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A plant proteinase, extracted from *Bromelia fastuosa*, as an alternative to proteinase K for DNA extraction.

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Abstract: We report the use of brofasin, a proteolytic enzyme extracted from the fruits of *Bromelia fastuosa*, as a protective agent in DNA extraction protocols. Brofasin at concentrations varying between 236 units/µg to 2,372 units/µg was used to extract DNA of human leukocytes and *Drosophila melanogaster* tissues. At brofasin concentration between 1,186 and 2,372 units/µg the resulting DNA concentrations are similar to those obtained by treatment with proteinase K. The good yield of extracted DNA and its intactness and availability for further analysis, verified by PCR and restriction enzyme digestion reactions, suggest the use of this enzyme as an alternative to proteinase K.

Introduction

Proteinases are hydrolytic enzymes used by industrial processing to transform proteins in shorter peptides for several purposes (Flynn, 1975). These enzymes act by hydrolizing peptide bonds and exhibit a wide range of cleavage preferences. Extraction and purification of several plant proteinases can be relatively easy and can be done with low-cost procedures. That is the example of papain, used in meat tenderization and beverage clarification (Flynn, 1975; Castro, 1981; Salunke and Desai, 1984; Poulter and Caygill, 1985).

Proteinases can be also used as protective agents during DNA extraction, by inactivating endogenous nucleases. Proteinase K, a Ca^{2+}-dependent enzyme extracted from *Tritirachium album* (Ebeling *et al.*, 1974) is largely used with that purpose, remaining substantially active during incubation above 50ºC in SDS-containing buffers (Blind and Stafford, 1976; Krusius and Finne, 1982).

Recently Genelhu *et al.* (1998) isolated by gel filtration and ion-exchange chromatography a 22-24 kD plant cysteine-proteinase from *Carica candamarcensis* which was named as E6870. The authors proposed the use of this enzyme as a good option as protective agent during isolation of bacterial genomic DNA.

The present work is part of a research involving the purification and characterization of proteolytic enzymes from the fruits of *Bromelia fastuosa* (known as gravatá). Preliminary studies performed with enzyme inhibitors (Mateus *et al.*, 2000; Cabral *et al.*, 2000; Romanelli *et al.*, 1994) classified this enzyme, named brofasin, as a cysteine-proteinase, a class of enzymes having a cysteine and a histidine residues in the active site (Dunn, 1996).

As low-cost alternative products are very welcome in molecular biology procedures, we report results obtained with brofasin which illustrate its use as a protective agent in DNA extraction protocols from human leukocytes and *Drosophila melanogaster* tissues in comparison with proteinase K.

Material and Methods

All purification procedures concerning brofasin were carried out at low temperature (0-4ºC). Purification of the proteinase started with salt precipitation using ammonium sulphate (AS) of the extract obtained from the pulp of unripe fruits. The precipitation procedure started with 50% AS, decreasing progressively to 30%. The supernatant, at this concentration, exhibited the highest level of proteolytic activity. After dialysis against buffer A (70 mM acetate buffer pH 3.6) the supernatant was applied to a 5 × 11 cm BioRad column containing CM-Sepharose equilibrated with buffer A. The elution was done by salt gradient against buffer A containing 2 M sodium chloride. The fractions containing proteolytic activity were pooled, concentrated by centrifugation on Amicon centriprep-10 and submitted to gel-filtration. This operation was performed using a 1.5 × 70 cm BioRad column and Sephadex G-50 (Sigma). The enzyme containing fractions were applied subsequently to isoelectric focusing performed in a Rotofor Cell (BioRad) using an EPS 3501 power supply (Pharmacia) and cooled with a refrigerated circulating water bath (Lauda WK-500).

Proteolytic activity was measured using 1% casein (Sigma), stopping the reaction with 10% trichloroacetic acid (TCA). Every test had its own blank. The increase of absorbance at 280 nm in the supernatant after centrifugation is proportional to soluble peptides that remain in solution (Sarath, 1996). A unit of enzyme activity was defined as the amount of enzyme required to produce release one µmol of Tyrosine per minute in the essay. To relate the absorbance reading at 280 nm with Tyrosine concentration we used an extinction coefficient of 0.005 ml μg^{-1} cm^{-1} conditions (Moyano-López *et al.*, 1999).

