 5 mM (final concentration) for 15 min at 0°C.

2.5. Caseinolytic Activity Assay

Caseinolytic activity was measured according to the method of Lopez *et al*. [33] with some modifications. Mixture of 50.0 µl of sample (1.0 to 3.0 mg/ml, depending on the sample) and 550.0 µl of 1% (w/v) casein in 0.05 M Tris-HCl buffer, pH 8.0, was incubated for 2 min at 37°C. The reaction was stopped with 900 µl of 5% (w/v) trichloroacetic acid (TCA), incubated for 10 min at 4°C and centrifugated at 7000x*g* for 10 min at room temperature. Blank solutions were prepared by adding the TCA solution to the sample before the casein solution. Absorbance of the supernatant was measured at 280 nm. An arbitrary enzyme unit (U_C) was used, which was defined as follows: one U_C is the amount of enzyme that produces an increase of 2 absorbance units per minute under the assayed conditions.

2.6. Action on Fibrinogen

Activity on fibrinogen was evaluated over 120 min by electrophoresis and by the fibrinogen polymerization assay.

2.6.1. Fibrinogen Hydrolysis Detected by Electrophoresis

Fibrinogen hydrolysates were obtained by incubating 0.8 µl of 1.0 U_C/ml sample and 200.0 µl of 1.2 mg/ml fibrinogen solution in 0.025 M Tris-HCl, pH 7.4, at 37°C. Aliquots were withdrawn and treated according to Errasti *et al*. [29]. Then, 5.0 µl of each sample and molecular weights standards were analyzed by tricine-SDS-PAGE according to Schägger [34] using 10% T, 3% C separating gel, 4% T, and 3% C stacking gel. Gels were stained with Coomassie brilliant blue G-250.

2.6.2. Fibrinogen Polymerization Assay

Mixtures of 700.0 µl of 5.0 mg/ml fibrinogen solution in 0.1 M Tris-HCl buffer, pH 7.4, and 70.0 µl of 3.5, 0.35, and 0.035 U_C/ml sample were incubated at 37°C in a 1 cm-path-length polystyrene cuvette while recording the absorbance at 540 nm as a turbidity measurement [29, 35]. A negative control assay was carried out in the same way but using sterile distiller water instead of sample. Thrombin 1.0 NIH U/ml (final concentration) was used as a positive standard control.

2.7. Action on Fibrin

Fibrinolytic activity was measured by the fibrin plate assay as previously described [29]. After placing 10.0 µl of sample on fibrin clot and incubating for 14 h at 37°C, two perpendicular diameters of the lysed area were measured. Several U_C/ml ranging from 1.0 to 10.0 were tested for the different samples. Fibrinolytic activity was expressed as the product of diameters (mm^2). Plasmin was used as a positive standard control.

2.8. Action on Blood Coagulation

2.8.1. Obtaining Blood Samples

Pool of platelet-poor plasma (*ppp*) was prepared and provided by the staff of the Public Health Laboratory of the Na-

tional University of La Plata (UNLP) from blood of 16 healthy individuals who had not ingested medication in the previous 10 days. This procedure was analyzed and endorsed by the Bioethical Committee of the UNLP (Record N° 59, File N° 0700-000017/19-000) according to requirements of Argentine laws and the Declaration of Helsinki. Platelet-poor plasma was obtained from supernatants after centrifuging a mixture of 9 parts of blood with 1 part of 3.8% (w/v) sodium citrate at 2500x*g* for 15 min at ambient temperature. The supernatants of each blood sample were pooled, aliquoted and stored at -80°C until use.

2.8.2. Coagulation Tests

Mixtures of 10.0 µl of sample and 100.0 µl of *ppp* were incubated for 2 min at 37°C and PT and APTT were immediately evaluated by using a commercial kit [29]. Several U_C/ml ranging from 0.01 to 1.0 (final concentration) were tested for the different samples. Control values (PT_C and $APTT_C$) were obtained by replacing sample with sterile distiller water. Assays were carried out at least in triplicate.

2.9. Statistical Treatment of the Data

Differences between slopes of linear regressions were evaluated by the analysis of covariance (ANCOVA). Significance in the differences between the mean values of each sample was determined by the One Way ANOVA followed by Tukey's test, while the comparison of the mean values of each sample with the control value was performed by the One Way ANOVA followed by Dunnett's test. The level of significance was established at $p<0.05$. The statistical evaluations were performed through the Graph Pad Prism software version 6.01.

3. RESULTS AND DISCUSSION

Four proteolytically active fractions, F1, F3, F4, and F5, were obtained from E_T after following the purification strategy described in the section 2.2 (Supplementary Figure **S1**). Fs are constituted by the S-methylthio-derivatives of: acidic bromelain forms 1 and 2 (F1), basic bromelain forms 1 and 2 (F3), ananain form 1 (F4), and ananain form 2 (F5) [7].

Caseinolytic units (U_C) of the Fs as well as of the S-methylthio-derivative of E_T were measured after activating them and immediately before performing the assays (Supplementary Table **S1**). Assuming that the effects of stem bromelain proteases investigated in this work are linked to their proteolytic activity, the data of different samples were compared considering the U_C, instead of the protein amounts.

Action on the fibrinogen subunits (Aα, Bβ, and γ) was analyzed by tricine-SDS-PAGE after incubating each sample with fibrinogen solution at the same enzyme/substrate ratio (Figure **1**). Electrophoretic profiles show that Aα and Bβ subunits were completely hydrolyzed within 2 min and 30 min, respectively, while the γ subunit was poorly hydrolyzed over 120 min. When the enzyme/substrate ratio was increased 10 times, the γ subunit was completely hydrolyzed after 120 min incubation by all the samples except F1, which increased the degradation anyway (Supplementary Figure **S2**). The higher proteolytic specificity towards Aα and Bβ subunits than towards the γ subunit is characteristic of the thrombin-like enzymes from snake venoms and from plant lattices [35, 36], that was also observed for extracts from stem bromelain and other bromeliad's species [29].

Thrombin converts fibrinogen into fibrin monomers by cleaving fibrinopeptides A (FpA) and B (FpB) from Aα and Bβ, respectively. The FpA and/or FpB release is enough to trigger the polymerization of fibrin monomers by several steps of self-assembly and aggregation that produce the clot, a three-dimensional network, which is then covalently cross-linked by the transglutaminase activity of the plasma Factor XIIIa [37]. The first products of Factor XIIIa activity are γ dimers that can be detected by SDS-PAGE under reducing conditions as a band of about 100 kDa [38]. In Figure **1**, the lanes corresponding to 1 and 2 min of the fibrinogen hydrolysis by F1, F4, and F5 show a broad band with molecular weights around the marker 97.4 kDa that could correspond to γ dimers, since Factor XIII is a usual contaminant of the commercial plasma fibrinogen [38]. When the plasma fibrinogen used in this assay was incubated with thrombin, γ dimers were detected (Supplementary Figure **S3**), corroborating the presence of Factor XIIIa. However, activity of Factor XIII requires activation by thrombin, which could also be attributed to the action of F1, F4, and F5.

