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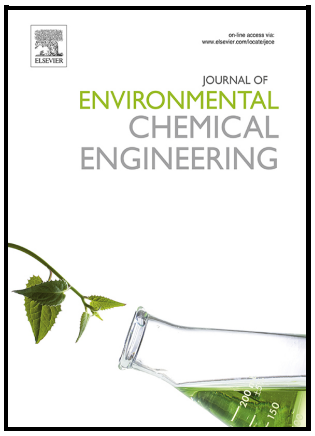

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Cloud point extraction based on non-ionic surfactants: an ecofriendly tool for recovering papain from papaya latex

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Abstract

The potential use of cloud point extraction with aqueous micellar two-phase systems (AMTPS), formed by the non-ionic biodegradable surfactants Triton X-114 (TX-114) and Genapol X-080 (GX-080), to recover papain (PAP) from fresh

papaya latex was evaluated. Binodal curves obtained for both surfactants, in absence and presence of PAP, showed cloud points below 40°C, the PAP presence slightly favoring the phase separation phenomenon.

Partitioning experiments showed that PAP migrated toward the micelle-poor phase independently of the surfactant and the working temperature assayed, this behavior being consistent with this enzyme hydrophilic character. PAP recoveries of over 60% in the poor-micelle phase were obtained when partitioned fresh papaya latex in both surfactant-based AMTPS. TX-114 systems (4.00% w/w, 33°C) allowed recovering 66% of PAP with a purification factor of 4.6. On GX-080 systems (4.00% w/w, 42°C) a yield of 78% PAP and a purification factor of 3.7 were achieved. This AMTPS also allowed the separation of PAP from reducing sugars present in papaya latex.

These results show that TX-114 and GX-080-based AMTPS could be potentially used as simple and economical tools at recovering PAP from papaya latex.

Keywords

Papain; Micellar systems, Sustainable methods, Biodegradable surfactants, Cloud point extraction, Aqueous two-phase systems

1. Introduction

Liquid-liquid extraction with aqueous two-phase systems (ATPS) constitutes a benign and non-destructive method to recover biological molecules from their natural/bioengineered resources [1, 2]. Simplicity, low cost, scalability, and an eco-friendly environment for labile molecules are advantages that make this strategy promising in downstream processes. A wide range of components (polymer/polymer; polymer/lyotropic salts, alcohol/salts, etc.) combined under

appropriate conditions (concentration, temperatures, pH, etc.), can render ATPS [3, 4]. Surfactants are amphiphilic molecules that, above a critical concentration, i.e. critical micelle concentration (CMC), are able to self-assemble in water into supra-molecular aggregates. These structures, called micelles (also termed “direct/normal” micelles), present a core formed by buried hydrophobic tails while hydrophilic heads are oriented toward the aqueous environment [5]. Some surfactant aqueous solutions, when heated at a critical temperature, i.e., cloud point, also result in biphasic systems with a micelle-rich (or surfactant-rich) and a micelle-poor (or surfactant-poor) phase that can be the upper or the lower layer, depending on the surfactant used [6]. They are named aqueous micellar two-phase systems (AMTPS) and have applications in environmental remediation, elemental speciation analysis [7-9] and liquid-liquid extraction, i.e. cloud point extraction, of a wide range of molecules [6, 10-14]. The most common surfactants employed to form AMTPS are non-ionic, usually belonging to the Triton X and Genapol series due to their low cloud points (25-40 °C) and eco-friendly characteristics. Genapol X-080 presents additional advantages such as minimal absorption at 280 nm, which prevents overlapping with protein absorption, and it is FDA approved.

In the protein purification field, after the pioneering work of Bordier [15], an abundant amount of work has been published concerning the use of AMTPS; however, most of them comprise the extraction of hydrophobic membrane proteins [16, 17]. Recovery of hydrophilic soluble proteins has been generally reported by using “reverse” micelles formed in organic solvent media, in which the hydrophilic groups are oriented toward inside and the hydrophobic groups are exposed to the outside. Extraction of proteins using reverse micelle strategy

includes two steps: -a forward extraction that solubilizes proteins into de aqueous core and -a backward extraction in which the protein is recovered from the reverse micelle [5, 18].

To the best of our knowledge, works on the recovery of soluble proteins by cloud point extraction with AMTPS (direct micelles) are scarce. Some of them report that proteins are recovered in the micelle-rich (surfactant-rich) phase due to the protein-surfactant hydrophobic interaction which is enhanced by the salt presence [19, 20]. In these cases, a back-extraction would be required to recover a surfactant-free protein for some final applications.