Suitability of brofasin was tested for genomic DNA extraction from insect and human cells.

For insects we tested two protocols for DNA extraction from *Drosophila melanogaster*, which differ only in terms of the buffer. The first protocol used an extraction buffer containing urea (7M urea, 350 mM NaCl, 20 mM EDTA, 1% n-lauryl sarcosine and 0.5 % SDS). The second method used a different buffer, without urea (10 mM Tris-HCl pH 8.0, 10 mM EDTA, 350 mM NaCl, 0.5 % SDS). For both experiments we used six experimental groups, each constituted by 15 specimens, which were squashed with the extraction buffers. The first two groups were the positive control (group 1), with 400 units/µg of proteinase K, and the negative control (group 2), without any enzyme. The brofasin containing groups (3 to 6) contained, respectively, 2,372 units/µg, 1,185 units/µg, 474 units/µg and 236 units/µg of brofasin.

The eppendorf tubes of each experimental group were incubated at 50ºC for 20 minutes, followed by a standard phenol/chloroform/isoamyl alcohol extraction and sodium acetate/absolute ethanol precipitation (Sambrook *et al.*, 1989). After washing with 70% ethanol, the dried pellet was dissolved in 20 µl TE (Tris-EDTA) and 10 µl of this solution was electrophoresed on a 1% agarose gel in TAE buffer containing 0.5µg of ethidium bromide.

A sample of this DNA was also spectrophotometrically quantified. To check for possible adverse effects on DNA, the intactness of DNA resulting from this extraction was evaluated by applying the PCR technique. The PCR reaction was carried out by standard methods using oligonucleotide primers that flank a 764 bp fragment of *P* transposable element, a middle repetitive sequence DNA of *D. melanogaster* genomes (primer 829: 5´-AACATAAGGTGGTCCCGTCG 3´ is complementary to nucleotides 12 to 31 of a complete *P* element and the primer 830: 5´-CGACTGGGAAAGGAAATCC 3´ is complementary to nucleotides 757 to 776). Temperature cycling was performed with the following profile: 92°C for 1 min; 50°C for 1 min; 72°C for 1 min, for a total of 30 cycles.

We also performed an additional evaluation of DNA intactness carrying out restriction enzyme digestion with endonuclease *Hin*dIII, performed according to manufacturers recommendation.

Table 1. Genomic DNA concentrations (ng/µL) produced in six experimental groups of *Drosophila melanogaster*, with two extraction buffers (with and without urea).

Experimental group	Enzyme concentration (units/µg)	DNA concentrations	
		Buffer with urea	Buffer without urea
1. Proteinase K	0.4	212.3	85.9
2. without proteinase	—	262.2	93.9
3. Brofasin	11.86	349.5	174.5
4. Brofasin	5.93	254.0	129.3
5. Brofasin	2.37	129.3	111.1
6. Brofasin	1.18	12.8	3.9

In a second group of experiments we evaluated the performance of brofasin to obtain human genomic DNA. Lymphocytes from collected blood samples were isolated using the Ficoll-Histopaque procedure. DNA was isolated from lymphocytes by the salting out extraction procedure (Abdel-Rahman *et al.*, 1994). To each eppendorf tube containing the isolated lymphocytes were added the lysis buffer (10 mM Tris-HCl, 400 mM NaCl, 2 mM EDTA) and 28.8 units/µg of RNase A, constituting three experimental groups: the negative control group (1), without any proteinase, the positive control group (2), to which was added 20 units/µg of proteinase K, and the brofasin group (3), to which was added 65.3 units/µg of the enzyme. Precipitation was performed with sodium chloride/absolute ethanol. After washing with 70% ethanol, the dried pellet was dissolved in 300 µl H_2O and 10 µl of this solution was electrophoresed on a 1% agarose gel in TAE buffer containing 0.5µg of ethidium bromide. DNA concentration was measured spectrophotometrically.