Polymerization of fibrin monomers can be followed by changes in turbidity [39]. Figure **2** shows turbidity measurements of a fibrinogen solution at different incubation times with E_T and Fs for three enzyme/substrate ratios. Interestingly, F1, F4, and F5 produced changes in the turbidity of the fibrinogen solution. According to the data reported for a stem bromelain total extract [29], E_T did not show changes, which could be explained by the lowest proportion of F1, F4, and F5 in the E_T sample [7]. F5 showed the highest polymerizing action, followed by F4 and then F1, according to values of polymerization rate (Table **1**) and of the maximum turbidity, which is related to the cross-sectional area of fibrils produced by the aggregation of fibrin monomers [39, 40]. Polymerizing action of F1, F4, and F5 can be attributed to their proteolytic activity, since turbidity was not detected when the fibrinogen polymerization assay was performed with the irreversibly inhibited fractions at the same enzyme/substrate ratio as that used for the respective active fractions (Figure **3**). Considering the electrophoretic profiles and the turbidity curves, it can be assumed that F5 is the most effective proteolytic fraction towards Aα and Bβ subunits to trigger the aggregation of the hydrolyzed fibrinogen, followed by F4, while F1 is the least effective fraction.

In the haemostasis, fibrin formed by thrombin is degraded by plasmin. In comparison with thrombin, turbidity curves obtained with the bromelain fractions were different since they did not have the plateau phase characterizing thrombin, with the exception of F1 at 0.007 U_C/mg fibrinogen. Furthermore, increasing the enzyme/substrate ratio causes an increase in the polymerization rate but also in the decreasing rate of turbidity, which could be due to plasmin-

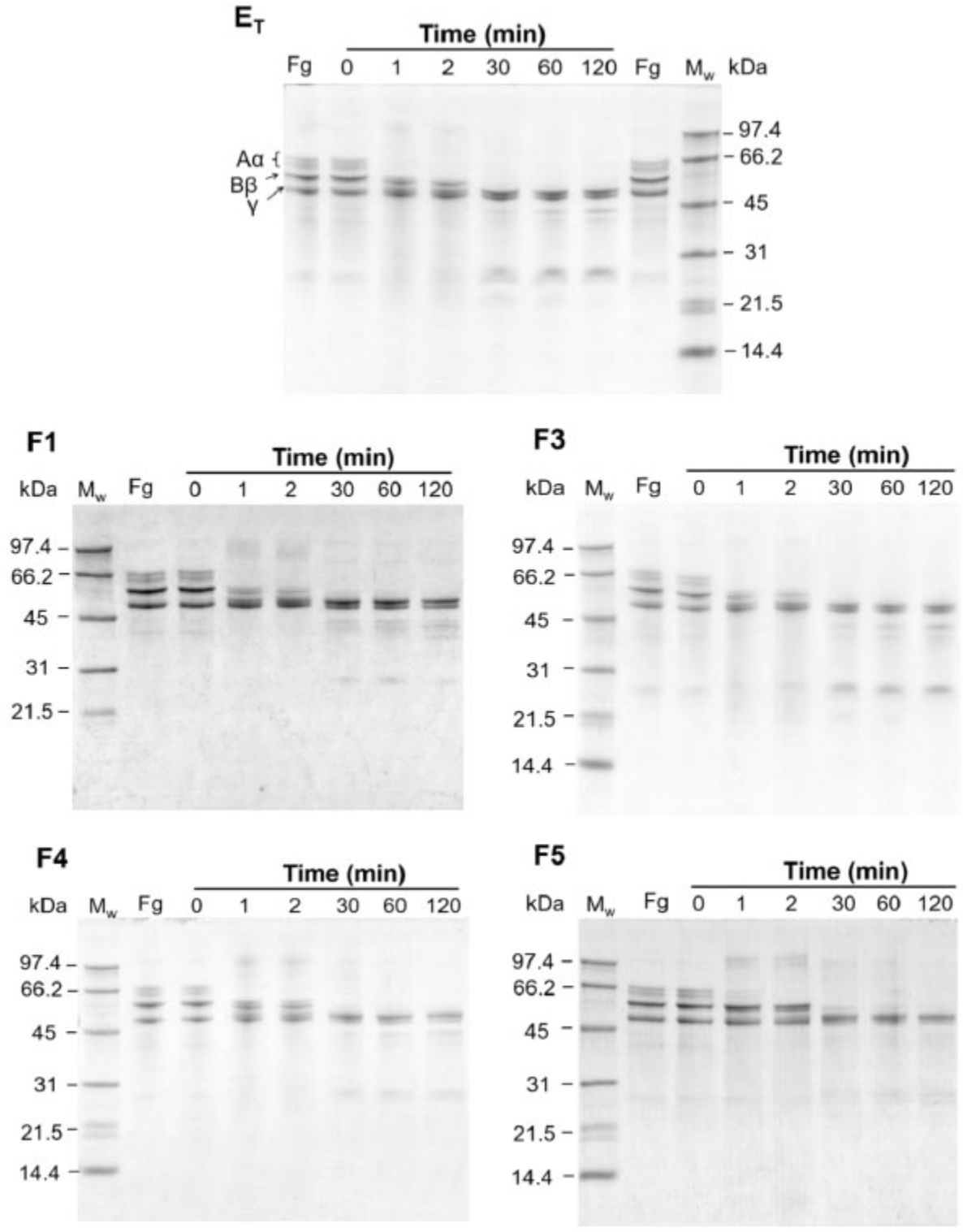

Figure (1). Tricine-SDS-PAGE of fibrinogen hydrolysates obtained with E_T, F1, F3, F4, and F5 for different times. The same enzyme/substrate ratio and equivalent to 0.003 U_C/mg fibrinogen was used for all the samples. Aα, Bβ, and γ indicate fibrinogen subunits; M_W, molecular weight markers; Lane Fg, fibrinogen (3 µg). *A higher resolution / colour version of this figure is available in the electronic copy of the article*.

