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Valorization of fruit by-products of *Bromelia antiacantha* Bertol.: Protease obtaining and its potential as additive for laundry detergents

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ABSTRACT

The aim of this work is to isolate and study the performance of an alkaline protease from fruit by-products of *Bromelia antiachanta* Bertol. for its potential application as an additive of laundry detergent formulations. Its behavior is compared with Pura Felt 4000 (Genencor Argentina). *Antiacanthain*, a cysteine protease from fruit by-products of *B. antiachanta*, showed a remarkable stability (50 ± 0.82% to 177 ± 0.82% residual activity) in buffer with non-ionic and cationic surfactants (0.1, 0.4 and 1.0%), metal chelation (1, 5 and 10 mM EDTA), antiredepositant (100 mM Na_2CO_3) and bleaching (5% H_2O_2) agents. In commercial detergents, *antiacanthain* retained 100 ± 1.25% to 120 ± 0.82% of its maximum activity at 60 °C and removed stains from cloths in a short time period. *Antiacanthain* fulfilled all the requirements as detergent additive: high activity and stability in a broad temperature range (25–60 °C) and alkaline pH (5–9), and very good compatibility with commercial detergents.

1. Introduction

Bromelia antiacantha Bertol. (Bromeliaceae) is a native plant of South America that measures up to 2 m, it has short stems, long leaves, reddish flowers and rosette-shape thorns. The fruits are yellow/orange and rich in fiber; they look like small bananas and have a sweet flavor (Vallés et al., 2007; Filippon et al., 2012a).

The fruits of *Bromelia antiacantha* Bertol. are used in human food and folk medicine (for the production of cough syrups), in Uruguay, Brazil and the northeast of Argentina. The scale of this promising agricultural activity and related industrial processes, as well as their economic scope has not been evaluated yet. The average yield of *Bromelia antiacantha* crops has been estimated in 150 kg of fruit/ha, equivalent to USD 25/ha per month. This value is higher than the ones of traditional crops in the region such as soybean or corn, which have been estimated in USD 20 or 15/ha per month, respectively (Filippon et al., 2012b). The processing of *Bromelia antiacantha* fruit to obtain pulp and juice leaves a large amount of unused waste consisting of peel (with pulp attached to it) and insoluble fiber. There are no reports on the extraction of phytochemical compounds and enzymes from these residues.

Enzymes are considered as green chemicals because they are friendly with nature and play an important role in biotechnology because of their specificity, selectivity, efficiency and sustainability (Binod et al., 2013). The world market for industrial enzymes is projected to reach USD 6.30 billion by 2022 (Industrial Enzyme Market-Transparency Market Research, 2018). Proteolytic enzymes represent two thirds of the enzymes sold in the world market, most of them of microbial origin (Barberis et al., 2018a). New sources of proteases are intensively searched, especially from native plants and exotic organisms, because they can be stable and active under extreme conditions, and more selective against chiral compounds (Barberis et al., 2018b; Origone et al., 2018). However, there are few reports on the isolation of proteases from plants that grow in South America (Liggieri et al., 2004; Morcelle et al., 2006; Bruno et al., 2010; Errasti et al., 2018).

Nowadays, proteases are frequently used in the chemical, pharmaceutical, leather and food industries; and in the growing market of laundry detergents (Illanes et al., 2009; Barberis et al., 2008, 2013; Feijoo-Siota and Villa, 2011). Alkaline proteases and other enzymes are now well-accepted ingredients in household detergents, stain removers,

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automatic dishwashing detergents, industrial and institutional cleaning products (Research and Markets, 2018). Most alkaline proteases used as additives for laundry detergents are nonspecific serine endoproteases such as *subtilisin* from *Bacillus* sp. (U.S. Patent Nº 1240058, 374971, 370482, and 4266031, and UK Patent Nº 13155937). *Subtilisins* have low operative stability due to the fact that methionine catalytic residue is easily oxidized by bleach-based detergent formulations. That residue was changed by a non-oxidizable amino acid but its catalytic power was significantly reduced (Saeki et al., 2007). Thiol proteases (e.g. *papain*) could be oxidized by the bleaching agents, and metalloproteases (e.g. *thermolysin*) could lose their metal cofactors due to their interaction with softening agents (Egmont, 1997). Nevertheless, promising results were obtained in our lab using a cysteine phytoprotease as additive of laundry detergents (Barberis et al., 2013).

The performance of a good detergent protease is defined by multiple parameters, such as protein stain degradation, compatibility with other detergent components (e.g. surfactants, complexing agents, perfumes, and other enzymes), stability in the presence of oxidizing agents as bleach, and half-life in detergent formulations. Besides, the leading enzyme suppliers and detergent manufacturers are actively pursuing new proteases that address consumer needs for improved cleaning, fabric care and antimicrobial properties, focusing on new natural sources of proteases and enzyme engineering (Vojcic et al., 2015; Barberis et al., 2018a).

The aim of this work is to isolate and study the performance of an alkaline protease from fruit by-products of *Bromelia antiachanta* Bertol. (a native South American plant of the Bromeliaceae family) for its potential application as an additive of laundry detergent formulations. Its behavior is compared with Pura Felt 4000, a commercial laundry detergent protease (Genencor International Argentina S.R.L.).