The viability of the resulting DNA was checked by amplification of a sequence of the CYP2E1 gene (locus 1370 - 1349: 5´-CCA GTC GAG TCT ACA TTG TCA and locus 999-978: 5´- TTC ATT CTG TCT AAC TGG) by the PCR reaction consisting of 25 cycles of denaturation at 95ºC (1 minute), annealing at 55ºC (1 minute) and extension at 72ºC (1 minute). The DNA intactness was also checked by a restriction enzyme digestion with endonuclease *Eco*RI performed according to manufacturers recommendation.

The extracted DNA from *Drosophila* and humans, their products of amplification and digested fragments were separated in a 1% agarose gel in TAE buffer containing 0.5µg of ethidium bromide.

Results and Discussion

Table 1 shows the concentrations of genomic DNA of *D. melanogaster* obtained with both kinds of extraction buffers. In those groups where extraction buffer containing urea was used, higher amounts of DNA were recovered. Group 3 (2,372 units/µg of brofasin) yield the higher amount of DNA (349.5 ng/µL), even higher than the proteinase K group (212.3 ng/µL). The amount in groups 1, 2 and 4 were similar and groups 5 and 6 showed a significant decrease in DNA yield. Without urea the concentration of 236 units/µg of brofasin (group 6) was shown to be inadequate for DNA extraction. Concerning the tests using extraction buffers without urea, the amount of recovered DNA was significantly inferior compared to the urea buffer, in all groups; in the three experimental groups containing brofasin (3, 4 and 5), DNA concentration varied between 111.1 and 174.5 ng/µL, being higher than those obtained with proteinase K (85.6 ng/µL). Samples of these genomic DNA (10 µL) that was run in 1% agarose gels are shown in Figures 1 A (DNA extracted with urea) and 2A (DNA extracted without urea).

More important than the amount of DNA is the quality and its availability for ulterior analysis. PCR amplification of *P* sequences produced the expected fragment with 764 bp, for almost all the concentrations of brofasin and the positive control as well (Figures 1B and 2B). The only failure on obtaining amplification occurred for group 6 in the absence of urea (Figure 2B-6), probably due to the

low template concentration. The DNA produced in the negative control (extraction without proteinase K) also did not show amplification (Figures 1B and 2B), independently of the buffer used.

As shown on Table 1 and Figures 1A and 1B, there was enough concentration for amplification in the samples of group 2; the band of available genomic DNA to be amplified can be