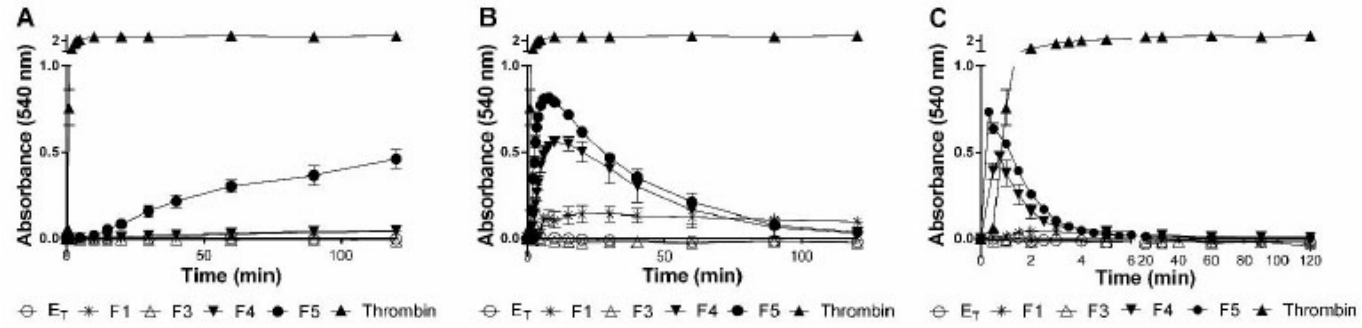

Figure (2). Turbidity curves of fibrinogen solutions incubated with E_T, F1, F3, F4, and F5. Enzyme/substrate ratios (U_C/mg fibrinogen) of 0.0007 (A), 0.007 (B), and 0.07 (C) were assayed. Thrombin was used as a positive standard control at 0.18 NIH U/mg fibrinogen. Values are expressed as the mean ± S.E.M of three independent experiments. *A higher resolution / colour version of this figure is available in the electronic copy of the article.*

Table 1. Fibrinogen polymerizing and fibrinolytic activities.

Sample	Polymerization rate (x min^{-1}) [a]	Plasmin mg/sample mg [b]
E_T	ND	2.40 (± 0.02)
F1	0.027 (± 0.008)	0.003 (± 0.001)
F3	ND	22 (± 2)
F4	1.9 (± 0.1)	0.30 (± 0.02)
F5	8.2 (± 0.2)	0.19 (± 0.04)
Thrombin	2700 (± 500)	ND

[a] Fibrinogen polymerizing activity. Polymerization rates were calculated as the maximum slopes of the turbidity curves for 1 mg/ml of sample. ND, not detected. Values are expressed as the mean (± S.D).

[b] Fibrinolytic activity is expressed as mg of plasmin per mg of sample. ND, not detected. Values are expressed as the mean (± E.P).

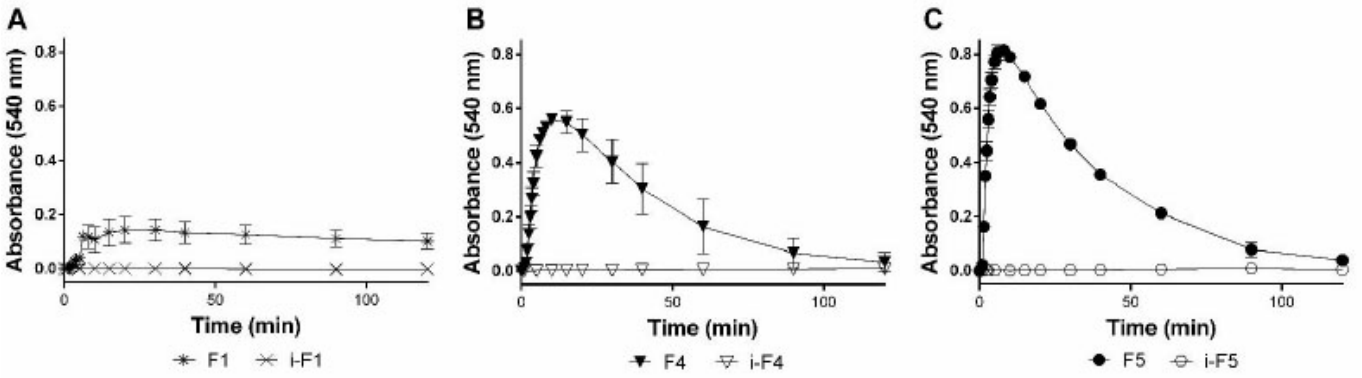

Figure (3). Effect of proteolytic activity on the turbidity curves for F1 (A), F4 (B), and F5 (C) after incubating catalytically competent (F) and irreversibly inhibited (i-F) fractions with fibrinogen solutions at the same enzyme/substrate ratio and equivalent to 0.007 U_C/mg of fibrinogen. Values are expressed as the mean ± S.E.M of three independent experiments. *A higher resolution / colour version of this figure is available in the electronic copy of the article.*

type activity. Thereby, the products formed from fibrinogen by the thrombin-type activity are then hydrolyzed by the plasmin-type activity. This assumption is supported by the electrophoretic profiles, where bands of around 100 kDa begin to disappear after an incubation period of 30 min instead of intensifying.

Plasmin-type activity was measured by the fibrin plate assay (Figure **4**). E_T and Fs showed fibrinolytic activity and a linear double logarithmic relationship between fibrinolytic activity and sample concentration (Supplementary Table **S2**). The slopes were not significantly different, which allowed to convert mg of E_T and Fs to mg of plasmin (Table

Table 2. Anticoagulant effects.

	E_T	F1	F3	F4	F5
U_C/ml for PT^{MAX}	0.76^{ab} (± 0.05)	ND	0.60^{b} (± 0.08)	0.84^{a} (± 0.06)	0.83^{a} (± 0.04)
U_C/ml for $APTT^{MAX}$	0.69^{ac} (± 0.07)	ND	0.42^{b} (± 0.05)	ND	0.82^{c} (± 0.07)

Comparison of proteolytic activity (U_C/ml) required to reach PT^{MAX} and $APTT^{MAX}$ of human plasma upon its incubation with E_T, F1, F3, F4, and F5. ND, not detected. Values are expressed as the mean (± S.D). Mean values of the same row with different superscript letters are significantly different (ANOVA followed by Tukey´s test, $p<0.05$).

Table 3. Procoagulant effects.

	E_T	F1	F3	F4	F5
Maximum percentage shortening of APTT (%)	22^{a} (± 3)	29^{a} (± 3)	29^{a} (± 8)	26^{a} (± 1)	26^{a} (± 1)
Concentration range with procoagulant effect (U_C/ml)	0.20 – 0.38	0.28 – 0.56	0.17 – 0.26	0.36 – 0.72	0.22 – 0.54

Values are expressed as the mean (± S.D). Procoagulant effect was defined when APTT values were shorter than that of the control (ANOVA followed by Dunnett´s test, $p<0.05$). Mean values with different superscript letters are significantly different (ANOVA followed by Tukey´s test, $p<0.05$).