2. Materials and methods

2.1. Crude extract preparation

Crude extract (CE) was obtained by chopping and homogenizing the fruit by-products of *Bromelia antiacantha* Bertol. using a manual mixer with ice bath, after 6 cycles of 2 min each. 50 g of peel (with pulp attached to it) and insoluble fiber were mixed with 100 ml of cold 0.1 M sodium acetate buffer pH 3.6 containing 5 mM EDTA and 5 mM cysteine, in order to avoid phenol oxidase activity and oxidation, respectively. Homogenates were filtered through a two-folded piece of gauze, and centrifuged for 15 min at 6,654 × *g* and 4 °C. Supernatants were collected, filtered and frozen at – 20 °C until analysis.

2.2. Preliminary purification of the crude extract

CE was treated with four volumes of cold acetone with gentle agitation, left to settle for 20 min at − 20 °C and centrifuged at 6,654 × *g* for 30 min. Pellets were dried in an air stream and suspended in 0.1 M Tris - HCl buffer pH 8; and protein concentration, sugar content and specific proteolytic activity were determined (Dubois et al., 1956; Vallés et al., 2007). This dissolved acetone precipitate was named *antiacanthain*, and was frozen until further use.

2.3. Reagents

Analytical grade chemical reagents were used (Sigma, St. Louis, USA). Surfactants and detergents were selected based on previous studies (Barberis et al., 2013).

2.4. Proteolytic activity assays

Proteolytic activity of *antiacanthain* was measured using 5 mg/ml of *N*-α-Benzyloxycarbonyl-L-arginine *p*-nitroanilide (BApNA) in 0.1 M Tris-HCl buffer pH 8 containing 20 mM cysteine. After 5 min at 37 °C, the absorbance of *p*-nitroaniline was measured (λ: 410 nm). BApNA is not a specific substrate for cysteine proteases, but it proved to be a suitable substrate for papain and other plant cysteine proteases (Gray et al., 1984; Quiroga et al., 2011; Origone et al., 2018). An international enzyme activity unit (IU) was defined as the amount of enzyme that releases 1 μmol of *p*-nitroaniline/min in the assay conditions (Quiroga et al., 2011).

2.5. Protein determination

Total protein content of *antiacanthain* was determined by the Bradford method, according to the protocol provided by BioRad Supplier, in the interval of 100 μg/ml and 1000 μg/ml (Bradford, 1976).

2.6. Effect of surfactants on the proteolytic activity of antiacanthain

The effect of some non-ionic surfactants (Triton ×100, Tween® 20 and Tween® 80) and ionic surfactants: cationic (Phospholipid® EFA) and anionic (SDS) on the proteolytic activity of *antiacanthain* was assessed at different concentrations (0.1, 0.4 and 1.0% (v/v)). This enzyme extract (50% (v/v), 12 IU/ml) was incubated in the surfactant solutions at 25, 40 and 60 °C, for 1 h. This enzyme concentration was selected based on the volumetric activity of *antiacanthain* in 0.1 M Tris-HCl buffer pH 8 at 37 °C, in order to compare the behavior of *antiacanthain* in each studied condition with reference to the mentioned buffer. Control samples without surfactants and incubated under similar conditions were taken as 100%. The residual proteolytic activity was determined.

2.7. Effect of detergent additives on the proteolytic activity of antiacanthain

Antiacanthain (50% (v/v), 12 IU/ml) was separately incubated for 1 h at 25, 40 and 60 °C to investigate the effect of bleaching (H_2O_2, 5 and 10% (v/v)), antiredepositant (Na_2CO_3, 100 mM), and chelation (EDTA, 1, 5 and 10 mM) agents on the residual proteolytic activity. *Antiacanthain* activity without any additive was taken as 100%.

2.8. Effect of commercial detergent on the proteolytic activity of antiacanthain

Compatibility of *antiacanthain* with different commercial laundry detergents (Woolite®, Ace® (Procter & Gamble), Skip®, Alamatic® and Drive® (Unilever)) was assessed. The detergents were diluted in distilled water (7 mg/ml) to simulate washing conditions, and the typical laundry detergent proteases were inactivated by freezing (24 h) and overheating (95 °C, 1 h). After this treatment, the proteolytic activity of the commercial laundry detergents was measured in order to confirm that the proteases had been fully inactivated in all cases. The enzyme (50% (v/v), 12 IU/ml) was incubated in detergent solutions for 1 h at 25, 40 and 60 °C, and the residual proteolytic activity was measured. Control samples without detergent and incubated under similar conditions were taken as 100%. Simultaneously, a commercial laundry protease (Pura Felt 4000, Genencor International Argentina S.R.L.) was assayed in the same conditions, using the above mentioned surfactants, additives and commercial detergents. The standard deviation of reported data by triplicate was lower than 2.5% (Solomon and Cox, 1992).

2.9. Testing to evaluate the efficiency of antiacanthain in removing stains from cloths

The efficiency of a formulated bioactive detergent containing the alkaline protease under study (*antiacanthain*) was evaluated on white cotton cloth pieces (3 cm × 3 cm) stained with human blood, egg yolk and chocolate. The stained cloth pieces were placed in separate flasks. One flask contained only tap water (10 ml); the second flask contained tap water (10 ml) with Alamatic® protease-free detergent (7 mg/ml);

whereas the third one contained tap water (5 ml) with Alamatic® protease-free detergent (7 mg/ml) added with 5 ml of *antiacanthain* solution (12 IU/ml). Besides, a fourth flask with a composition similar to that in the third one, but with Pura Felt 4000 (Genencor International Argentina S.R.L.) instead of *antiacanthain*, was also assessed. After 1 h incubation at 25, 40 and 60 °C, using a Digital Orbital Shaker (GFL, Model 3031) at 200 rpm, the cloth pieces were taken out, rinsed with distilled water and dried. The stain removal capability of *antiacanthain* was examined visually by looking at the pieces of dried cloth. Untreated cloth pieces stained with blood, egg yolk and chocolate were taken as control.