Among proteins, those with catalyzing function, i.e., enzymes, are increasingly demanded in industrial processes due to economic and environmental advantages [3, 21]. Proteases are the most important type of commercialized enzymes, comprising more than 60% of the global market share [22]. Microbial proteases are preferred to those of plant and animal origin due to their broad biochemical diversity, the susceptibility to be engineered, and the feasibility of being produced at a large scale with low costs [23]. However, several plant proteases are still extensively used because they develop proteolytic activity on various protein substrates under a broad range of operating conditions. Particularly, papain (PAP), from papaya plant (*Carica papaya*), is still used in dairy, brewing, and baking industries, meat tenderization and the production of protein hydrolysates due to its temperature stability and high half-life [24, 25].

Natural PAP is a minor component (5-8%) in the latex of papaya fruit among other cysteine-endopeptidases, such as chymopapain, glycyl endopeptidase, and caricain [25]. Papaya enzymes commercially available are obtained by oven- or spray-drying following the latex collection. Frequently, this operation

renders a dried latex with reduced activity, which becomes brown easily due to oxidation processes [26]. Further purification of the enzyme from dried latex has been developed by different methods such as the classical salt precipitation and various types of chromatography (ion-exchange, affinity, covalent), which require a clarifying step before the latex is loaded onto the chromatography columns [27]. Different scalable strategies that allow purification without requiring a drying stage have been reported in the last decade. He et al. [28] developed an efficient recovery method from non-clarified papaya juice feedstock through reversed-phase expanded bed adsorption chromatography, thus achieving an enzyme with purity close to 75%. Extraction with aqueous two-phase systems consisting of the polymer polyethylene glycol (PEG) and ammonium sulfate salt [27] and three phase system of crude extract, t-butanol and ammonium sulfate were successfully used to recover PAP from latex [29]. Our research group proposed to use alginate (0.1% w/w) as a macro-ligand in PEG/sodium citrate (NaCit) ATPS to recover PAP from latex [30]. Alginate was demonstrated to be a successful affinity macro-ligand due to several reasons: -it partitioned toward the polymer-rich phase; -it bound papain reversibly, thus driving the protein partitioning to the polymer-rich phase and -it could be later precipitated by an appropriate stimulus, i.e. addition of calcium ions. These features allowed us to design a novel strategy that, although exhibiting lower purification indicators (a yield of 72% and a purification factor of 2.41) than those extractions that use ammonium sulfate, represented an environmentally friendlier alternative. It allowed the separation of the target molecule and the recycling of the polymer phase, minimizing the environmental impact caused by the salt disposal.

In this context, developing other more sustainable tools to recover PAP directly from papaya latex with improved performance indicators represents an attractive challenge. The main goal of this work was to evaluate the suitability of AMTPS, formed by the biodegradable surfactant Triton X-114 and Genapol X-080, to recover PAP from fresh papaya latex. The effect of both surfactants on the thermal stability and activity of PAP was analyzed. Recoveries and purification factors at different incubation temperatures were calculated to select the system and operating conditions with the best performance. Additionally, a comparison of main features, achieved by the method here proposed and other ones based on phase equilibrium, is presented.

2. Materials and methods

2.1. Reagents and raw materials

Genapol X-080 (GX-080) and Triton X-114 (TX-114) of analytical grade and commercial papain (PAP_{com}) were purchased from Sigma-Aldrich (St. Louis, MO, USA, product codes 48750, 9036-19-5 and P3375). Reagents were used as received without further purification. Solutions were prepared by adding deionized water to an appropriate amount of reagent. A PAP_{com} stock solution (100 mg/mL) was prepared by dissolving the enzyme in 50 mM sodium phosphate pH 6.00. This solution was stored at -18°C until use. Fresh latex was collected from locally grown *C. papaya* trees. Several longitudinal incisions, 2-3 mm in depth, were made on the surface of unripe fruits with a stainless-steel knife. Exuded latex was collected in a glass beaker, placed in ice, and stored at -18 °C. A 100 mg/mL latex suspension was prepared by mixing thawed latex

with phosphate pH 6.00 buffer. The suspension was then fractionated and stored at -18°C until use.

2.2. Surfactant binodal curves

Binodal curves of Genapol X-080 and Triton X-114, defining the boundary between the micelle-poor and micelle-rich phases, were determined by the cloud point method firstly reported by Watanabe and Tanaka (1978) with modifications [31]. The temperature at which a surfactant solution of a given concentration becomes cloudy is the cloud point (CP). Buffer solutions of GX-080 and TX-114 in 50 mM sodium citrate (NaCit) pH 5.20 were prepared, in triplicate, at final concentrations in the range of 0.1-7.0% w/w. At room temperature (23 °C), solutions were clear with no turbidity. When placed in a transparent thermostat CT52 (SI Analytics GmbH), and after raising the temperature in stepwise 0.01 °C increments, each solution became cloudy at its corresponding CP. CP's visual determination could not be achieved when the surfactant concentration was lower than 0.50% w/w. A spectrophotometric procedure was employed in these systems based on the drastic increase of solution absorbance at 400 nm due to turbidity. Binodal curves were obtained by plotting the CP mean values corresponding to three independent determinations against the surfactant concentration. The standard deviations were calculated in each case.