1). Interestingly, samples with the highest fibrinolytic activity (E_T and F3) are the same ones that did not show fibrinogen polymerizing activity. On the other hand, fibrinolytic activity of F4 and F5 were similar and about two orders of magnitude higher than that of F1, which could explain the differences observed in the turbidity curves. Thus, fibrinolytic activity of F4 and F5 would be high enough to hydrolyse faster than F1 the products formed from fibrinogen by the thrombin-type activity.

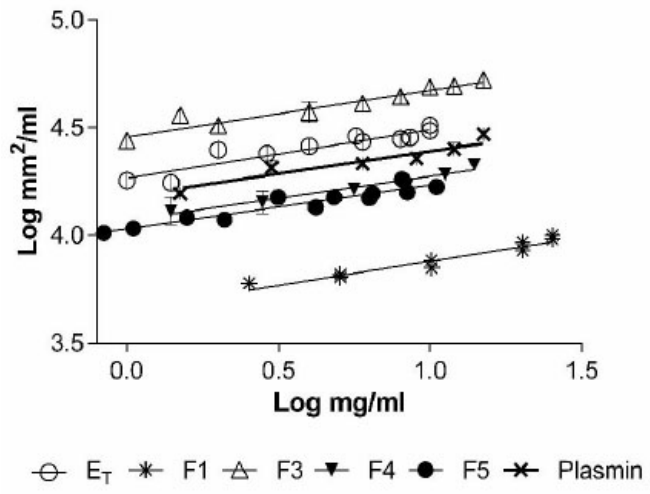

Figure (4). Fibrinolytic activity of E_T, F1, F3, F4, and F5. Double-logarithmic relationship between fibrinolytic activity (mm^2/ml) and sample concentration (mg/ml). Values are expressed as the mean ± S.E.M of three independent experiments. *A higher resolution / colour version of this figure is available in the electronic copy of the article.*

In the blood coagulation process, transformation of fibrinogen into fibrin clot by thrombin is the last reaction of the coagulation cascade, which can be divided into intrinsic, extrinsic and common pathways. PT reflects the enzymatic activations of extrinsic and common pathways, while APTT represents those of the intrinsic and common ones. Anticoagulant effects of E_T and Fs on PT were detected from around 0.5 U_C/ml (Figure **5**). Major differences were found in the U_C/ml required to prolong PT to more than 180 s, which was considered a maximum anticoagulant effect (PT^{MAX}). Thus, F1 did not show PT^{MAX}, F4 and F5 required the highest concentrations to reach it, F3 the lowest one, while E_T required an intermediate concentration value (Table **2**).

APTT curves (Figure **6**) show similar shapes to those reported for stem bromelain and proteolytic extracts of other bromeliad's species [29]. Samples showed anticoagulant effects on APTT, with the exception of F4, and some reached maximum effect with APTT values higher than 180 s ($APTT^{MAX}$). F1 did not show $APTT^{MAX}$, F3 required the lowest concentration to reach it, F5 the highest one, while E_T required an intermediate concentration value (Table **2**). In addition, procoagulant effects were observed since APTT measured for the samples ($APTT_S$) were significantly shorter than that of the control ($APTT_C$). In these cases, percentage shortening of APTT was calculated as $100*(APTT_C-APTT_S)/APTT_C$ and similar maximum values were obtained for E_T and Fs (Table **3**). However, differences were observed in the concentration range showing the procoagulant effect, being wider (about 3 times) for F1, F4, and F5 than for F3, while that of E_T was intermediate (Table **3**). Pro- and anticoagulant effects of E_T and Fs are linked to the proteolytic activity since no changes in PT and APTT were detected when assays were performed with the irreversibly inhibited samples (data not shown).

As the procoagulant effect was only observed in the APTT assay, it could be hypothesized that both, E_T and Fs interfere with some of the plasma factors belonging to the intrinsic pathway. This assumption could have some relationship with the action of bromelain on the kallikrein-kinin system by activation of the Factor XII (the first coagulation factor involved in the intrinsic pathway) that was detected from *in vivo* and *in vitro* assays [41, 42]. In addition to our previously published work [29], only Kaur *et al*. [25] have reported the procoagulant action of bromelain. In this work [25], an increase in the coagulability was detected by thromboelastography in mice treated with stem bromelain extract, which was paradoxical since stem bromelain behaved as anticoagulant in the *in vitro* assays. One of the explanations was that