2.10. Statistical analysis

Residual proteolytic activity was obtained from three independent trials which were done by duplicate, and data were reported as mean ± SD. The linear range of proteolytic activity reaction was previously determined for each assay. IBM® SPSS® Statistics V22.0 software was used for statistical analysis. Kruskal-Wallis method was used to test the significant differences ($p < 0.05$) between the proteolytic activity of both *antiacanthain* and Pura Felt 4000 (Genencor International Argentina S.R.L.) in 0.1 M Tris-HCl buffer pH 8 versus each studied condition. Kruskal-Wallis test was selected because it is a non-parametric test that is applied to more than two independent samples, with a reduced number of repetitions ($N < 50$) (McDonald, 2014). Mann-Whitney method was used to test the significant differences ($p < 0.05$) between the proteolytic activity of *antiacanthain* and Pura Felt 4000 (Genencor International Argentina S.R.L.) at each studied condition (Mann and Whitney, 1947). Mann-Whitney *U* test was selected because it is a non-parametric test that is applied to two independent samples, with a reduced number of repetitions ($N < 50$) (Mann and Whitney, 1947).

3. Results and discussion

3.1. Preliminary purification and characterization of antiacanthain

Peel and fiber represent 65% (w/w) of the fruit of *Bromelia antiacantha* Bertol., and until now these by-products are not used. The United Nations Food and Agriculture Organization (FAO) have warned on the losses and waste in fresh fruits and vegetables processing industry. They are the highest among all types of foods, and may reach up to 60% (Sagar et al., 2018).

In this work, a preliminary purification step was applied to the CE obtained by chopping and homogenizing the fruit by-products of *B. antiacantha*, in order to remove several pigments, sugars and other water soluble compounds of phenolic nature. This purification step was evaluated as regards the protein concentration, the sugar content and the specific proteolytic activity (Dubois et al., 1956; Vallés et al., 2007). Table 1 shows a representative acetone precipitation of the proteolytic crude extract of fruit by-products of B. *antiacantha*. After 4 cold acetone volumes were applied to CE, increases in both the amount of recovered protein and the proteolytic activity were observed. The sugar content was reduced to 90% under this condition.

Table 1
Preliminary purification of the proteolytic crude extract of fruit by-products of *Bromelia antiacantha* Bertol. with different volume of cold (−20 °C) acetone (P-1 to P-4).

Sample	Protein content mg/ml %		Volumetric activity IU/ml %		Specific activity IU/mg
Crude extract	1.98 ± 0.0	100	33.35 ± 0.2	100	16.86 ± 0.1
P-1	1.35 ± 0.1	68	22.32 ± 0.0	67	16.47 ± 0.0
P-2	1.67 ± 0.0	84	27.42 ± 0.1	82	16.42 ± 0.0
P-3	1.66 ± 0.0	84	27.82 ± 0.2	83	16.76 ± 0.1
P-4	1.83 ± 0.2	96	31.28 ± 0.1	94	16.88 ± 0.2

The specific proteolytic activity of *antiacanthain* from the by-products was 97% higher than that obtained from the fruit pulp of *B. antiacantha* in previous studies (Vallés et al., 2007). Nevertheless, both *antiacanthain* extracts showed similar properties. They were inhibited by a specific inhibitor of cysteine protease such as E64 and activated with the addition of cysteine. They showed high specific proteolytic activity within a pH range from 5 to 9 and were highly active and stable from 35 to 65 °C.

3.2. Performance of antiacanthain as an additive of laundry detergents

3.2.1. Effect of surfactants on the proteolytic activity of antiacanthain

Results of residual proteolytic activity of *antiacanthain* in pure non-ionic and cationic surfactants (0.1, 0.4 and 1% (v/v)), after 1 h at 25, 40 and 60 °C ranged from 59 ± 0.47% to 177 ± 0.82% of its maximum proteolytic activity in buffer (pH 8) (Fig. 1 a-c). These results are quite good if considering that *antiacanthain* is a cysteine protease. For instance, *asclepain* (*Asclepias curassavica* L.) was severely affected by the increase in temperature and the concentration and nature of surfactants (Barberis et al., 2013).

Particularly, *antiacanthain* in Phospholipid® EFA-aqueous solutions (0.1% (v/v)) expressed from 131 ± 1.25% to 177 ± 0.82% of its maximum proteolytic activity in buffer (pH 8). This behavior may be related to ionic and hydrophobic interactions between the surfactant and *antiacanthain*, which allow the enzyme to be activated. The good performance of *antiacanthain* in surfactant solutions may also be due to the presence of an interface area.

On the other hand, when a commercial laundry detergent protease, Pura Felt 4000 (Genencor International Argentina S.R.L.) was incubated for 1 h at 25, 40 and 60 °C with several non-ionic and cationic surfactant solutions a very high residual proteolytic activity was observed at all concentrations tested, which ranged from 85 ± 0.00% to 300 ± 0.82% of its proteolytic activity in buffer (Fig. 1(d–f)). Nevertheless, anionic surfactants such as SDS, which is known to be a strong protein denaturant (Otzen, 2002) completely inhibited both the commercial enzyme and *antiacanthain* at any concentration tested.