2.3. Surfactant critical micelle concentration determination

Critical micelle concentrations (CMC) of TX-114 and GX-080 in buffer solutions were determined by fluorescent measurements using the fluorescent probe

1-anilino-8-naphthalenesulfonate (ANS) [32]. Surfactant solutions 0.40% w/w were prepared by adding an appropriate amount of pure TX-114 or GX-080 to a 50 mM NaCit buffer solution. Growing volumes of this solution (0-150 µL) were added to a 0.1 mM ANS and 20 µM commercial PAP solution (in 50 mM NaCit buffer). This experiment was also performed in the absence of enzyme. Fluorescence emission at 480 nm (excitation wavelength 360 nm) was measured in triplicate. The mean values corresponding to each surfactant concentration were plotted against surfactant concentration, thus resulting in a graph with two approximately linear sections. Data in each linear section were fitted using the least-squares approach and CMC was obtained from the intersection between the curves.

2.4. Effect of surfactant media on PAP stability

To evaluate the effect of surfactant on enzyme stability, PAP_{com} solutions (840 µM), were incubated in GX-080 and TX-114 (0.1-1.0% w/w) for 2 h at different temperatures (20-50 °C). Samples were withdrawn at 60 min intervals for determining enzyme stability against time. Changes in functionality were evaluated by monitoring enzyme activity. All determinations were carried out in triplicate. Results were expressed as residual activity, calculated as the current activity after incubation, divided by its initial value in the absence of surfactant at 20 °C. Enzyme activity measurements were carried out according to the protocol described below (section 2.7.).

2.5. Partitioning experiments

Small aliquots (0.250 g) of stock solutions (100 mg/mL) of PAP_{com} were added to graduated glass tubes containing 4.750 g of surfactant (GX-080 or TX-114) solution (4% w/w) in 50 mM NaCit pH 5.20. The resulting systems were homogenized (30 rpm) in a tube rotator apparatus (Bioelec®, Argentina) for 1 h at room temperature. Each system was then placed in a thermostated water bath and incubated at a given temperature (a few degrees above the respective CP) for 2.5 h to achieve the phase separation equilibrium. After incubating, both phases were separated by centrifuging at 4000 rpm (1500 xg) for 10 min at constant temperature (incubation temperature ± 0.1 °C) in a thermostated centrifuge (Sigma Laborzentrifugen, 3-18KS). Finally, enzyme activity was determined in samples of the top and bottom phases.

The partition coefficient of PAP_{com} (Kp_{PAPcom}) was calculated using the following equation:

$$Kp_{PAP_{com}} = \frac{(Act_{PAP})_{poor}}{(Act_{PAP})_{rich}} \quad (1)$$

where $(Act_{PAP})_{rich}$ and $(Act_{PAP})_{poor}$ are the enzyme activities at the micelle-rich and micelle-poor phases, respectively. In the GX-080 AMTPS, the micelle-rich phase was the top phase, while in the TX-114 AMTPS, the micelle-rich phase was the bottom phase.

Papaya latex was also partitioned by following the protocol described for PAP_{com} with slight modifications. In this case, 0.500 g of “latex suspension” and 4.500 g of surfactant solution were mixed to form the systems. After homogenization, incubation, and phase separation, PAP activity in each phase was determined, and $Kp_{PAPlatex}$ was calculated using equation 1. Additionally, reducing sugars (RS) and total protein (TP) content at the starting latex, micelle-

rich and micelle-poor phases were determined to calculate the purifying indicators.

All partition experiments were performed in triplicate. The mean Kp values and their estimated standard deviations were presented. Mass balance (%) was also calculated for each molecule after partitioning.

2.6. Monitoring of the extraction process from latex

2.6.1. Performance indicators

When partitioning the papaya latex, the purification factor (PF) and the recovery percentage (R%) of PAP at a given phase (micelle-rich/-poor) were calculated as follows:

$$PF_{rich/poor} = \frac{(Act_{PAP})_{rich/poor} / [TP]_{rich/poor}}{(Act_{PAP})_{latex} / [TP]_{latex}} \quad (2)$$

$$R_{rich/poor}\,\% = \frac{(Act_{PAP})_{rich/poor}\ V_{rich/poor}}{(Act_{PAP})_{latex}\ V_{latex}} \quad (3)$$

where Act_{PAP} is enzyme activity; [TP] is total protein concentration; V is the volume corresponding to a given phase (either micelle-rich or micelle-poor) or to the latex loaded into the AMTPS.

