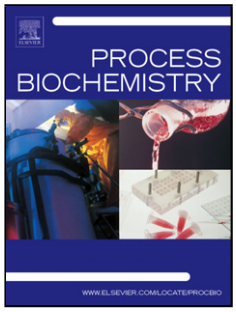

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Recovery of Bromelain from pineapple stem residues using aqueous micellar two-phase systems with ionic liquids as co-surfactants

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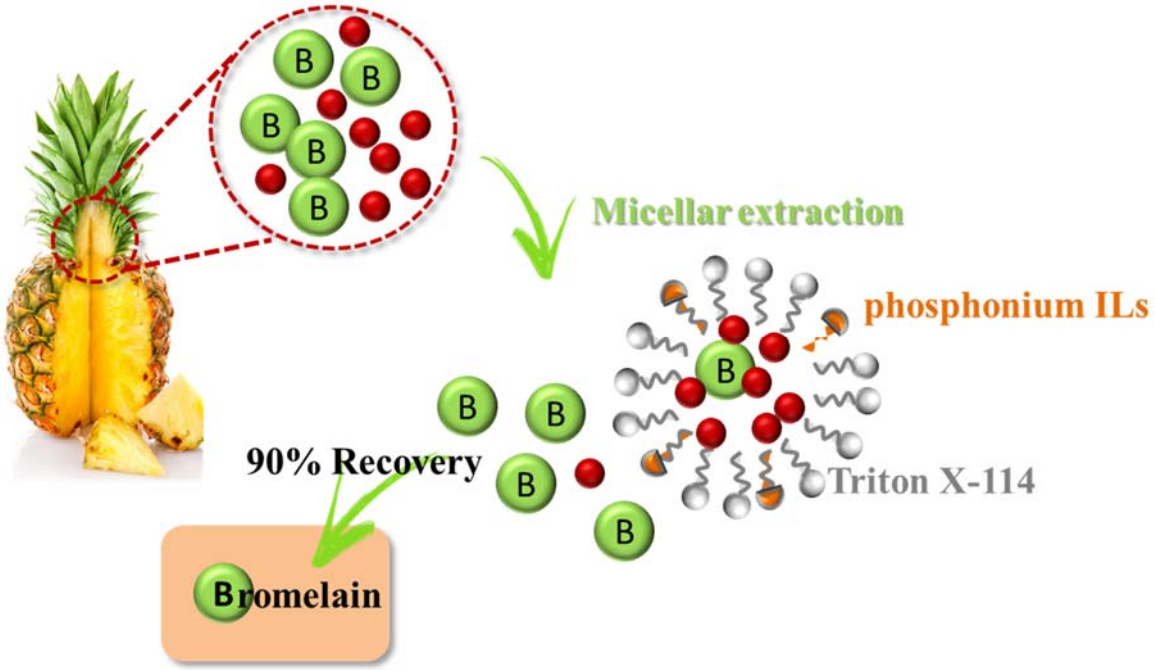
Graphical Abstract



Highlights

- Bromelain is a set of cysteine proteinases crucial in the pharmaceutical field.
- Bromelain purification was tested using AMTPS based in ILs as co-surfactants.
- It is notorious the stabilizing effect of phosphonium-based ILs on the bromelain.
- High bromelain recovery (90%) for the micelle-poor phase was found for all systems.

Abstract

Pineapple processing industries produce a large amount of residues; for example, the pineapple stem is rich in bromelain. Bromelain is a group of cysteine proteinases that have major importance in the pharmaceutical field due to, among others, their anti-inflammatory, antithrombotic, and fibrinolytic activities. Since Brazil is one of the largest producers of pineapples in the world, the development of an ecofriendly and cost-effective process to extract and purify bromelain from food/fruit residues would be significant. In this study, aqueous micellar two-phase systems composed of ionic liquids as co-surfactants were evaluated in the extraction of bromelain from the pineapple stem crude extract. The main results showed that bromelain partitions preferentially towards the micelle-poor phase, with enzyme recoveries above 90% for the systems studied. Moreover, a stabilizing effect of the ionic liquids towards the enzyme was observed.

Keywords: Bromelain, aqueous micellar two-phase system, Triton X-114, ionic liquids, co-surfactant, purification

1. Introduction

Brazil is the third largest producer of pineapples due to its vast territory and appropriate climate conditions [1]. Not only are pineapples of interest in the food industry, but they are also a rich source of valuable enzymes such as ananain, phosphatases, glucosidades, peroxidases and, mainly, bromelain [2], the production of which would allow the valorization of an abundant resource. Bromelain is a mixture of cysteine endopeptidases [3,4] and is mainly composed of pineapple stem bromelain (80%), pineapple fruit bromelain (10%) and ananain (5%), in which the pineapple stem is rich. However, it may also be found in lower concentrations, in pineapple wastes, namely peel, core, leaves and crown [2,5,6]. This group of proteases has been shown to be absorbed in the gastrointestinal tract [7] and to be safe to humans [2,4], therefore, it may be used as a food supplement and in pharmaceutical formulations [3,5]. Within its medical applications, it has been reported that bromelain has the ability to inhibit both platelet aggregation and the proliferation of different tumour cells, in addition to its anti-inflammatory, antithrombotic, antiedemateous and fibrinolytic activities (data reviewed in [7,8]). In this sense, the purification of bromelain from pineapple stem (the richest part of the fruit on bromelain) is highly recommended because this is a residue of very low applicability. The current methodologies that are applied extract bromelain from pineapple stems are ethanol, polyethylene glycol [9] and ammonium sulphate [9,10] precipitations. For bromelain purification the literature has described the use of reverse micellar systems [10–16], conventional polymer-salt [3,5,6,17–21] and polymer-polymer [22] aqueous two-phase systems (ATPS), and finally, some chromatographic techniques [4,23]. However, only the first two techniques act in mild operation conditions and are economically advantageous. Furthermore, polymeric-based ATPS display some restrictions related to the limited range of polarities between the