Antiacanthain and Pura Felt 4000 (Genencor International Argentina S.R.L.) in Tris-HCl buffer alone (0.1 M, pH 8), and in solutions of Tris-HCl buffer (0.1 M, pH 8) containing surfactants for laundry detergents at different concentrations and at different temperatures, showed significant differences ($p < 0.05$) according to the Kruskal-Wallis test. Surfactant solutions increased, decreased or maintained the residual proteolytic activity of both enzymes when compared to the one obtained in buffer alone under the same conditions.

According to the Mann-Whitney *U* test, the residual proteolytic activity of both *antiacanthain* and Pura Felt 4000 (Genencor International Argentina S.R.L.) in solutions of Tris-HCl buffer (0.1 M, pH 8) containing surfactants for laundry detergents have no significant differences ($p < 0.05$) in 56% of the 36 cases studied at usual concentrations and washing temperatures. In addition, *antiacanthain* expressed greater activity than the commercial enzyme in only 1 of the 16 remaining cases; so in 58% of the studied cases, *antiacanthain* showed similar or better behavior in solutions of Tris-HCl buffer (0.1 M, pH 8) and surfactants than the commercial enzyme.

3.2.2. Effect of detergent additives on the proteolytic activity of antiacanthain

When *antiacanthain* was incubated for 1 h at 25, 40 and 60 °C in solutions containing several laundry additives, such as bleaching (H_2O_2), antiredepositant (Na_2CO_3) and chelation (EDTA) agents, it was observed that the enzyme was not inactivated, but it was affected in different ways, depending on the additive used (Fig. 2 a-c). *Antiacanthain* in EDTA solutions (1, 5 and 10 mM) expressed between 74 ± 1.63% and 115 ± 0.82% of their residual proteolytic activity in buffer (pH 8) at 25, 40 and 60 °C. These results suggest that metal

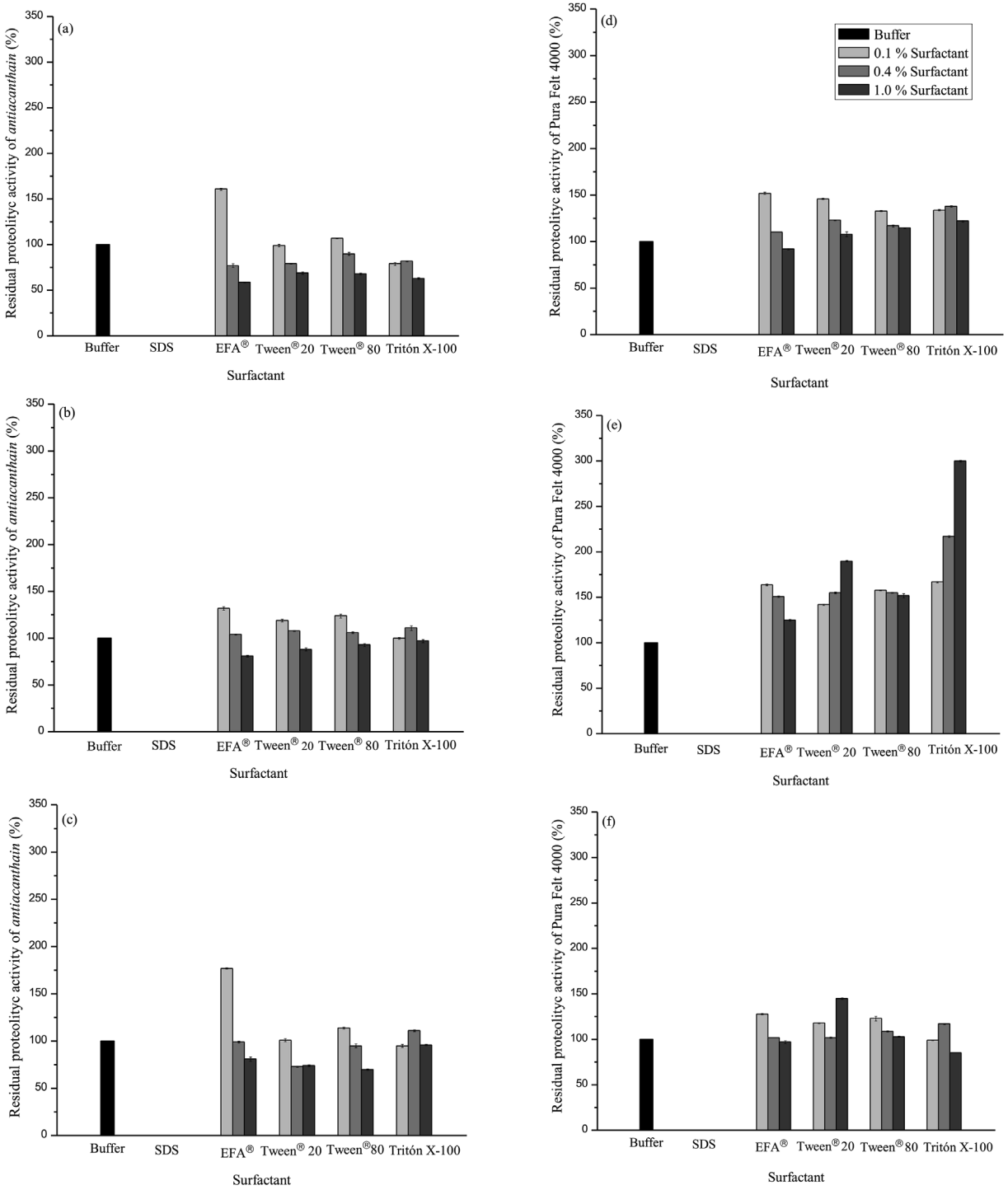

Fig. 1. Residual proteolytic activity of *antiacanthain* and Pura Felt 4000 (Genencor International Argentina S.R.L.) in surfactant solutions: non-ionic (Tween®20, Tween®80 and Triton X-100), cationic (Phospholipid® EFA) and anionic (SDS) at 25 °C (a, d); at 40 °C (b, e); and at 60 °C (c, f).

cofactors did not influence *antiacanthain* activity.