The ability of AMTPS of separating PAP from reducing sugars (RS) was also evaluated through the calculation of the separation/selectivity coefficient, β, defined as:

$$\beta = \frac{Kp_{PAP}}{Kp_{RS}} \quad (4)$$

where Kp_{RS}, the partition coefficient of RS, was calculated as the ratio of RS concentration at the micelle-poor and micelle-rich phases.

2.6.2. Electrophoretic analysis

PAP purification was also evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) according to the Laemmli method [33]. The assay was carried out using 4% stacking gel and 10% running gel. Latex suspension, PAP_{com} stock solution, and phases were treated with iodoacetamide to maintain the protein sulfhydryls groups. In addition, phases from partitioning were previously precipitated by trichloroacetic 20% acid to concentrate the proteins. Electrophoresis was performed at a constant current of 15 mA during the stacking stage and followed by 17 mA for 1 h. After electrophoresis, proteins in the separating gel were made visible by staining with Coomassie Brilliant Blue.

2.7. Analytical determinations

PAP amidase activity was determined using alpha-N-benzoyl-dl-arginine-p-nitroanilide (BAPNA) as substrate [30, 34]. BAPNA final concentration was 0.95 mM in 50 mM Tris–HCl buffer pH 8.20, cysteine 100 mM (enzyme activator). The reaction was followed by measuring the absorbance of the reaction product p-nitroanilide at 400 nm for 4 min. The assay was performed at a constant temperature of 25 °C by placing the reaction mixture in a Jasco V-550 spectrophotometer with a thermostated (± 0.1 °C) 10 mm path length quartz cuvette. Enzyme activity was calculated from the initial linear portion of the absorbance vs. time plot.

TP concentration in the latex suspension and phases was estimated by the Warburg and Christian method [35].

RS concentration in the latex suspension and phases were also determined by the 3,5-dinitrosalycilic acid (DNS) colorimetric method [36]. Glucose solutions (0-100 mM), dissolved in different media (buffer and micelle-rich/poor phases), were used as standard.

3. Results and discussion

3.1. Binodal curves and critical micelle concentration

Characterization of phase equilibrium is the first step when using aqueous micellar two-phase systems for purifying purposes. Knowledge of the working conditions required to form two-phase systems, like temperature, surfactant concentration, ionic strength, buffer composition, etc., is essential to perform partitioning experiments. Binodal curves depend on the kind of surfactant and salt used (type of salt and concentration). Additionally, slight displacements in the binodal curve of a given surfactant could be found when surfactants of different suppliers or batches are used. Consequently, obtaining the binodal curve is a mandatory first step to characterize the system, irrespective the corresponding binodal curve being available in literature.

Figure 1 shows the phase diagrams corresponding to TX-114 and GX-080 in 50 mM NaCit buffer pH 5.20. Different binodal curves were obtained for each surfactant considered; however, both have the U/V-shaped pattern [37], typical for non-ionic surfactants. GX-080 and TX-114 systems (1-7% w/w) presented cloud points (CP) at 38-40°C and 21-24°C, respectively, these being comparable values to those obtained previously [31]. A low NaCit concentration (50 mM) was selected to perform this work in order to guarantee a pH regulation without favoring hydrophobic interactions between the target protein

(papain) and micelles, which are enhanced at high salt concentrations [20]. While the cloud points of GX-080 are higher than those of TX-114, they are low enough to be below the denaturing or decomposition temperature of a wide range of molecules, including those of biological nature. Figure 1 also shows the effect of PAP presence on the phase-separation behavior of both surfactants. In the presence of PAP, the binodal curves moved to a slightly lower (≅1 °C) CP. This decrease depends on PAP concentration. This result agrees with previous reports on the effect of *Pichia pastoris* fermentation supernatants on Triton X-114 AMTPS [13] and changes in the behavior of surfactant solutions produced by the introduction of other biomass samples [38]. The highest PAP concentration, here assayed (280 μM), is similar to the usual protein content present in the latex suspension.

Table 1 shows critical micelle concentration (CMC) values obtained for TX-114 and GX-080 according to section 2.3. The CMC values are low and similar to those reported in the literature [31], suggesting that both surfactants can readily form micelle aggregates in the operating conditions. This also implies that micelles are present even in the poor-surfactant phase of a AMTPS. When PAP 20 μM is present, a slight decrease in CMC is observed for both surfactants, these results being compatible with the weak interactions reported in literature for proteins and non-ionic surfactants [39].