coexisting phases (13), the high cost of some polymers usually employed (e.g. dextran), and their high viscosities, which all limit the scale-up process [24]. When salt-based ATPS employing inorganic salts (often corrosive) are used, additional requirements regarding the equipment maintenance and the wastewater treatment appear [25]. Thus, there is a need for alternative purification systems that are capable of separating bromelain from the remaining contaminants present in the stem pineapple natural composition. Although ATPS are claimed to be promising fractionation/purification processes [26,27], the need for more biocompatible phase forming agents that can minimize their potential negative interaction with proteins (denaturation and conformational alterations), and more benign from an environmental point of view, is required.

Aqueous micellar two-phase systems (AMTPS) have been proposed as alternative purification techniques when proteins and/or enzymes are involved [28], principally due to their outstanding ability to maintain the native conformations and biological activities of the target molecules [29]. Recently, AMTPS with ionic liquids (ILs) acting as co-surfactants were reported [30] to combine the advantages of the previously mentioned micellar systems, with the benefits conferred by the ILs' unique properties, including their high chemical and thermal stabilities, low melting points and "designer" solvent status, as the numerous cation/anion possible combinations cover a wide range of polarities [31,32], in addition to the surfactant character of the long alkyl chain ILs. These mixed AMTPS showed an enhanced ability to selectively fractionate and purify (bio)molecules [30]. Thus, these systems are here evaluated as an environmental friendly alternative to the purification of bromelain. For that purpose we performed the extraction and purification of bromelain from the pineapple stem using different

combinations of AMTPS based on Triton X-114 and McIlvaine buffer with imidazolium and phosphonium ILs as co-surfactants.

2. Experimental section

2.1. Materials

Commercial bromelain from pineapple stem (3-7 units/mg protein) was acquired at Sigma-Aldrich (St. Louis, MO, USA, product code B4882), and the pineapple (*Ananas comosus*) used was purchased at a local market in São Paulo, Brazil. In addition to bromelain, its substrate, azocasein, and its activator cysteine (purity ≥ 97%) was obtained, and the nonionic surfactant was employed, Triton X-114 (laboratory grade, structure present in **Table 1**) and bicinchoninic acid (BCA, product code B9643) were also obtained from Sigma-Aldrich. The McIlvaine buffer components, *i.e.* sodium phosphate dibasic anhydrous Na_2HPO_4 (purity ≥ 99%) and citric acid anhydrous $C_6H_8O_7$ (purity = 99.5%) were supplied by Fisher Chemical and Synth, respectively. The studied imidazolium-based ILs, 1-decyl-3-methylimidazolium chloride [C_{10}mim]Cl (purity > 98 wt%), 1-dodecyl-3-methylimidazolium chloride [C_{12}mim]Cl (purity > 98 wt%) and 1-methyl-3-tetradecylimidazolium chloride [C_{14}mim]Cl (purity > 98 wt%) were acquired at Iolitec (Ionic Liquid Technologies, Heilbronn, Germany), and the phosphonium-based ILs: trihexyltetradecylphosphonium bromide [$P_{6,6,6,14}$]Br (purity = 99.0 wt%), trihexyltetradecylphosphonium decanoate [$P_{6,6,6,14}$][Dec] (purity = 99 wt%) and trihexyltetradecylphosphonium bis (2,4,4-trimethylpentyl)phosphinate [$P_{6,6,6,14}$][TMPP] (purity = 93.0 wt%) were kindly supplied by Cytec. The chemical

structures of the cations and anions composing the list of ILs herein investigated are depicted in **Table 1**.

2.2. Methods

2.2.1. Preparation of bromelain stock solution

To prevent the bromelain denaturation, a specific buffer [21] was prepared containing 50 mM of McIlvaine buffer (composed of 82.35 mL of 0.2M Na_2HPO_4, 17.65mL of 0.1M $C_6H_8O_7$ and 264.7 mL of distilled water) at pH 7, 100 mM of EDTA and 300 mM of KCl. A bromelain stock solution of 20 mM was prepared in this specific buffer using the commercial enzyme (high purity level), which was used in the following studies.

2.2.2. Stability studies of commercial bromelain in Triton X-114 and ILs

The experiments of bromelain stability were performed in the McIlvaine buffer at pH 7 and at 20 ± 1°C (temperature at which the solution is still clear, *i.e.* below the T_{cloud}, 30.57 ± 0.17ºC for 10 wt% of Triton X-114).