Furthermore, the residual proteolytic activity of *antiacanthain* decreased between 29% and 39% in 100 mM Na_2CO_3 solution regard to buffer (pH 8) at 25, 40 and 60 °C. This was probably due to the fact that *antiacanthain* in buffer pH 11 showed nearly 65% of residual proteolytic activity at pH 8 (Vallés et al., 2007). The effect of H_2O_2 depended on the concentration used. At 5% (v/v) H_2O_2, *antiacanthain* retained between 50 ± 0.82% and 67 ± 1.25% of its proteolytic activity in buffer (pH 8). These residual activity values are acceptable taking into account that very few wild-type cysteine proteases have proved to be active in the presence of bleaching agents (Haddar et al., 2009; Barberis et al., 2013). However, at 10% (v/v) H_2O_2 the enzyme retained only between 25 ± 1.63% and 43 ± 1.25% of its proteolytic activity in buffer (pH 8).

As shown in Fig. 2(d–f), the commercial protease as well as *antiacanthain* showed a decrease of 22%–24% in residual proteolytic activity when incubated with 100 mM Na_2CO_3 solutions, at 25, 40 and 60 °C. Besides, the commercial enzyme retained from 48 ± 1.63% to 100 ± 0.95% of its proteolytic activity in buffer (pH 8) when was incubated in (1, 5 and 10 mM) EDTA at 25, 40 and 60 °C. These values