For GX-080, CMC is lower than for TX-114 with/without PAP. To explain this finding, it should be considered that when surfactant molecules are dissolved in an aqueous medium, water molecules form highly ordered layers, ice-like structures, around the surfactant hydrophobic tails in order to minimize the contact area. This mechanism, termed “hydrophobic effect”, depends on the

structural characteristics of hydrophobic group (number of carbons, shape, etc.) [40]. During the micelle formation, the hydrophobic tails self-assemble and the ordered water molecules are released into the bulk, thus resulting in an entropy increase which contributes to the thermodynamic favorability of micellization (Supplementary Figure S1 A) [41]. The greater the hydrophobic effect, the greater is the entropy increase which favors the micelle formation and reduces the CMC. When comparing TX-114 and GX-080 chemical structures, it is noticed that their polar heads are similar in structure and size while, their hydrophobic groups, although possessing a similar number of carbons, differ each other in their structure (Supplementary Figure S1 B). The hydrophobic tail in GX-080 is an aliphatic linear chain while it consists of an aromatic ring and a branched alkyl chain in TX-114. According to literature, aliphatic organic molecules have stronger hydrophobic effect than aromatic and branched alkyl structures. Consequently, it is reasonable that the surfactant owing the tail with the greatest hydrophobic effect, i.e. the GX-080, exhibits a major tendency to form micelles and the lowest CMC.

3.2. Effect of surfactant media on functional PAP behavior

Several surfactants, particularly those of ionic structure, are known to denature proteins, thus causing the loss of their structural integrity and functionality. This effect is dependent on surfactant concentration, and therefore it is necessary to evaluate protein stability before using any surfactant for extracting purposes. Although TX-114 and GX-080, both non-ionic surfactants, have been reported to exhibit low denaturing capability, their incidence on PAP activity was tested.

The effect of both surfactants at different concentrations on enzyme activity over 120 min is shown in Figure 2A. In the 0.1-1.0% w/w concentration range, neither TX-114 nor GX-080 significantly affects PAP amidase activity with BAPNA as substrate. After 2 h of incubation, a slight decay (5-10%) of enzyme activity was observed in the absence and presence of surfactant. Thus, the surfactant presence in these concentrations does not affect the enzyme activity. It is worth noting that the surfactant concentration assayed largely exceeds the concentration usually present in the micellar-poor phase of AMTPSs. PAP is a hydrophilic protein; therefore, it is expected to migrate to the aqueous (micelle-poor) phase. Above mentioned experiments were carried out at 20 °C; however, phase separation of surfactant systems occurs at higher temperatures; therefore, thermal denaturation and loss of PAP activity under potential working temperatures were also evaluated [42]. Figure 2B shows the effect of surfactant presence and incubation temperature on residual enzyme activity. Surfactant concentration was 1% w/w, and incubation time was 2.5 h. Residual enzyme activity remained close to 100% (reference value obtained at zero time in the absence of surfactant) at temperatures between 20 to 35°C, irrespective of surfactant presence. At higher temperatures, moderate decays in the enzymatic activity were observed. The enzyme retained most of its initial activity (74-83%) up to 48 °C. A further loss of amidase activity of 28% was observed at 50°C in absence of surfactant, while activity diminished to 50% of the initial values in presence of TX-114 or GX-080. These findings set an upper limit to the working temperature for the recovery of PAP by AMTPS of GX-080 and TX-114 to 48 °C. To assess the reversibility of these effects, enzyme incubated at 50 °C was cooled in a bath at 20°C for 1 h. No regain of activity was observed, but a

further loss was found on all systems, even those without surfactant, suggesting the process is irreversible. These findings could be explained by favorable interactions between surfactant molecules and hydrophobic moieties present in the enzyme active site that are exposed by heating at sufficiently high temperatures. Taken together, these results indicate that PAP is stable to perform the aqueous micellar two-phase extraction in the range of 0-1% GX-080 or TX-114 and between 20 to 48°C.

3.3. Partitioning profile of commercial PAP in AMTPS

Once the binodal curves were obtained, three different incubation temperatures were selected to study the enzyme partitioning behavior on each surfactant system. A total surfactant concentration of 4% for all the systems was assayed to achieve phase equilibrium volumes that were easily manageable. Note that too low/high surfactant concentrations would lead to minimal micelle-rich/micelle-poor phase volumes that are hard to measure and separate for quantifying purposes. The temperatures to assay PAP partitioning were determined by successive temperature rises of either 5 or 3°C steps above the CP of TX-114 and GX-080 4% w/w solutions (23 and 39°C respectively) to assure that the phase separation takes place. For GX-080 lower temperature rises were applied to avoid high temperatures that could compromise PAP stability. Table 2 summarizes assayed working conditions and the composition and volume of each equilibrium phase.