For all the stability experimental tests, falcon tubes were weighed with 0.25 g of the bromelain stock solution (20 mM). The tubes were completed with: (i) 0.25 g of Triton X-114 (final mass composition of 10 wt%) and 2.00 g of McIlvaine buffer to perform the enzyme stability tests in the surfactant; and (ii) 0.0075 g of each IL (final mass composition of 0.3 wt%) and 2.2425 g of McIlvaine buffer for the stability tests to assess the effect of the different ILs under study. All tubes with a final weight of 2.5 g were rapidly homogenized by hand-mixing, and then they were placed in a thermostatic bath at a controlled temperature of 20°C. A control solution was prepared that comprise

0.25 g of bromelain stock solution and 2.25 g of McIlvaine buffer, exposed to the same conditions of the previous solutions (temperature and time of exposition), though without the presence of Triton X-114 or ILs. The activity of the enzyme was thus monitored over a 24 hour period, with samples being taken at 0 h, 1 h, 2 h, 4 h, 6 h and 24 h of exposure, and it was determined as described in section 2.2.3. The experiments were performed in triplicate and the respective standard deviations were calculated.

The relative bromelain activity was determined for all systems regarding the ratio between the enzyme activity (U/mL) in the presence of surfactant or IL and the enzyme activity (U/mL) in the buffer solution (without the presence of surfactant or IL), as described by Eq. 1.

$$Relative\ Activity = \frac{\text{Act}_{solv.}}{\text{Act}_{buffer}} \quad \text{(Eq. 1)}$$

where $\text{Act}_{solv.}$ is the bromelain activity in each solvent at a specific time (0 h, 1 h, 2 h, 4 h, 6 h or 24 h) and Act_{buffer} is the enzyme activity in the buffer solution at the same time.

2.2.3. Protein quantification and protease assay

The total protein content was determined by the Bicinchoninic Acid (BCA, product code B9643, Sigma Aldrich, St. Louis, MO, USA) method [33]. Proteolytic activity was measured by the digestion of the chromogenic substrate azocasein according to Charney and Tomarelli [34] with some modifications. Briefly, the appropriate dilution of the commercial enzyme (150 µL), or the enzyme present in each of the phases of the AMTPS was incubated for 20 min at 37 ºC with 150 µL of 0.5% (w/v) of the azocasein solution in 50 mM of the McIlvaine buffer (pH 7, guaranteeing no effects of pH in terms of bromelain activity [18] with 0.96 mM of cysteine. The reaction was stopped by

adding 150 µL of 10% (w/v) of trichloroacetic acid (TCA). After centrifugation (5 min at 13000 rpm) of the reaction mixture, 100 µL of supernatant was mixed with 100 µL of 0.5 M of KOH, and the absorbance was measured at 430 nm against the appropriate control sample, *i.e.* an identical system was prepared for each AMTPS, but without the presence of the enzyme to remove any possible interference with the components of the AMTPS systems in the absorbance experimental data (blank control). Samples were assayed in triplicate, and the activity was expressed in units of enzyme activity (U/mL). One U was defined as the amount of enzyme that led to an increase in the absorbance measured of 0.0001, under the assay conditions. The total protein and enzyme activity data were determined using a microplate spectrophotometer, model Spectra Max Plus 384 (Molecular Device, Sunnyvale, CA, USA).

When assays were determined using the pineapple crude extract, the enzymatic activity was evaluated accordingly to the bromelain specific activity (SA), according to Eqs. 2 and 3:

$$SA_{Top} = \frac{Act_{Top}}{c_{proteins.Top}} \quad \text{(Eq. 2)}$$

$$SA_{Bot} = \frac{Act_{Bot}}{c_{proteins.Bot}} \quad \text{(Eq. 3)}$$

where SA_{Top} and SA_{Bot}, Act_{Top} and Act_{Bot}, and $c_{proteins.Top}$ and $c_{proteins.Bot}$ are the bromelain specific activity (U/mg), the bromelain activity in U/mL and the total proteins concentration in mg/mL, in both the top and bottom phases, respectively.

2.2.4. Partitioning studies of pure stem bromelain by applying AMTPS

The AMTPS binodal curves used in this work were recently reported by us [30]. An AMTPS mixture point corresponding to 10 wt% of Triton X-114, 0 wt% or 0.3 wt% of each IL tested, 10 wt% of a 20 mM bromelain stock solution, completed with McIlvaine

buffer at pH 7 (final volume of 10 mL), was gravimetrically prepared in a glass tube for each studied system. Then, the tubes were left for 2 hours at 4 ºC in a tube rotator apparatus model 270 from Fanem® at 25 rpm, to guarantee the complete homogenization of the system. Since the AMTPS is not formed at 4 ºC (monophasic solution), the systems were homogenized without bromelain partition since the two phase were not formed at this temperature. Subsequently, the systems were moved to a water bath at 37 ºC (guaranteeing no effects of temperature towards the bromelain activity [18]) for 3 hours to reach thermodynamic equilibrium thus, completing the phases separation and the partition of bromelain. With these conditions, the systems resulted in a micelle-rich and a micelle-poor phases as the bottom and top layers, respectively. Both phases were carefully separated, and then collected for the volume measurement with a tube of 10 mL, and bromelain activity was determined by the azocasein method (see section 2.2.3). The analytical quantifications were performed in triplicate. Any interference from the AMTPS components (Triton X-114, McIlvaine buffer or IL, when present) with the analytical quantification method was investigated and prevented by routinely applying blank controls. Therefore, the partition coefficient for bromelain ($K_{bromelain}$) was calculated as the ratio between the protease activity (U/mL) in the micelle-poor (top) and the micelle-rich (bottom) phases, as described in Eq. 4.

$$K_{bromelain} = \frac{Act_{Top}}{Act_{Bot}} \quad \text{(Eq. 4)}$$

where Act_{Top} and Act_{Bot} are, respectively, the bromelain activity in the top and bottom phases.