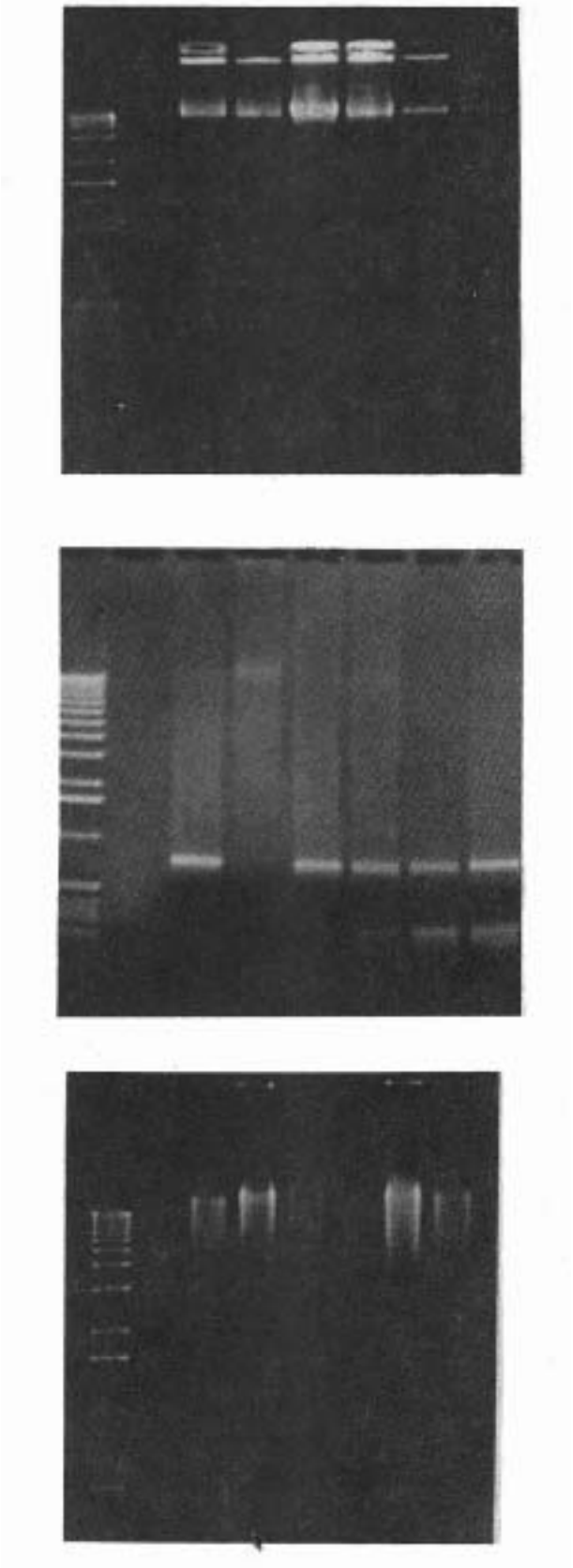

Figure 1 (A, top; B, middle; C, bottom). DNA from 15 *D. melanogaster* specimens which were squashed with extraction buffer containing urea (7M urea; 350 mM NaCl; 20 mM EDTA; 1% n-lauryl sarcosine; 0.5 % SDS) to which it was added proteinase K, brofasin or no enzyme, followed by phenol extraction (phenol: chlorophorm: isoamylalchool = 25:24:1). A: Genomic DNA extracted with different concentraions of proteases, or without enzyme. Lanes: 1- with 0.4 units/µg of proteinase K; 2 - without any enzyme; 3- with 11.86 units/µg of brofasin; 4- with 5.93 units/µg of brofasin; 5- with 2.37 units/µg of brofasin; 6- with 1.18 units/µg of brofasin; B: Amplification of 764 bp of P-element transposon; C: Genomic Hind III digests.