To evaluate the partitioning behavior of commercial PAP in both AMTPS (Supplementary, Figure S2), the partition coefficient Kp_{PAPcom} was calculated according to the equation 1, i.e. the ratio between PAP concentration in the

poor- micelle and the rich-micelle phases. All the Kp_{PAPcom} values are above 1 in the temperature range assayed, independently of the surfactant used, indicating that most PAP is partitioned into the aqueous phase (micelle-poor one). Bordier [15] and Rangel-Yagui et al. works [43] demonstrated that protein partitioning in AMTPS is driven by the interaction between hydrophobic proteins and surfactant micelles and excluded-volume interactions between micellar structures and hydrophilic proteins. Therefore, the asymmetrical distribution of PAP toward the poor-micelle phase is a predictable behavior consistent with its hydrophilic character. Kp_{PAPcom} values are higher in TX-114 (Kp values ranging from 2.0 to 3.2) than in GX-080 systems (from 1.2 to 1.8). On TX-114 systems, a slightly higher Kp_{PAPcom} was observed at 38°C compared to the other temperatures. On GX-080 systems, Kp_{PAPcom} values were similar at all temperatures assayed. Besides, the profile Kp_{PAPcom} vs. incubation time was analyzed. As shown in supplementary Figure S2, 2.5 h showed to be an incubation time long enough to achieve both a complete phase separation and the partition equilibrium.

3.4. PAP extraction from papaya latex

3.4.1. Partitioning profile of PAP from papaya latex in AMTPS

Figure 3 shows the partition coefficients obtained for commercial and papaya latex PAP in AMTPS. As a general trend, $Kp_{PAPlatex}$ values over 1 were obtained, similarly to Kp_{PAPcom}, this evidencing that PAP is partitioned preferentially to the more hydrophilic phase, that is, the micelle-poor one, independently of the enzyme source. All the $Kp_{PAPlatex}$ values, except that from GX-080 at 45°C, were lower than the Kp_{PAPcom}. Similar results were reported by

Malpiedi et al. on the partitioning of bovine trypsinogen in PEG/sodium tartrate aqueous two-phase systems [44]. They proposed that certain compounds, present in a complex matrix (crude extract) together to the target molecule (trypsinogen) can interact with the latter and alter its partitioning behavior. In addition, $Kp_{PAPlatex}$ exhibited slightly higher values in TX-114 AMTPS than in GX-080 ones.

3.4.2. Performance indicators of the extraction process

To evaluate the extraction process from papaya latex using AMTPs, two parameters, recovery percentage, R% (Figure 4A), and purification factor, PF (Figure 4B), were determined. The recovery in all aqueous phases (micelle-poor ones) was 60% or higher, in agreement with the $Kp_{PAPlatex}$ values (>1) previously obtained, confirming an asymmetrical distribution of the enzyme between the phases. A mass balance close to 100% was obtained, indicating that enzyme loss due to interfacial adsorption was non-significant. On TX-114 systems, the highest recovery, 66%, was obtained at 33°C, which also showed the highest $Kp_{PAPlatex}$ value of 1.63. Purification factors were higher than 4 at all the assayed temperatures, achieving a value of 4.6 at 33 °C. On GX-080 systems, the recoveries on aqueous phases were on average higher than those obtained with TX-114, reaching a value of 78% at 42°C ($Kp_{PAPlatex}$ 2.06). Purification factors were close to or higher than three at the poor-micelle phases, reaching a value of 3.7 at 42 °C. These findings demonstrate a successful performance of AMTPS at recovering PAP from papaya latex since extracts with good recoveries and notorious purification factors are achieved. Purification of PAP from papaya latex for industrial purposes, has routinely