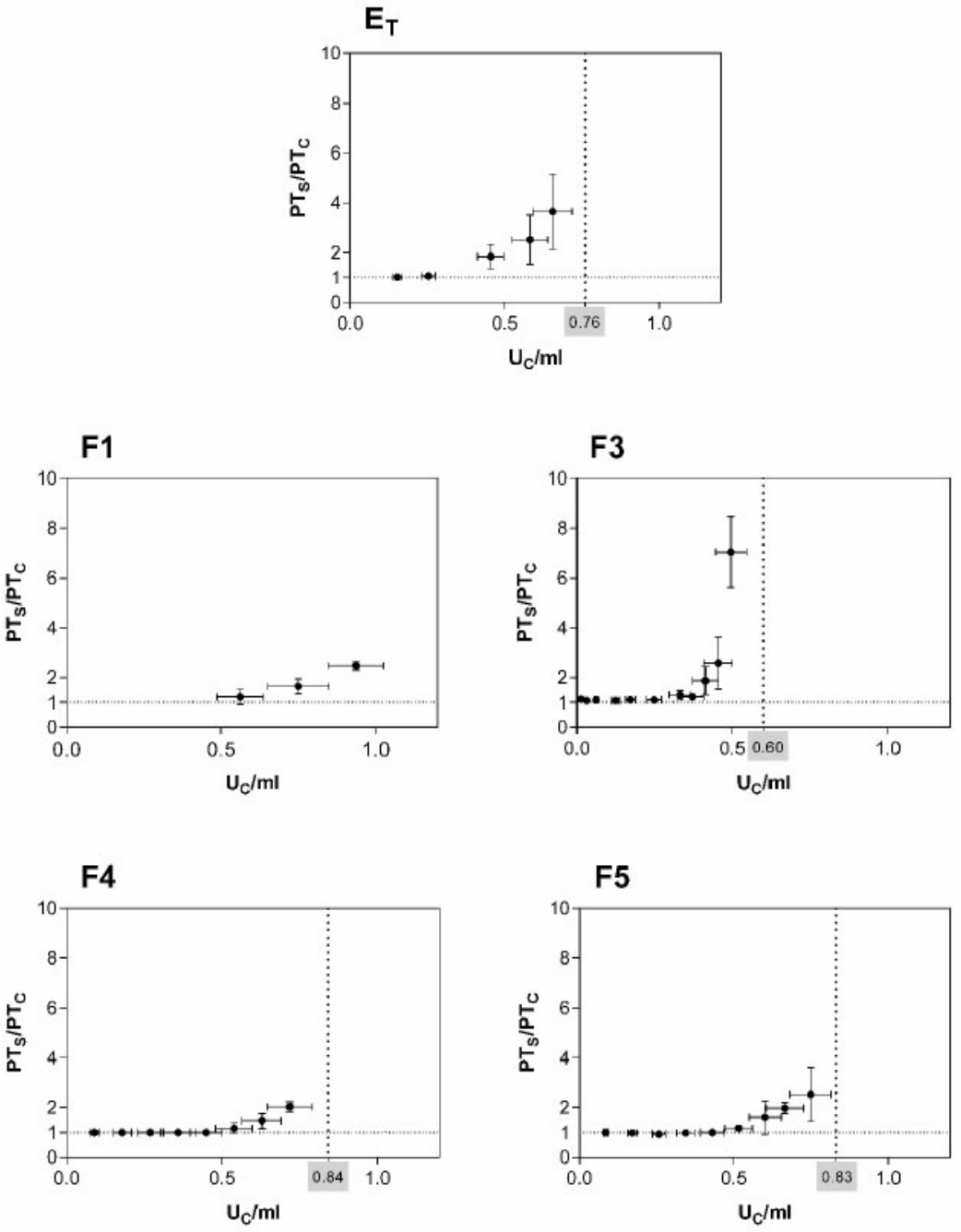

Figure (5). Relative PT for E_T, F1, F3, F4, and F5. Dotted vertical lines indicate the proteolytic activity (U_C/ml) of each sample required to reach PT^{MAX}. PT_S, PT of enzymatically treated human plasma; PT_C, PT of the control. Values are expressed as the mean ± S.D. *A higher resolution / colour version of this figure is available in the electronic copy of the article*.

bromelain concentration reached in the mice blood was lower than that of the *in vitro* assay. Even lowering the dose, the blood coagulability increased in the *in vivo* assay. The analgesic effect of stem bromelain also showed similar relationship with the doses in clinical and animal studies, which was explained by the presence of proteases with different proportions and specificities in the stem bromelain total extract [43]. Thereby, proteases with the specificity to release the active product predominate at low doses, while other proteases able to digest it predominate at high concentrations. In the present work, the dual action of the stem bromelain extract detected by the APTT test would depend on the concentration of active proteases rather than on a particular proteolytic component.

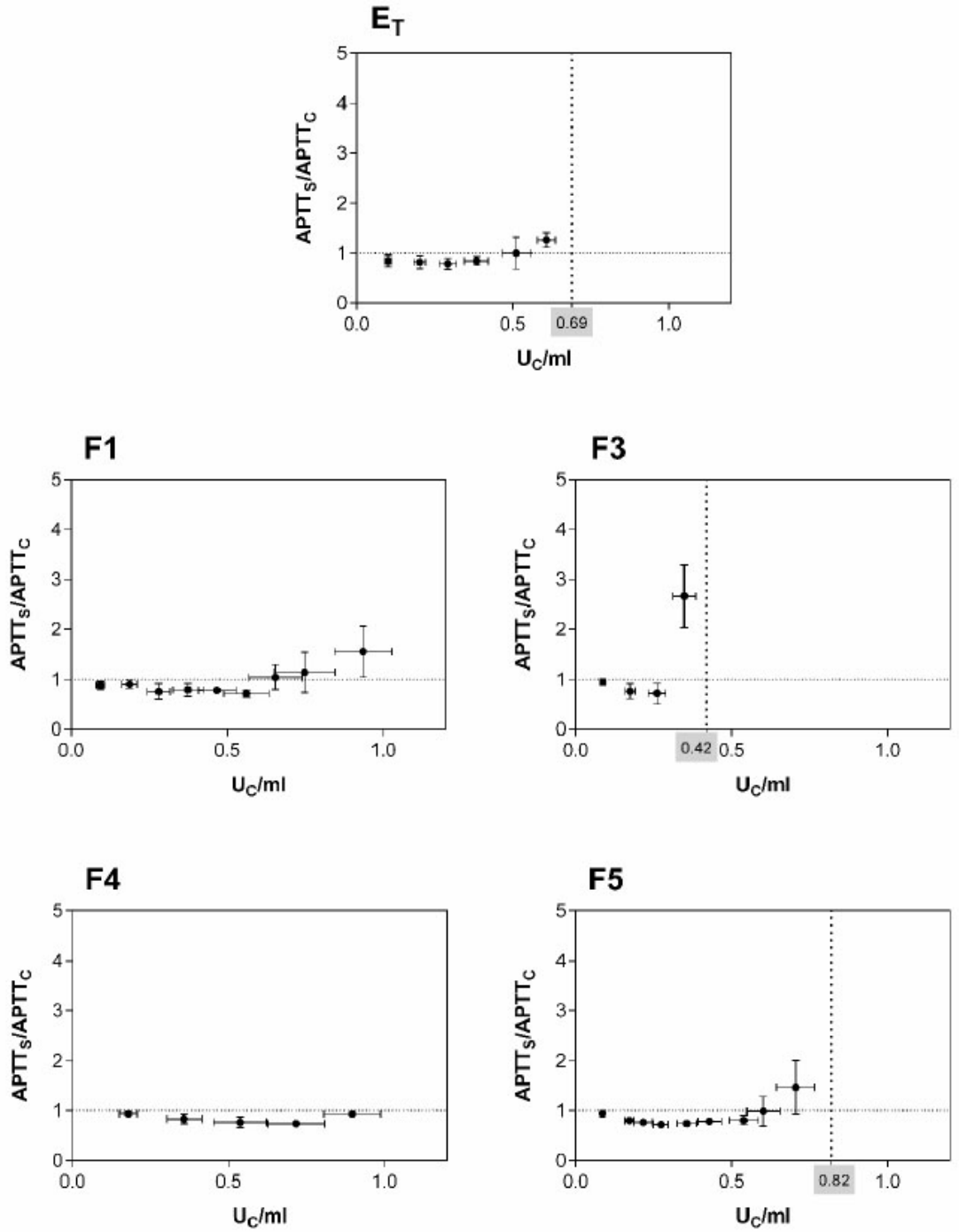

Figure (6). Relative APTT for E_T, F1, F3, F4, and F5. Dotted vertical lines indicate the proteolytic activity (U_C/ml) of each sample required to reach $APTT^{MAX}$. $APTT_S$, APTT of enzymatically treated human plasma; $APTT_C$, APTT of the control. Values are expressed as the mean ± S.D. *A higher resolution / colour version of this figure is available in the electronic copy of the article*.