The recovery (R) parameters of each molecule (bromelain and total proteins) towards the top (R_{Top}) and the bottom (R_{Bot}) phases were determined following Eqs. 5 and 6:

$$R_{Top} = \frac{100}{1 + \left(\frac{1}{R_v \times K}\right)} \quad \text{(Eq. 5)}$$

$$R_{Bot} = \frac{100}{1 + R_v \times K} \quad \text{(Eq. 6)}$$

where R_v stands for the ratio between the volumes (mL) of the bottom and top phases and K represents the enzyme partition coefficient. It should be stressed that these equations are only valid when there is no protein precipitation in the interface, which is the case for all the studied AMTPS.

2.2.5. Partition studies of bromelain extracted from the pineapple stem by applying AMTPS

To obtain the bromelain crude extract, the pineapple's leaves were cut off and the stem (central part with *circa* of 7 g) was cut into pieces and milled using a mortar and pestle in ice. Then, 10 mL of bromelain buffer was added and the crude extract obtained was centrifuged for 10 min at 4000 rpm. The supernatant used in these experiments was diluted for comparison purposes, aiming at its preparation and extraction with an initial similar specific enzymatic activity to that obtained from the pure commercial bromelain and was used in the partition experiments (~5000 U/mg).

For the purification/fractionation studies, a similar experimental procedure to the one described in the partition experiments in section 2.2.4 was used, but now with 10 wt% of the diluted supernatant. In these tests, the same parameters described in section 2.2.4 were calculated.

In contrast, the partition coefficient for total proteins was determined as the ratio between the total protein concentration (mg/mL) in the micelle-poor (top) and micelle-rich (bottom) phases, as described in Eq. 7.

$$K_{total\ proteins} = \frac{[TProtein]_{Top}}{[TProtein]_{Bot}} \quad \text{(Eq. 7)}$$

where $[TProtein]_{Top}$ and $[TProtein]_{Bot}$ are, the total protein concentration in the top and bottom phases, respectively.

The selectivity was also evaluated using the ratio of the bromelain and the total protein partition coefficients following Eq. 8:

$$S_{bromelain/proteins} = \frac{K_{bromelain}}{K_{totalproteins}} \quad \text{(Eq. 8)}$$

where $K_{bromelain}$ and $K_{totalproteins}$ are the bromelain and total proteins partition coefficient, respectively.

2.2.6. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The surfactant used interferes with running the SDS-PAGE, therefore, a previous protein precipitation was carried out to eliminate the solvent interferences in the SDS-PAGE experiments. Thus, 300 µL of each phase or bromelain crude extract or pure bromelain solution (~0.4 mg/mL) was mixed with 300 µL of TCA 100% (w/v) and 300 µL of acetone. These mixtures were left overnight at 4 ºC and were then centrifuged for 15 min at 13000 rpm at 4 ºC, with the supernatant discarded. The precipitate was washed twice with acetone to remove residual TCA and the centrifugation step was repeated and followed by a 30 min dry step in vacuum to eliminate the residual acetone. Subsequently, the precipitate was re-suspended in 60 µL of McIlvaine buffer, pH 7 followed by the addition of 20 µL of sample buffer (composed of 3 µL of sample and 1 µL of buffer) [62.5 mM Tris-HCl, 2% (w/v) sodium dodecyl sulfate (SDS), 5% (v/v) β-

mercaptoethanol (β-Me) and 0.002% (w/v) bromophenol blue]. Then, the samples were boiled at 100 °C for 5 min and subjected to SDS-PAGE, as described elsewhere [35]. A protein Ladder (Bio-Rad, Richmond, CA, USA), furnishing 12 bands from 2 kDa to 250 kDa, was used as the standard. The proteins were visualized by staining with the Coomassie Brilliant Blue R-250 and G-250.

3. Results and discussion

3.1. Stability studies

Because proteins are very sensitive macromolecules preliminary studies of the stability bromelain in the presence of all of the micellar systems components used during the extraction and purification are mandatory. The bromelain relative activity for each component with several times of exposure was determined in accordance with the protease activity in the buffer, which was used as a control. The results obtained are depicted in **Figure 1** and show the stability screening for all the ILs studied and the surfactant Triton X-114.

Figure 1 clearly shows a distinct tendency between the ILs families, *i.e.*, the phosphonium family leads to a slight increase in the bromelain activity, whereas the imidazolium interacted negatively with the enzyme, which resulted in an abrupt loss of activity, reaching values lower than 0.200. This increase in the bromelain activity undoubtedly resulted from a positive effect of the phosphonium-based ILs on the bromelain stability using only 0.3 wt% of IL, a behavior that we have reported as the superactivity phenomenon [36]. Moreover, the non-ionic surfactant Triton X-114 displayed a reduction of enzymatic activity to values approximately 0.600, representing 40% of the enzyme activity loss. Therefore, the partitioning studies were carried out

using both the conventional AMTPS (composed only of the non-ionic surfactant) and the mixed AMTPS with phosphonium-ILs, despite the traditional AMTPS' lower enzyme stability, for comparison.