carried out by salt precipitation which allows recovering 50% of PAP with a 39% purity of enzyme [25]. Additional chromatographic steps (ion-exchange and affinity) are required to improve the enzyme quality; however, a pre-treatment of latex which removes particulate matter is needed before applying it to chromatographic columns. Use of aqueous two phase systems to extract PAP from papaya latex has shown to be an interesting strategy for industrial applications since it integrates clarification, concentration and purification in only one unit operation. Several attempts have been made to apply these systems at PAP recovering. Nitsawang et al. [27] recovered 88% of highly pure PAP at the polymer-enriched phase of a PEG/ammonium sulfate ATPS; however, the separation of the PAP from polymer and the environmental impact caused by the salt disposal constitutes significant disadvantages of the reported method. To overcome these drawbacks, Rocha et al. [30] proposed the use of biodegradable sodium citrate as phase-forming salt with PEG and the inclusion of sodium alginate as affinity macro-ligand to recover a polymer-free PAP and recycle the PEG. This strategy allowed extracting 72% of PAP from papaya latex with a purification factor of 2.4. More recently, Hafid et al. [27] used three phase systems formed by crude latex, ammonium sulfate and t-butanol to recover PAP with excellent process indicators (PF 11.45 and R% 134); however, 40% w/w of ammonium salt is required to form the system constitute an environmental disadvantage. The AMTPS, here proposed, conserve the advantages of two/three phase systems, such as simplicity, low cost, easy scalability and present the additional benefit of using eco-friendly and biodegradable phase forming components such as TX-114, GX-080 and NaCit. Besides, the target molecule, PAP is recovered at the aqueous phase, whose

surfactant concentration is low (< 0.05% w/w). Consequently, the obtained extract can be used directly or subjected to chromatographic steps without a pre-treatment requirement.

3.4.3. Reducing sugars partitioning from latex

Papaya latex is a complex matrix that contains proteins (cysteine proteases) over other components such as moisture, ash, fat, fiber and sugar. It should be noticed that carbonyl groups on reducing sugars can readily interact with amino acids in proteins through a complex process named Maillard reaction which affects organoleptic properties, color and protein functionality [45]. Therefore, removing reducing sugars in PAP extract will result in preventing this phenomenon and the consequent loss of enzyme activity during processing and storage.

Figure 5 shows results from partitioning experiments of reducing sugars present in latex. On TX-114 systems, Kp_{RS} were higher than one at all studied temperatures, indicating these compounds migrate to the aqueous phase similarly to PAP. This finding suggests that PAP and reducing sugars cannot be separated each other by using these systems. On GX-080 systems, Kp_{RS} values were close to one at 45°C and 48°C, thus indicating that reducing sugars are distributed evenly between phases; however, at 42°C, a preferential migration toward the micellar phase was observed (Kp_{RS}= 0.64). Clearly, GX-080 systems exhibit a better performance at separating PAP from reducing sugars than TX-114 ones. To quantify these differences and compare them, the separating/selectivity coefficients (β), calculated as Kp_{PAP}/Kp_{RS}, were plotted together with the values of $Kp_{PAPlatex}$ and Kp_{RS} for all the surfactant systems at

the three temperatures assayed (Supplementary Figure S3). The β obtained for the TX-114 systems were close to one (0.8-1.0) at all the temperatures. This indicates that a high concentration of reducing sugars will be present at the poor-micelle extracts together to PAP, even at that corresponding to the system with the best performance (33 °C) which showed a recovery of 66% with a purification factor of 4.6. On GX-080 systems, higher selectivity was observed. The system incubated at 42°C (the lowest temperature assayed for this surfactant) with a recovery of 78% and a PF of 3.7 exhibited the best separating capability with a β value of 2.6.

The partitioning trend, here observed, was similar to that reported by Ritter et al. [46] for D-glucose in aqueous micellar systems formed by Triton X-114 and Tergitol 15-S-7 surfactants. Authors found that the glucose distribution in the mentioned AMTPS depended on the operating temperature. At temperatures slightly above the corresponding cloud point, the difference of the D-glucose concentrations between both phases was small (Kp close to one), while at higher temperatures the sugar distribution into the aqueous phase was more pronounced (Kp>>>1). In our case, TX-114 system was assayed at temperatures (28, 33 and 38 °C) markedly above the cloud point (23 °C); therefore, reducing sugars showed a one-sided distribution toward the micelle-poor phase (Kp_{RS}>>>1). On the other side, GX-080 system, whose working temperatures (42, 45, 48°C) were selected slightly higher than the cloud point (39 °C), showed a more even distribution of reducing sugars (Kp_{RS} close to one). This trend could be explained taking into account that at increasing temperatures, the water content of the micelle-poor phase increases. This

promotes hydrogen bond interactions between sugars and water and favors the distribution of sugar to the micelle-poor phase.