According to data of Tables **1** and **2**, the fraction with the highest fibrinolytic activity, F3, required the lowest concentration to show PT^{MAX} and $APTT^{MAX}$, the reverse was observed for F1, F4, and F5, with F1 exhibiting the lowest fibrinolytic and anticoagulant actions. In this sense, and without considering the possibility of other blood components being targeted by the samples, it could be hypothesized that the formation of a macroscopic fibrin clot is delayed or prevented when the sample concentration exhibits enough fibrinolytic activity to degrade the fibrin that is forming. Furthermore, it

can be assumed that the fibrinogen polymerizing activity of F1, F4, and F5 counteracts the already low fibrinolytic activity of these fractions, thus decreasing their potency to act as anticoagulant agents, compared to F3.

CONCLUSION

Acidic bromelains, basic bromelains, and ananains isolated from stem bromelain were compared with respect to their action on fibrinogen, fibrin, and blood coagulation. Analysis of the obtained data allows us to conclude that two sub-populations of cysteine proteases exhibiting different biological effects are present in the stem bromelain extract. On the one hand, the basic bromelain forms 1 and 2 (F3) without thrombin-type activity and with the highest plasmin-type activity, being the most effective fibrinolytic and anticoagulant agents present in the stem bromelain extract. On the other hand, the acidic bromelain forms 1 and 2 (F1) as well as the ananain form 1 (F4) and ananain form 2 (F5), which showed thrombin-type activity and the lowest plasmin-type activities, with acidic bromelains being the least effective as fibrinolytic and anticoagulant agents.

As a corollary, changes in both, proteases composition and proteolytic activity of the stem bromelain extracts could lead to diametrically opposite results regarding blood coagulability. The present study highlights the importance of using a well-characterized stem bromelain extract with respect to its proteolytic system for a better understanding of the mechanisms underlying the bromelain action and hopefully will constitute a worthwhile subject for its further, more detailed investigation.

LIST OF ABBREVIATIONS

APTT	=	Activated Partial Thromboplastin Time
APTT^{MAX}	=	APTT value higher than 180 s
E_T	=	stem bromelain total extract
Fs	=	proteolytic fractions chromatographically separated from E_T
PT	=	Prothrombin Time
PT^{MAX}	=	PT value higher than 180s
U_C	=	caseinolytic Unit

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Pool of platelet-poor plasma was prepared and provided by the staff of the Public Health Laboratory of the National University of La Plata (UNLP) from blood of 16 healthy individuals. This procedure was analyzed and endorsed by the Bioethical Committee of the UNLP (Record N° 59, File N° 0700-000017/19-000).

HUMAN AND ANIMAL RIGHTS

Prior to initiation of the research work, the procedure was analyzed and endorsed by the Bioethical Committee of the UNLP (Record N° 59, File N° 0700-000017/19-000) according to requirements of Argentine laws (25326 and 26529) and the Declaration of Helsinki.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

FUNDING

This work was financially supported by the Universidad Nacional de La Plata [grant number X-834] and by grant from the Comisión de Investigaciones Científicas de la Provincia de Buenos Aires (CICPBA).

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

MEE is member of the CICPBA Researcher Career. The authors gratefully acknowledge Professor Mariela Bruno and the staff of the Laboratorio de Salud Pública of the UNLP for obtaining the blood samples and Miss L. Bolle for her technical contribution to the preparation and characterization of the proteolytic fractions of stem bromelain.

SUPPLEMENTARY MATERIAL

Supplementary material isavailable on the publisher's web site along with the published article.

REFERENCES

[1] Heinicke, R.M.; Gortner, W.A. Stem Bromelain—a New Protease Preparation from Pineapple Plants. *Econ. Bot.,* **1957**, *11*(3), 225-234. [http://dx.doi.org/10.1007/BF02860437]

[2] Harrach, T.; Eckert, K.; Maurer, H.R.; Machleidt, I.; Machleidt, W.; Nuck, R. Isolation and characterization of two forms of an acidic bromelain stem proteinase. *J. Protein Chem.,* **1998**, *17*(4), 351-361. [http://dx.doi.org/10.1023/A:1022507316434] [PMID: 9619588]

[3] Harrach, T.; Eckert, K.; Schulze-Forster, K.; Nuck, R.; Grunow, D.; Maurer, H.R. Isolation and partial characterization of basic proteinases from stem bromelain. *J. Protein Chem.,* **1995**, *14*(1), 41-52. [http://dx.doi.org/10.1007/BF01902843] [PMID: 7779262]

[4] Napper, A.D.; Bennett, S.P.; Borowski, M.; Holdridge, M.B.; Leonard, M.J.; Rogers, E.E.; Duan, Y.; Laursen, R.A.; Reinhold, B.; Shames, S.L. Purification and characterization of multiple forms of the pineapple-stem-derived cysteine proteinases ananain and comosain. *Biochem. J.,* **1994**, *301*(Pt 3), 727-735. [http://dx.doi.org/10.1042/bj3010727] [PMID: 8053898]

[5] Ritonja, A.; Rowan, A.D.; Buttle, D.J.; Rawlings, N.D.; Turk, V.; Barrett, A.J. Stem bromelain: amino acid sequence and implications for weak binding of cystatin. *FEBS Lett.,* **1989**, *247*(2), 419-424. [http://dx.doi.org/10.1016/0014-5793(89)81383-3] [PMID: 2714443]

[6] Rowan, A.D.; Buttle, D.J.; Barrett, A.J. The cysteine proteinases of the pineapple plant. *Biochem. J.,* **1990**, *266*(3), 869-875. [PMID: 2327970]

[7] Matagne, A.; Bolle, L.; El Mahyaoui, R.; Baeyens-Volant, D.; Azarkan, M. The proteolytic system of pineapple stems revisited: Purification and characterization of multiple catalytically active forms. *Phytochemistry,* **2017**, *138*, 29-51.