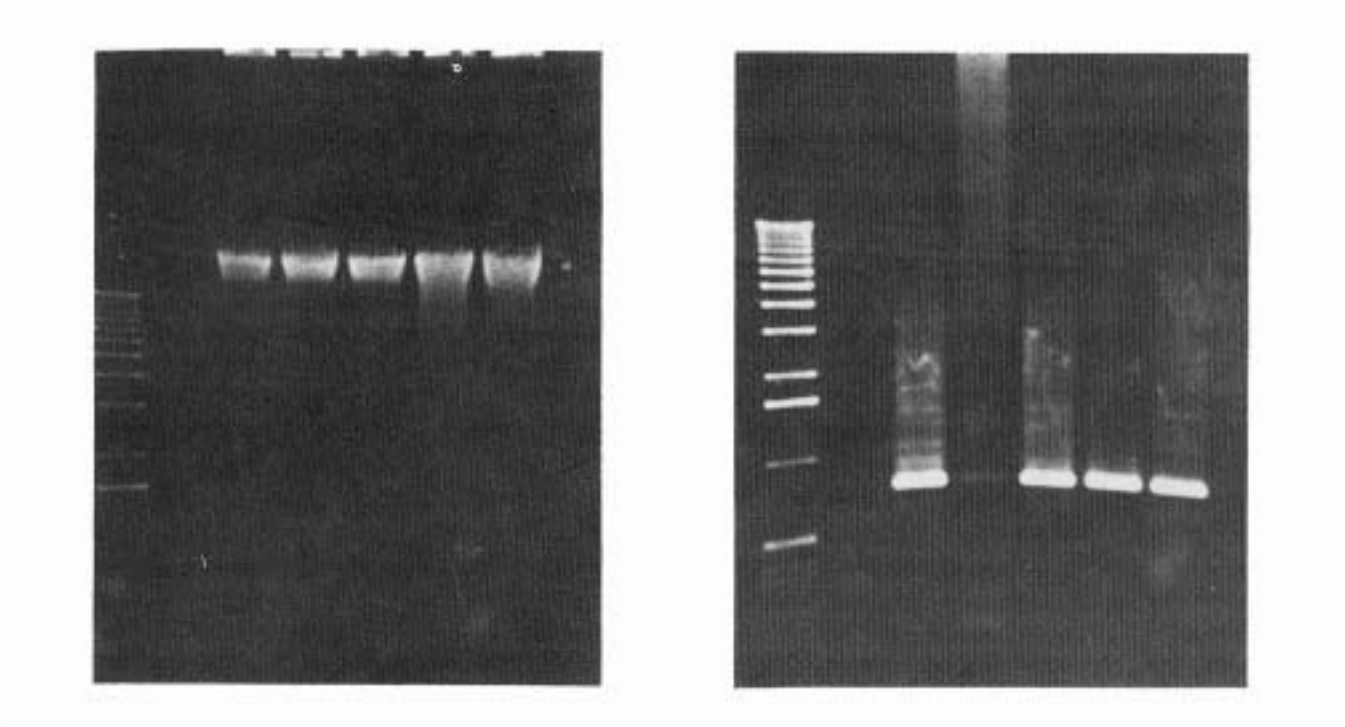

Figure 2 (A, left; B, right). DNA from 15 *D. melanogaster* specimens which were squashed with extraction buffer without urea (10mMTris-HCl pH 8,0; 10 mM EDTA; 350mM NaCl; 0.5 % SDS) to which it was added proteinase K, brofasin or no enzyme, followed by phenol extraction (phenol:chlorophorm:isoamylalchool = 25:24:1). A: Genomic DNA extracted with different concentraions of proteases, or without enzyme. Lanes: 1- with 0.4 units/µg of proteinase K; 2 - any enzyme; 3- with 11.86 units/µg of brofasin; 4- with 5.93 units/µg of brofasin; 5. with 2.37 units/µg of brofasin; 6- with 1.18 units/µg of brofasin; B: Amplification of 764 bp of P-element transposon; C: Genomic Hind III digests.

observed intact in the gel. This suggests that, in the negative control, the released DNA would have been degraded by endogenous nucleases, which destroy most of DNA templates, jeopardizing subsequent amplification.

Another procedure used to test the intacteness of DNA resulting from these ectractions was digestion with restriction endonucleases, a necessary operational step in many techniques, specially in Southern Blot analysis. The DNA extracted in the presence of urea was suitable for digestion with restriction enzyme *Hin*dIII, resulting in total digestion of the DNA in experimental groups 1, 3 and 4, and partially in the others (Figure C).

Human genomic DNA was obtained with the following concentrations: 309.3 ng/µL for the negative control, 462.6 ng/µL for the positive control (proteinase K) and 498.6 ng/µL for the brofasin containing group (Figure 3). The effects of brofasin and proteinase K were quite similar. The DNA yield in group 1, without proteinase, were high, however, note in Figure 3A that the band corresponding to it shows a smear suggesting degraded DNA. The DNA quality and its suitability for molecular techniques was verified by the PCR reaction, where for all the samples, the sequence of the CYPE1 gene with about 400 bp was amplified. Digestion with restriction endonuclease *Eco*RI was equally suitable for the groups containing proteinase K (2) and brofasin (3), but not for the negative control (1).

Proteinase K is frequently used for DNA extraction due its efficiency for digests of nuclei or whole cells and release of DNA for the action of polymerases. It has the advantage of being stable on relatively high temperatures (55 to 60ºC), being easily inactivated at 95ºC, an important characteristic during PCR reaction.