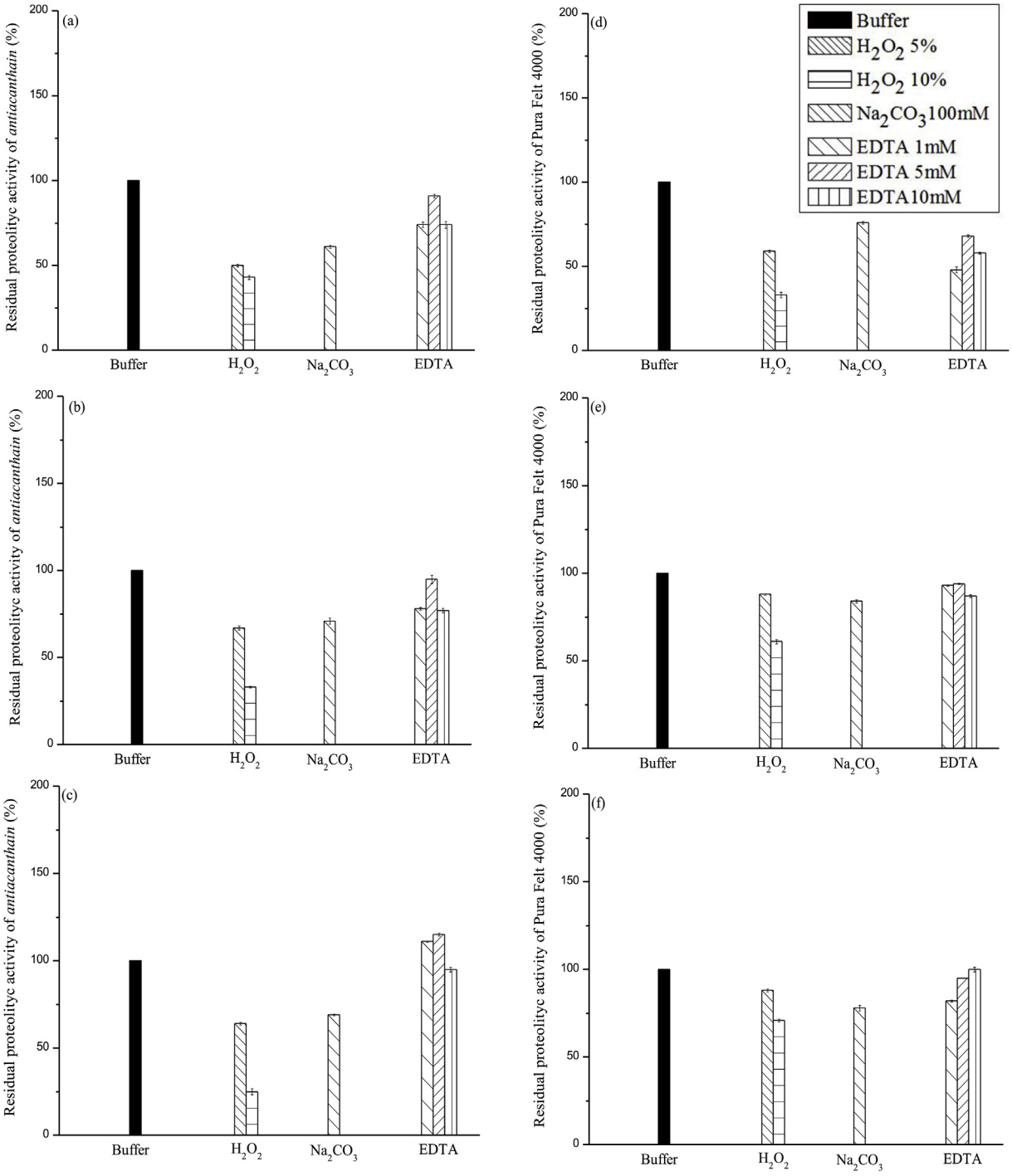

Fig. 2. Residual proteolytic activity of *antiacanthain* and Pura Felt 4000 (Genencor International Argentina S.R.L.) in detergent additive solutions: bleaching (H_2O_2), antiredepositant (Na_2CO_3) and chelation (EDTA) agents at 25 °C (a, d); at 40 °C (b, e); and at 60 °C (c, f).

were slightly lower than those shown by *antiacanthain* under the same conditions. Pure Felt 4000 was also affected by an oxidant agent as H_2O_2 (10% v/v), expressing between 33 ± 1.63% and 71 ± 0.82% of its proteolytic activity in buffer (pH 8).

Antiacanthain and Pura Felt 4000 in Tris-HCl buffer alone (0.1 M, pH 8), and in solutions of Tris-HCl buffer (0.1 M, pH 8) containing additives for laundry detergents, at different concentrations and temperatures, showed significant differences ($p < 0.05$) according to the Kruskal-Wallis test. Unlike surfactant solutions, the solutions containing detergent additives tended to reduce the residual proteolytic activity of both enzymes when compared to the ones obtained in buffer alone under the same conditions.

According to Mann-Whitney *U* test, the residual proteolytic activity of both *antiacanthain* and Pura Felt 4000 in solutions of Tris-HCl buffer (0.1 M, pH 8) containing additives for laundry detergents, have no significant differences ($p < 0.05$) in 56% of the 18 cases studied, at usual concentrations and washing temperatures. In addition, *antiacanthain* expressed greater activity than the commercial enzyme in 4 of the 8 remaining cases; so in 78% of the studied cases, *antiacanthain* showed similar or better behavior in solutions of Tris-HCl buffer (0.1 M, pH 8) and additives for laundry detergents than the commercial enzyme.