[http://dx.doi.org/10.1016/j.phytochem.2017.02.019] [PMID: 28238440]

[8] Hatano, K.; Kojima, M.; Tanokura, M.; Takahashi, K. Solution structure of bromelain inhibitor IV from pineapple stem: structural similarity with Bowman-Birk trypsin/chymotrypsin inhibitor from soybean. *Biochemistry,* **1996**, *35*(17), 5379-5384. [http://dx.doi.org/10.1021/bi952754+] [PMID: 8611527]

[9] Lenarcic, B.; Ritonja, A.; Turk, B.; Dolenc, I.; Turk, V. Characterization and structure of pineapple stem inhibitor of cysteine proteinases. *Biol. Chem. Hoppe Seyler,* **1992**, *373*(7), 459-464. [http://dx.doi.org/10.1515/bchm3.1992.373.2.459] [PMID: 1515075]

[10] Rowan, A.D.; Buttle, D.J. Pineapple Cysteine Endopeptidases.*Methods in Enzymology*; Elsevier, **1994**, Vol. 244, pp. 555-568. [38]

[11] Azarkan, M.; Feller, G.; Vandenameele, J.; Herman, R.; El Mahyaoui, R.; Sauvage, E.; Vanden Broeck, A.; Matagne, A.; Charlier, P.; Kerff, F. Biochemical and structural characterization of a mannose binding jacalin-related lectin with two-sugar binding sites from pineapple (Ananas comosus) stem. *Sci. Rep.,* **2018**, *8*(1), 11508. [http://dx.doi.org/10.1038/s41598-018-29439-x] [PMID: 30065388]

[12] de Lencastre Novaes, L.C.; Jozala, A.F.; Lopes, A.M.; de Carvalho Santos-Ebinuma, V.; Mazzola, P.G.; Pessoa Junior, A. Stability, purification, and applications of bromelain: A review. *Biotechnol. Prog.,* **2016**, *32*(1), 5-13. [http://dx.doi.org/10.1002/btpr.2190] [PMID: 26518672]

[13] Maurer, H.R. Bromelain: biochemistry, pharmacology and medical use. *Cell. Mol. Life Sci.,* **2001**, *58*(9), 1234-1245. [http://dx.doi.org/10.1007/PL00000936] [PMID: 11577981]

[14] Kasemsuk, T.; Saengpetch, N.; Sibmooh, N.; Unchern, S. Improved WOMAC score following 16-week treatment with bromelain for knee osteoarthritis. *Clin. Rheumatol.,* **2016**, *35*(10), 2531-2540. [http://dx.doi.org/10.1007/s10067-016-3363-1] [PMID: 27470088]

[15] de Souza, G.M.; Fernandes, I.A.; Dos Santos, C.R.R.; Falci, S.G.M. Is bromelain effective in controlling the inflammatory parameters of pain, edema, and trismus after lower third molar surgery? A systematic review and meta-analysis. *Phytother. Res.,* **2019**, *33*(3), 473-481. [http://dx.doi.org/10.1002/ptr.6244] [PMID: 30484910]

[16] Fusini, F.; Bisicchia, S.; Bottegoni, C.; Gigante, A.; Zanchini, F.; Busilacchi, A. Nutraceutical supplement in the management of tendinopathies: a systematic review. *Muscles Ligaments Tendons J.,* **2016**, *6*(1), 48-57. [http://dx.doi.org/10.32098/mltj.01.2016.06] [PMID: 27331031]

[17] Marzin, T.; Lorkowski, G.; Reule, C.; Rau, S.; Pabst, E.; Vester, J.C.; Pabst, H. Effects of a systemic enzyme therapy in healthy active adults after exhaustive eccentric exercise: a randomised, two-stage, double-blinded, placebo-controlled trial. *BMJ Open Sport Exerc. Med.,* **2017**, *2*(1)e000191 [http://dx.doi.org/10.1136/bmjsem-2016-000191] [PMID: 28879033]

[18] Seamont, D.; Vrcek, I.; Nakra, T.; Mancini, R. Arnica and Bromelain for Blepharoplasty-Associated Ecchymosis. *Am. J. Cosmet. Surg.,* **2018**, *35*(3), 130-134. [http://dx.doi.org/10.1177/0748806817743904]

[19] Baumueller, M.; Rau, S. Efficacy and Tolerance of Systemic Enzyme Therapy in the Treatment of Acute Thrombophlebitis–a Randomised Double-Blind Controlled Trial. *J. Phlebol. Lymphology,* **2018**, *11*(1) [http://dx.doi.org/10.14303/1983-8905.1000050]

[20] Loo, Y.L.; Goh, B.K.L.; Jeffery, S. An Overview of the Use of Bromelain-Based Enzymatic Debridement (Nexobrid®) in Deep Partial and Full Thickness Burns: Appraising the Evidence. *J. Burn Care Res.,* **2018**, *39*(6), 932-938. [http://dx.doi.org/10.1093/jbcr/iry009] [PMID: 29579268]

[21] Beuth, J. Proteolytic enzyme therapy in evidence-based complementary oncology: fact or fiction? *Integr. Cancer Ther.,* **2008**, *7*(4), 311-316. [http://dx.doi.org/10.1177/1534735408327251] [PMID: 19116226]

[22] Lee, J-H.; Lee, J-T.; Park, H-R.; Kim, J-B. The potential use of bromelain as a natural oral medicine having anticarcinogenic activities. *Food Sci. Nutr.,* **2019**, *7*(5), 1656-1667. [http://dx.doi.org/10.1002/fsn3.999] [PMID: 31139378]

[23] Ley, C.M.; Tsiami, A.; Ni, Q.; Robinson, N. A review of the use of bromelain in cardiovascular diseases. *J. Chin. Integr. Med.,* **2011**, *9*(7), 702-710. [http://dx.doi.org/10.3736/jcim20110702] [PMID: 21749819]

[24] Lotz-Winter, H. On the pharmacology of bromelain: an update with special regard to animal studies on dose-dependent effects. *Planta Med.,* **1990**, *56*(3), 249-253. [http://dx.doi.org/10.1055/s-2006-960949] [PMID: 2203073]

[25] Kaur, H.; Corscadden, K.; Lott, C.; Elbatarny, H.S.; Othman, M. Bromelain has paradoxical effects on blood coagulability: a study using thromboelastography. *Blood Coagul. Fibrinolysis,* **2016**, *27*(7), 745-752. [http://dx.doi.org/10.1097/MBC.0000000000000244] [PMID: 25517253]

[26] Cirelli, M.G.; Smyth, R.D. Effects of bromelain anti-edema therapy on coagulation, bleeding, and prothrombin times. *J. New Drugs,* **1963**, *3*(1), 37-39. [http://dx.doi.org/10.1002/j.1552-4604.1963.tb00060.x] [PMID: 14021409]

[27] Pirotta, F.; de Giuli-Morghen, C. Bromelain: Antiinflammatory and Serum Fibrinolytic Activity after Oral Administration in the Rat. *Drugs Exp. Clin. Res.,* **1978**, *4*, 1-20.