Our results show that the enzyme brofasin is equally efficient for nuclease inactivation during DNA extraction. The good yield of extracted DNA and the intactness and availability of DNA resulting from the protective action of brofasin for ulterior analysis, verified by PCR and restriction enzyme digestion reactions, suggest the use of this enzyme as an alternative to proteinase K for procedures involving DNA extraction.

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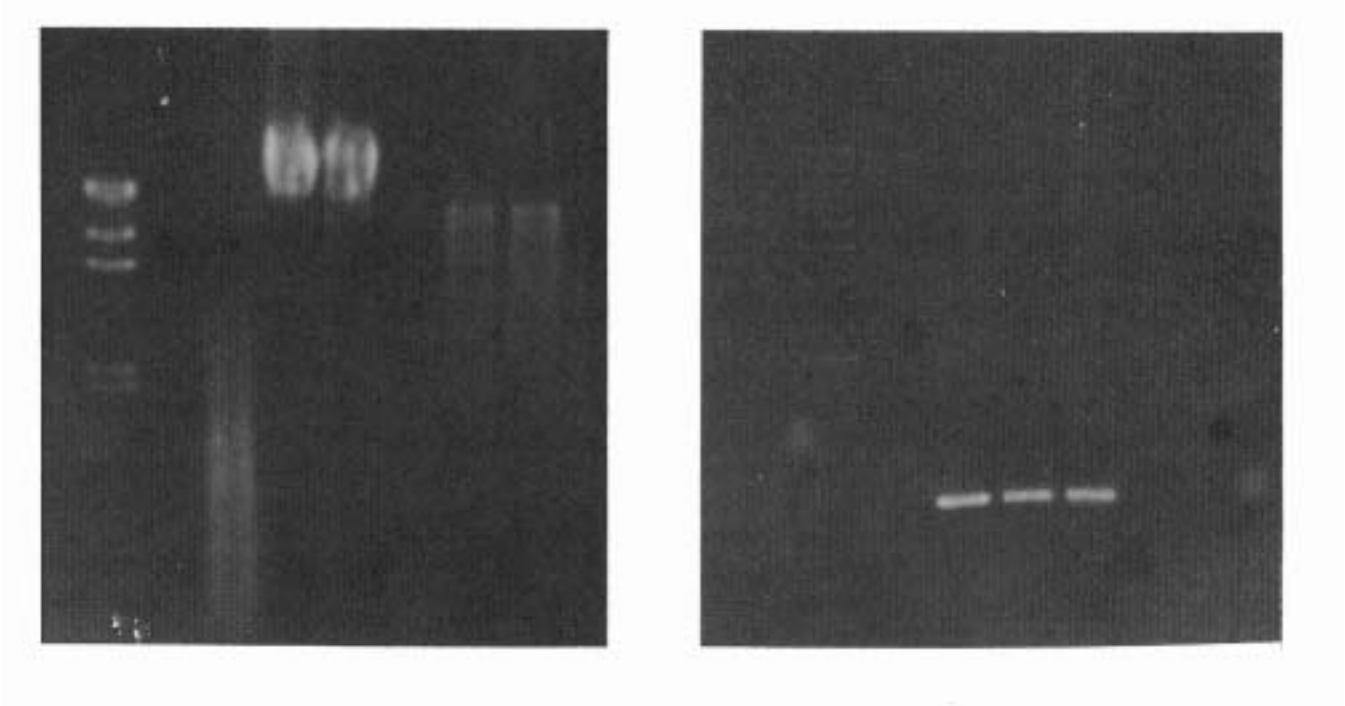

Figure 3 (A, left; B, right). Human genomic DNA extracted from lymphocytes be the salting out extraction procedure, using as lysis buffer 10 mM Tris-HCl, 400 mM NaCl, 2 mM EDTA and 28.8 units/µg of RNase A, to which was added proetinase K, brofasin or no enzyme. A: Genomic DNA extracted with different concentrations of proteases, or without enzyme (a), and genomic DNA EcoRI digests (b): Lanes: 1- withou any enzyme; 2- with 20 units/µg of proteinase K; 3- with 65.3 units/µg of brofasin; B: Amplification of 400 bp of the CYP2E1 gene.

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