3.2. Partitioning studies with the commercial bromelain

As mentioned above, the pineapple stem crude extract is a complex medium with different classes of contaminants, such as pigments and other proteins that can interfere with the bromelain extraction and purification. Therefore, an optimization study using commercial bromelain (high purity) was carried out. Thereby, the bromelain activity in the presence and absence of cysteine as an activator to evaluate the enzyme linearity range of operation was analyzed. The same procedure was implemented to determine the appropriate duration for the enzymatic reaction. From these preliminary studies, a 20 mM bromelain stock solution and 40 minutes of enzymatic reaction were chosen for use in further studies in presence of cysteine. Furthermore, the partitioning studies were evaluated determining the total bromelain specific enzymatic activity, expressed in U/mg protein and the bromelain partition coefficient (K) and the enzyme recovery (R in %) in both phases. The results are displayed in **Figures 2.A)** and **B)**, respectively.

These results showed that this type of cysteine endopeptidases partitions preferentially towards the micelle-poor (top) phase due to the bromelain hydrophilic character, in addition to the slight repulsion between the positive charges of bromelain (the medium pH below bromelain p*I* that is 9.5 [2]) and the micelles' charges [12] that are also positive as a result of the ILs incorporation. The enzymatic activity in the top phase is much higher than that in the bottom layer, indicating the higher concentration of bromelain in this phase, which is further corroborated by the high partition coefficient values, the recovery in the top phase approximately 100% and by the SDS-PAGE

results (**Figure S.1**). Since bromelain partition is complete for the top phase and is above 97% for all systems, the results found for the partition coefficients are similar between the different AMTPS, as confirmed by the similarity of the SDS-PAGE gels between the various micellar systems (**Figure S.1**). It should be stressed that the total amount of proteins was not determined during this optimization step because the bromelain used was a commercial sample and is supposed to be pure or to present a minor content of impurities, which was further proven by the SDS-PAGE results depicted in **Figure S.2**. Herein, the gel showed that the commercial bromelain is in a pure state, with a molecular weight approximately 24 kDa, which is in agreement with both the literature (23.8 kDa [8] and 24.5 kDa [18]), and the bromelain found in the crude extract. As commonly noned, at least two proteins other than bromelain are present in the pineapple stem; these are likely ananain (23.46 kDa) and comosain (24.50 kDa) [8], which are both visible in the SDS-PAGE results (**Figure S.2)** and can be identified at approximately 26 and 19 kDa, respectively, because these proteins are always present in bromelain stems. However, when the biomass varies, the molecular weight of these proteins can also change slightly as verified in the literature data for bromelain, in which it can achieved 28 kDa [5], 29 kDa [6] and 30 kDa [5].

3.3. Purification of bromelain extracted from the pineapple stem

After the optimization study, in which it was possible to identify the preferential partition of the commercial bromelain for the micelle-poor phase, practically independent of the micellar system applied, partitioning studies were also carried out with bromelain extracted from the pineapple stem crude extract. The pineapple stems were used as the raw material due to their low value and high bromelain content (80%). Therefore, a crude extract rich in bromelain was prepared (see section 2.2.5) and used

instead of the pure commercial bromelain solution. The results achieved in the purification study are displayed in **Figure 3** and include the bromelain specific enzymatic activity, the partition coefficients of bromelain and contaminant proteins as well as the protein and bromelain recovery in both the top and bottom phases. As expected, the enzyme partitions preferably towards the micelle-poor phase, with a recovery higher than 90% for all systems. Comparing **Figures 2.A)** and **3.A)**, it can be observed that there was an inverse tendency in the bromelain's enzymatic activity because in the assays with pure bromelain, aside from the $[P_{6,6,6,14}]Br$, which was the system with the highest enzymatic activity, the remaining systems displayed an identical activity. On the contrary, in the assays with the real matrix, the bromelain activity followed a decreasing tendency: without IL > $[P_{6,6,6,14}]Br$ > $[P_{6,6,6,14}][TMPP]$ > $[P_{6,6,6,14}]Dec$. These results made patent the effect of all the other components within the pineapple crude extract, because the stabilizing effect of the IL on the bromelain was no longer as strong as it was for the commercial sample (**Figure 1)**. Moreover, the standard deviation in the bromelain partition coefficients of both pure bromelain and crude extract assays led to an identical partition for the AMTPS studied, which was approximately 35 and 15, respectively. This considerable decrease in the partition coefficient can be attributed to the complexity of the crude extract and the presence of other proteins that negatively interact with bromelain. Concerning the selectivity (**Figure 4**), the traditional AMTPS showed the highest value at *circa* 20, while the mixed AMTPS displayed identical results approximately 10. Nonetheless, when these results are combined with the recovery studies present in **Figure 3.B)**, it can be seen that the proteins recovery in both top and bottom phases regarding the conventional systems and $[P_{6,6,6,14}]Dec$ exhibited similar results, namely a total protein recovery in the bottom phase slightly above 50%. Thus, although the selectivity of the traditional

AMTPS is much higher than that of the AMTPS with [$P_{6,6,6,14}$]Dec, this latter is also able to purify the bromelain in the micelle-poor phase due to the slightly higher partition of contaminant proteins towards the micelle-rich phase, *i.e.* the bottom phase [results also justified by the SDS-PAGE (**Figure S.3**) of all AMTPS systems and both phases]. This behavior may indicate that, with the proper optimization of the extraction conditions (namely the pH), it may be possible to successfully separate bromelain from the main contaminants, thus significantly increasing the purity of the bromelain extracted from pineapple stem. In summary, though the mixed AMTPS are not the most selective systems, they are able to stabilize the enzyme, as previously shown, whereas this was not observed in the system composed only of Triton X-114.