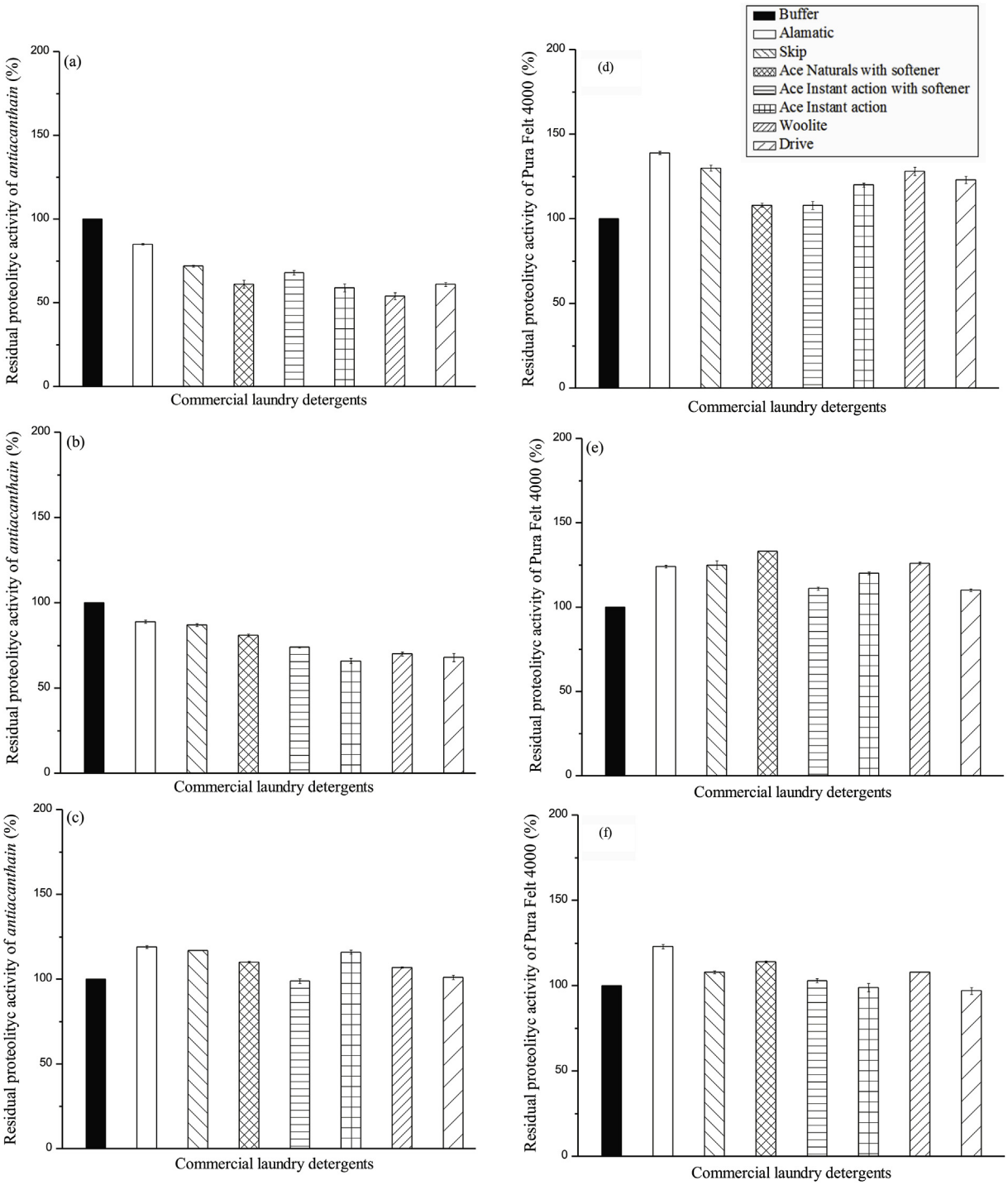

Fig. 3. Residual proteolytic activity of *antiacanthain* and Pura Felt 4000 (Genencor International Argentina S.R.L.) in commercial laundry detergents at 25 °C (a, d); at 40 °C (b, e); and at 60 °C (c, f).

3.2.3. Effect of commercial detergents on the proteolytic activity of antiacanthain

Fig. 3 (a,b) shows that *antiacanthain* was able to retain between 54 ± 2.16% and 89 ± 0.81% of its proteolytic activity in buffer (pH 8) when incubated with seven different commercial laundry detergents for 1 h at 25 and 40 °C. The decrease observed in *antiacanthain* proteolytic activity was attributed to detergent components other than the typical laundry detergent proteases, as these were previously inactivated by freezing (24 h) and overheating (95 °C, 1 h). However, at 60 °C *antiacanthain* expressed between 100 ± 1.25% and 120 ± 0.82% of its maximal proteolytic activity in buffer (pH 8) (Fig. 3 (c)). Besides, *antiacanthain* incubated with seven commercial laundry detergents for 1 h at 60 °C showed higher residual proteolytic activity than other proteases reported in the literature (Table 2).

Fig. 3 d-f compares the behavior of *antiacanthain* as well as Pura Felt 4000 in both 0.1 M Tris-HCl buffer pH 8 and commercial laundry detergents at 7 mg/ml diluted in distilled water (in order to simulate washing machine conditions).

According to the Kruskal-Wallis test, the proteolytic activity of *antiacanthain* in Tris-ClH buffer alone (0.1 M, pH 8) and in 7 mg/ml commercial laundry detergents showed significant differences ($p < 0.05$) under all studied conditions, except in the case of Ace

Table 2
Comparative performance of alkaline proteases in commercial laundry detergents (expressed as residual proteolytic activity, %).

Protease	Alamatic®	Skip®	ACE Instant Action	Woolite®	Drive®	References
Antiacanthain (*Bromelia antiacantha* B.) pH 8, 60 °C	120	117	116	107	101	
Serine protease (*Paecilomyces lilacinus* LPS 876) pH 10, 50 °C	66.5	64.6	98.1	–	88.8	Cavello et al. (2012)
β-keratinase (*Brevibacillus* sp. AS-S10-II) pH 12.5, 45 °C	104	–	96	–	–	Sudhir and Mukherjee (2011)
SPVP Protease (*Aeribacillus pallidus* VP3) pH 10, 40 °C	93	100	–	–	–	Mechria et al. (2017)
Alcalase ultra 2.5 L (*Bacillus licheniformis,* Novozymes Biopharma DK A/S) pH 10, 40 °C	100	76	–	–	–	Mechria et al. (2017)
Alkaline protease (*Bacillus pumilus* ATCC 7061) pH: 10, 50 °C	–	–	97.4	–	–	Gomaa (2013)
Carboxymethyl-cellulase (CMCase) (*Bacillus pumilus* ATCC7061) pH: 10, 50 °C	–	–	72.13	–	–	Gomaa (2013)
Asclepain (*Asclepias curassavica L.*) pH: 8.5, 37 °C	5	2	4	41	–	Barberis et al. (2013)
Araujiain (*Araujia hortorum Fourn.*) pH: 8.5, 37 °C	71	92	106	141	–	Barberis et al. (2013)

Instant Action with Softener and Drive® at 60 °C. With Pura Felt 4000 significant differences ($p < 0.05$) were observed under all studied conditions, except in the case of Ace Instant Action at 60 °C.

According to Mann-Whitney *U* test, the residual proteolytic activity of both *antiacanthain* and Pura Felt 4000 in 7 mg/ml commercial laundry detergents previously diluted in distilled water, have no significant differences ($p < 0.05$) in 57% of the 21 cases studied, at usual concentrations and washing temperatures. In addition, *antiacanthain* expressed greater activity than the commercial enzyme in only 1 of the 9 remaining cases; so in 62% of the studied cases, *antiacanthain* showed similar or better behavior in commercial laundry detergents than the commercial protease. However, at 60 °C, in 86% of the commercial laundry detergents studied, *antiacanthain* showed similar or better performance than the commercial enzyme.

3.2.4. Evaluation of the efficiency of antiacanthain in removing stains from cloths

Some authors have studied the ability of several laundry proteases to remove stains. Nevertheless, some of them have had to be preserved from the detergent additives before packaging due to the fact that the proteolytic activity was gradually lost after 30 min of incubation with laundry detergents (Abidi et al., 2008; Paul et al., 2014; Tanmay et al., 2014). *Antiacanthain* was active and stable without any protection in commercial detergent solutions during at least 1 h at 25, 40 and 60 °C as was previously demonstrated. Fig. 4 shows the efficiency of *antiacanthain* to remove human blood, chocolate and egg yolk stains from white cotton cloths.