[28] Martín, N.; Guilabert, P.; Abarca, L.; Usua, G.M.; Serracanta, J.; Colomina, M.J. Coagulation Abnormalities Following NexoBrid Use: A Case Report. *J. Burn Care Res.,* **2018**, *39*(6), 1067-1070. [http://dx.doi.org/10.1093/jbcr/irx044] [PMID: 29931322]

[29] Errasti, M.E.; Prospitti, A.; Viana, C.A.; Gonzalez, M.M.; Ramos, M.V.; Rotelli, A.E.; Caffini, N.O. Effects on fibrinogen, fibrin, and blood coagulation of proteolytic extracts from fruits of Pseudananas macrodontes, Bromelia balansae, and B. hieronymi (Bromeliaceae) in comparison with bromelain. *Blood Coagul. Fibrinolysis,* **2016**, *27*(4), 441-449. [http://dx.doi.org/10.1097/MBC.0000000000000531] [PMID: 26886361]

[30] Hale, L.P.; Greer, P.K.; Trinh, C.T.; James, C.L. Proteinase activity and stability of natural bromelain preparations. *Int. Immunopharmacol.,* **2005**, *5*(4), 783-793. [http://dx.doi.org/10.1016/j.intimp.2004.12.007] [PMID: 15710346]

[31] Azarkan, M.; Maes, D.; Bouckaert, J.; Thi, M.H.D.; Wyns, L.; Looze, Y. Thiolpegylation facilitates purification of chymopapain leading to diffraction studies at 1.4 Å resolution. *J. Chromatogr. A,* **1996**, *749*(1-2), 69-72. [http://dx.doi.org/10.1016/0021-9673(96)00360-3] [PMID: 8680598]

[32] Azarkan, M.; Matagne, A.; Wattiez, R.; Bolle, L.; Vandenameele, J.; Baeyens-Volant, D. Selective and reversible thiol-pegylation, an effective approach for purification and characterization of five fully active ficin (iso)forms from Ficus carica latex. *Phytochemistry,* **2011**, *72*(14-15), 1718-1731. [http://dx.doi.org/10.1016/j.phytochem.2011.05.009] [PMID: 21665232]

[33] López, L.M.; Sequeiros, C.; Natalucci, C.L.; Brullo, A.; Maras, B.; Barra, D.; Caffini, N.O. Purification and characterization of macrodontain I, a cysteine peptidase from unripe fruits of Pseudananas macrodontes (Morr.) harms (Bromeliaceae). *Protein Expr. Purif.,* **2000**, *18*(2), 133-140. [http://dx.doi.org/10.1006/prep.1999.1165] [PMID: 10686143]

[34] Schägger, H. Tricine-SDS-PAGE. *Nat. Protoc.,* **2006**, *1*(1), 16-22. [http://dx.doi.org/10.1038/nprot.2006.4] [PMID: 17406207]

[35] Shivaprasad, H.V.; Rajaiah, R.; Frey, B.M.; Frey, F.J.; Vishwanath, B.S. 'Pergularain e I'--a plant cysteine protease with thrombin-like activity from Pergularia extensa latex. *Thromb. Res.,* **2010**, *125*(3), e100-e105. [http://dx.doi.org/10.1016/j.thromres.2009.10.002] [PMID: 19853890]

[36] Viana, C.A.; Oliveira, J.S.; Freitas, C.D.; Alencar, N.M.; Carvalho, C.P.; Nishi, B.C.; Ramos, M.V. Thrombin and plasmin-like ac-

tivities in the latices of Cryptostegia grandiflora and Plumeria rubra. *Blood Coagul. Fibrinolysis,* **2013**, *24*(4), 386-392. [http://dx.doi.org/10.1097/MBC.0b013e32835d540b] [PMID: 23314383]

[37] Weisel, J.W.; Litvinov, R.I. Fibrin formation, structure and properties.*Fibrous Proteins: Structures and Mechanisms*; Springer, **2017**, pp. 405-456. [http://dx.doi.org/10.1007/978-3-319-49674-0_13]

[38] Gorkun, O.V.; Veklich, Y.I.; Weisel, J.W.; Lord, S.T. The conversion of fibrinogen to fibrin: recombinant fibrinogen typifies plasma fibrinogen. *Blood,* **1997**, *89*(12), 4407-4414. [http://dx.doi.org/10.1182/blood.V89.12.4407] [PMID: 9192765]

[39] Weisel, J.W.; Nagaswami, C. Computer modeling of fibrin polymerization kinetics correlated with electron microscope and turbidity observations: clot structure and assembly are kinetically controlled. *Biophys. J.,* **1992**, *63*(1), 111-128. [http://dx.doi.org/10.1016/S0006-3495(92)81594-1] [PMID: 1420861]

[40] Zubairova, L.D.; Nabiullina, R.M.; Nagaswami, C.; Zuev, Y.F.; Mustafin, I.G.; Litvinov, R.I.; Weisel, J.W. Circulating Microparticles Alter Formation, Structure, and Properties of Fibrin Clots. *Sci. Rep.,* **2015**, *5*, 17611. [http://dx.doi.org/10.1038/srep17611] [PMID: 26635081]

[41] Oh-ishi, S.; Uchida, Y.; Ueno, A.; Katori, M. Bromelian, a thiol-protease from pineapple stem, depletes high molecular weight kininogen by activation of Hageman factor (Factor XIII). *Thromb. Res.,* **1979**, *14*(4-5), 665-672. [http://dx.doi.org/10.1016/0049-3848(79)90121-X] [PMID: 483261]

[42] Oh-ishi, S. Fluid phase activation of Hageman factor (factor XII) in citrated human plasma by bromelain: an application to the indirect enzymatic assay for Hageman factor. *Thromb. Res.,* **1982**, *27*(5), 619-623. [http://dx.doi.org/10.1016/0049-3848(82)90309-7] [PMID: 6983741]

[43] Orlandi-Mattos, P.E.; Aguiar, R.B.; da Silva Vaz, I.; Moraes, J.Z.; de Araujo Carlini, E.L.; Juliano, M.A.; Juliano, L. Enkephalin related peptides are released from jejunum wall by orally ingested bromelain. *Peptides,* **2019**, *115*, 32-42. [http://dx.doi.org/10.1016/j.peptides.2019.02.008] [PMID: 30836111]