As previously stated, this is the first time that these mixed AMTPS were used for bromelain extraction and purification so there are not any data in the literature to be used in comparison. Nonetheless, several studies applying conventional polymer-salt [3,5,6,17–21] and polymer-polymer [22] ATPS have been reported as have reverse micellar systems [10–16]. In the common polymer-salt ATPS, the enzyme migrated prominently to the organic phase, *i.e.*, the polymeric phase, and in the polymer-polymer ATPS, it partitioned towards the polyethylene glycol phase. These results were explained as being due to the *salting-out* effect of the salt combined with the volume of the exclusion effect of the polymer [5,6,17,19,20], hydrogen bonding with the polymer [21], hydrophobic interactions [21,22] and electrostatic repulsions [22]. However, in the present study, the bromelain partition occurred towards the micelle-poor phase, *i.e.* the aqueous phase; therefore, it is obvious that this behavior is probably driven by the hydrogen bond interaction that is created between the bromelain and the water molecules, and by the electrostatic repulsion between the bromelain positive residues charge and the micelles' positive charge. Furthermore, the bromelain partition

coefficient attained with these ATPS varied greatly, ranging from 0.5 to 69 [17,19–22]; consequently, there is also a wide range of purification factors, from 0 to 28 [17,19–22]. The bromelain was also studied considering the application of reverse micellar systems, with the purification factor reported, which was generally calculated after different types of extraction: batch, continuous, affinity-based and back extractions. Despite the large differences between the, AMTPS and reverse micellar systems, purification factors of the reverse micellar systems ranging from 0 to 10 were obtained [10–16]. The AMTPS herein reported remains more advantageous than these last reported systems due to the absence of organic solvents in their composition and is, thus a more benign and biocompatible system for biomolecules extraction and purification. Additionally, a bromelain extraction using the conventional AMTPS with Triton X-114 was recently reported [37], for which it was concluded that the bromelain partition between the phases was dependent on the system temperature and the surfactant concentration, with the enzyme migration towards the micelle-poor phase dependent on higher temperatures and surfactant concentrations.

4. Conclusions

The valorization of bromelain from pineapple stem by the purification of bromelain from the fruit residue with AMTPS with ILs as co-surfactants was studied in this work. The stabilizing effect of phosphonium ILs on bromelain is well known, as are the distinct effects of the various IL families that were studied. Moreover, we showed high bromelain recovery; *i.e.* above 90%, for the micelle-poor phase for all of the AMTPS studied. Additionally, selectivity of the AMTPS in the enzyme extraction was observed, with the conventional system and $[P_{6,6,6,14}]$Dec found to be the most selective mixed AMTPS. Despite the higher selectivity of the conventional AMTPS, it did not present a

stabilizing effect on the enzyme, which the phosphonium ILs displayed, thus justifying the advantageous nature of mixed AMTPS.

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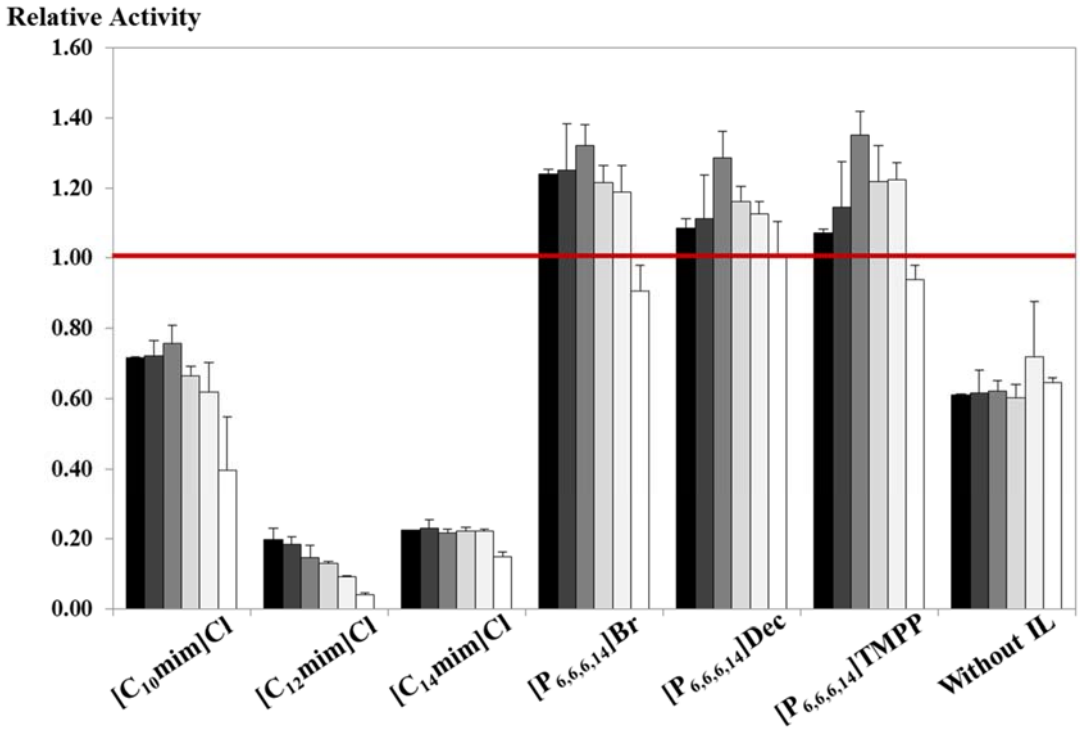

Figure 1. Relative activity of the commercial bromelain in the presence and absence of the different ILs or Triton X-114 at several hours of exposure: ■ – 0h, ■ – 1h, ■ – 2h, ■ – 4h; ■ – 6h; and □ – 24h.