The addition of *antiacanthain* solution (12 IU/ml) to tap water with Alamatic® protease-free detergent significantly improved the cleaning of stains from cotton cloth pieces as compared to stain removal by Alamatic® protease-free detergent or tap water alone. *Antiacanthain* protease showed high capability to remove protein stains very easily from cotton cloths, such as blood or egg yolk, but chocolate stains were slightly retained in the cotton pieces. These results were similar to those obtained with the commercial laundry detergent (Pura Felt 4000).

4. Conclusions

This work reports an original valorization way of fruit processing by-products of *Bromelia antiacantha* Bertol. as additive of laundry detergents. It can help offset growing environmental problems of the industries concerned and offers sustainable use of an available natural resource.

Many efforts have been made to find new proteases which are active and stable in extreme conditions to be used as laundry detergent additives. In this context, obstacles such as the enzyme purification steps, the time consuming factors (enzyme extract preparations), the low enzyme efficacy at low temperature, the gradual loss of proteolytic activity during the washing process and the enzyme protection requirements from detergent additives have been found. These obstacles

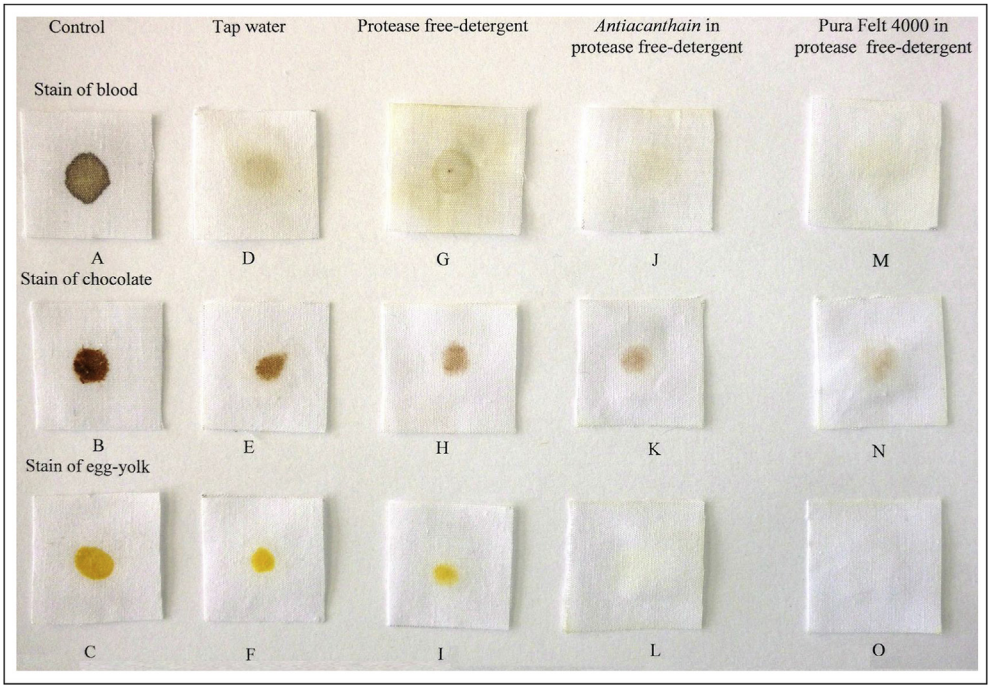

Fig. 4. Washing performance of *antiacanthain* and a commercial laundry protease (Pura Felt 4000, Genencor International Argentina S.R.L.) at 60 °C. Control cotton cloths with (A) blood stain, (B) chocolate stain and (C) egg-yolk stain. Cotton cloths washed with tap water after stained with blood (D), chocolate (E) and egg-yolk (F). Cotton cloths washed with Alamatic® protease-free detergent after stained with blood (G), chocolate (H) and egg-yolk (I). Cotton cloths washed with Alamatic® protease-free detergent supplemented with *antiacanthain* after stained with blood (J), chocolate (K) and egg-yolk (L). Cotton cloths washed with Alamatic® protease-free detergent supplemented with Pura Felt 4000 after stained with blood (M), chocolate (N) and egg yolk (O).

should be reduced or removed in order to get viable and sustainable products for the growing industrial demand of laundry detergents. The alkaline proteases obtained from the fruit by-products of *B. antiacantha* fulfill all the requirements for their application in detergent formulations: high activity and stability in a broad temperature range (25–60 °C) at alkaline pH (7–9) during washing processes (without protection), and good compatibility with commercial detergent additives. Besides, that enzymatic extract is very simple and cheap to obtain, and it avoids the use of expensive purification steps, fermentation and genetic engineering.

Contributors

Diego Vallés and Ana María Cantera participated in the preliminary purification steps applied to the CE obtained by chopping and homogenizing the fruit processing by-products of *B. antiacantha*.

Grisel Bersi participated in the experimental trials related with the performance of *antiacanthain* as an additive of laundry detergents.

Fabricio Penna participated in the statistical analysis of data.

Sonia Barberis participated in the experimental design, analysis of the results and writing of this paper.

All authors have approved the final article and agree with the submission to Biocatalysis and Agricultural Biotechnology. They confirm that this manuscript has not been published elsewhere and is not under consideration by another journal.

Declaration of interest

None.

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