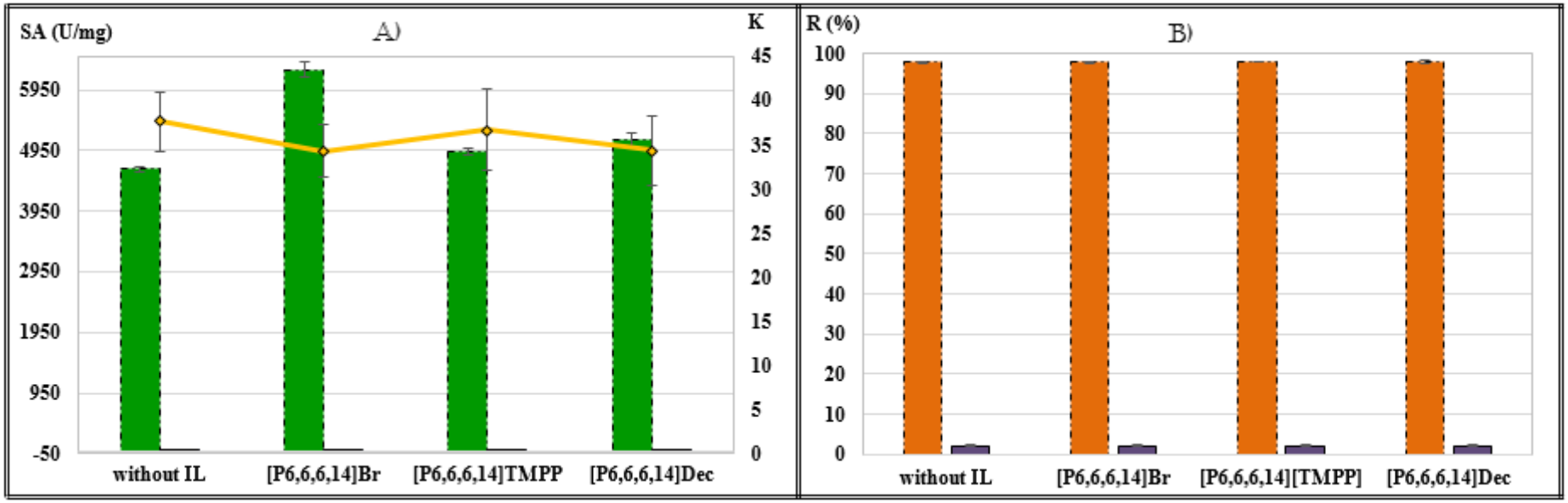

Figure 2. A) Bromelain specific enzymatic activity (U/mg protein) of the AMTPS without and with the phosphonium family, for both top (■) and bottom (■) phases; and the enzyme partition coefficient (◆); **B)** bromelain recovery for both top (■) and bottom (■) phases. The lines connecting the K values are only for eye guide.

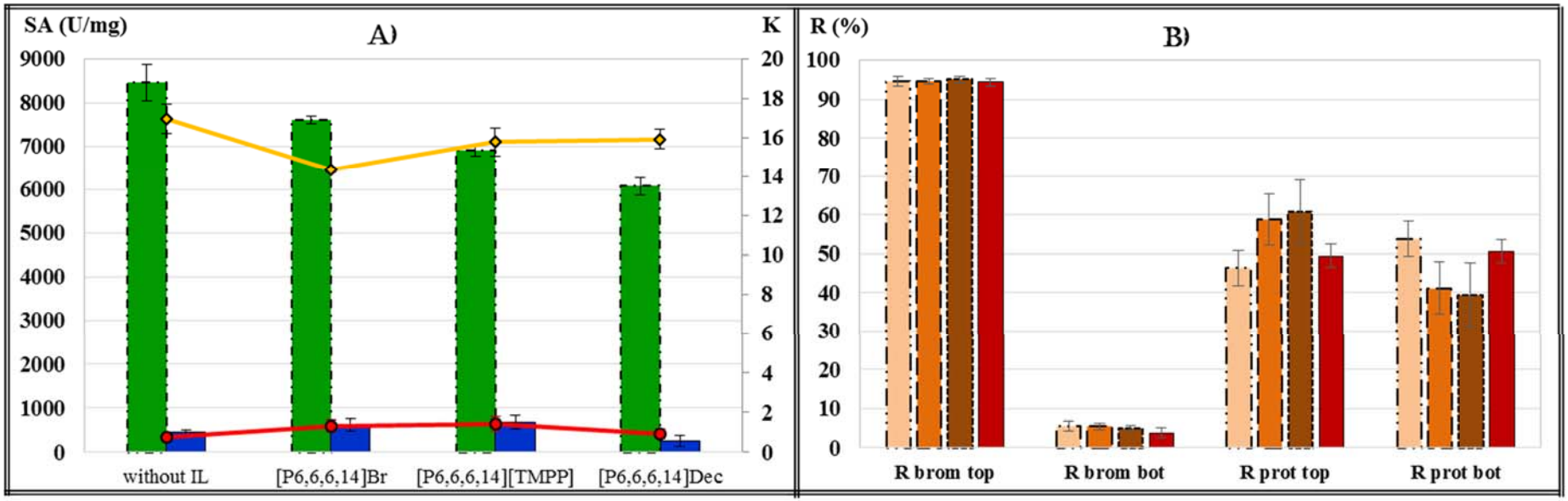

Figure 3.A) Bromelain specific enzymatic activity (U/ mg protein) at the top (■) and bottom (■) phases of the traditional AMTPS (without IL) and the mixed ones with the phosphonium family; the enzyme partition coefficient: ($K_{bromelain,}$ ◆), and the total protein coefficient: ($K_{total\ proteins}$, ●); **B)** Bromelain recovery (R, %) for top and bottom phases for the same AMTPS and for the enzyme (Rbrom) and the total proteins (Rprot): (■) without IL, (■) $[P_{6,6,6,14}]$Br, (■) $[P_{6,6,6,14}]$[TMPP] and (■) $[P_{6,6,6,14}]$Dec. The lines connecting the K values are only for eye guide.

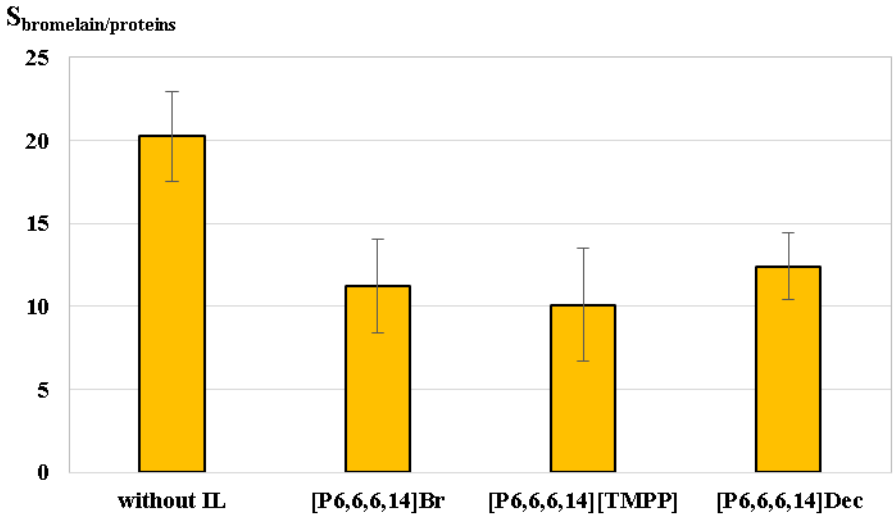

Figure 4. Description of the selectivity parameter considering the partition coefficient ratio of bromelain and the total proteins for the AMTPS with the ILs under study and the AMTPS without IL presence.

Table 1. The non-ionic surfactant, Triton X-114 and the ILs as co-surfactants used.

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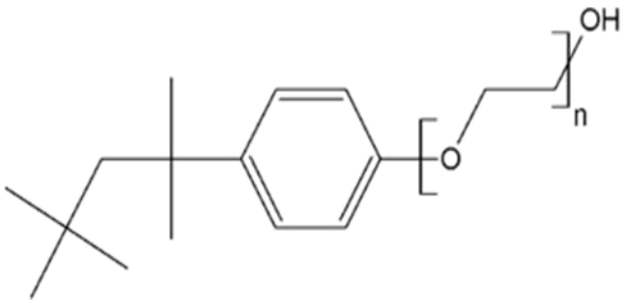
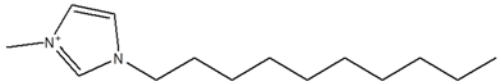
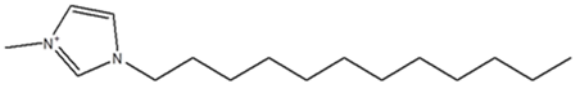
<tr><td rowspan="5"> 
Triton X-114, n = 7-8</td><td> 
$[C_{10}mim]^+$</td><td rowspan="3">Cl^-</td></tr>
<tr><td> 
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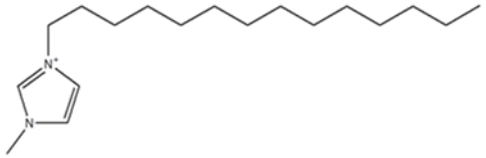
<tr><td> 
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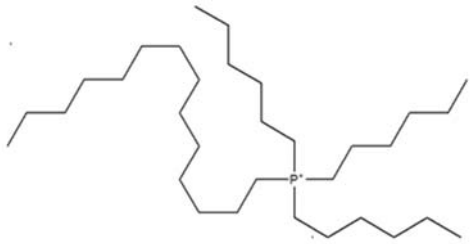
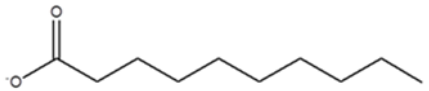
<tr><td rowspan="3"> 
$[P_{6,6,6,14}]^+$</td><td>Br^-</td></tr>
<tr><td> 
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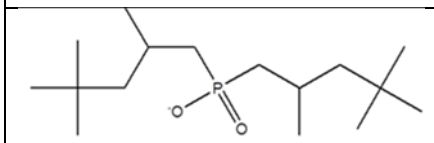
<tr><td> 
$[TMPP]^-$</td></tr>
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