3.4.4. Polyacrylamide gel electrophoresis under denaturing conditions

Taking into consideration the good performance of GX-080 AMTPS (4% w/w, 42 °C), we selected their corresponding extracts to analyze the distribution of papaya latex proteins by SDS PAGE (Figure 6). For comparing purposes, phases from PAP_{com} partitioning at the same systems were also analyzed. It can be seen that raw papaya latex (lane 1) shows several bands characteristic of a complex mixture of enzymes. Micelle-poor phases from partitioning experiments with latex and commercial PAP (lanes 5 and 6, respectively) show a similar pattern, i.e., an intense band corresponding to approximately 20,1 kDa compatible with cysteine proteases. On the other hand, lanes (3 and 4) corresponding to micelle-rich phases show a faint band at this location, thus suggesting the low content of these proteins in the loaded samples. These results confirm the asymmetric distribution of PAP (both commercial and from papaya latex) to the micelle-poor phase, just evidenced by their Kp values lower than one. The rest of the proteins present in the raw papaya latex were not detected on samples from either the micellar or the aqueous phases of AMTPS. For these samples, the protein precipitation performed by trichloroacetic precipitation, according to methodology described in section 2.6.2, could not have been effective enough to achieve a complete precipitation of those proteins with molecular weights around 14.2 kDa due to the surfactant presence.

4. Conclusions

Two biocompatible and biodegradable surfactants, Triton TX-114 and Genapol X-080, were selected to prepare aqueous micellar two-phase systems to recover PAP from papaya latex. Binodal curves obtained for both surfactants showed similar profiles and phase separation temperatures below 40°C, a convenient feature for separating temperature-sensitive molecules such as enzymes. The PAP presence slightly favored both phase separation and micelle formation phenomena. Partitioning experiments showed that the enzyme migrated toward the micelle-poor phase independently of the surfactant used. Yields of over 60% in the aqueous phase for the enzyme extracted from raw papaya latex were obtained. On TX-114 systems (4.00% w/w, 33°C) a yield of 66% in the aqueous phase was achieved with a purification factor of 4.6. On GX-080 systems (4.00% w/w, 42°C), a yield of 78% and a purification factor of 3.7 were achieved, with the additional benefit of a lower reducing sugar content preventing undesirable reactions with the enzyme. These results suggest that the cloud point extraction with AMTPS could be successfully applied as an economical and straightforward early step in the purification process of PAP from papaya latex. The development of a platform to recover PAP directly from fresh papaya latex by a safe, low cost and simple method could avoid latex drying operations which frequently cause loss of enzyme activity. Besides, a new insight into the use of AMTPS to recover soluble hydrophilic proteins in addition to hydrophobic membrane proteins is introduced. Several features of this methodology such as −mild working temperatures that prevent enzyme denaturation, −separation from reducing sugars that could minimize further purification steps and avoid undesirable reactions and −reuse of the micellar

phase that could reduce operating costs and decrease the environmental impact of the process represent noticeable advantages that make the AMTPS a powerful tool whose application could be extended to proteins of interest other than PAP.

Captions for Tables and Figures

Table 1. Critical micelle concentrations of TX-114 and GX-080, without and with PAP 20 μM.

Table 2. Surfactant concentration and phase volume of TX-114 and GX-080 AMTPS at different temperatures.

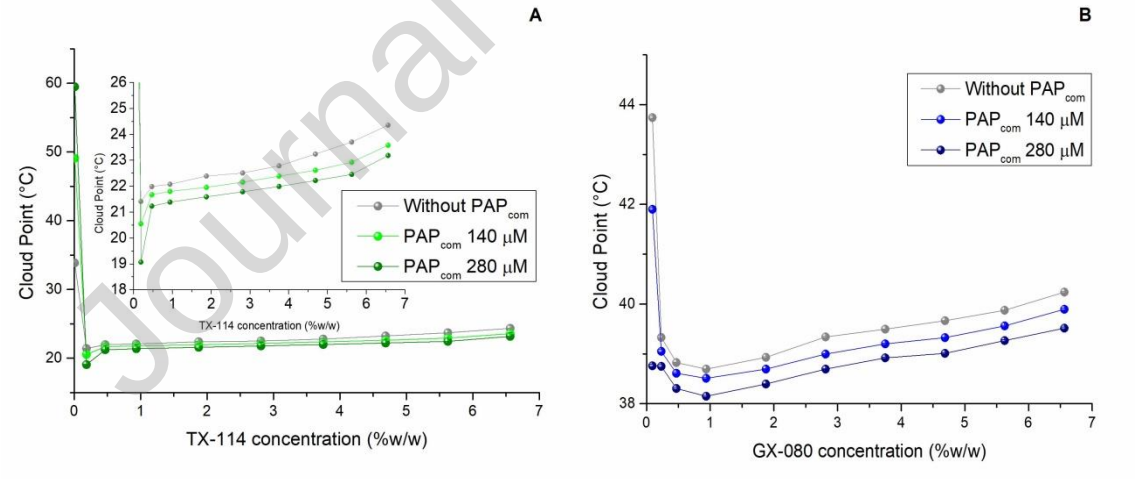


Figure 1. Binodal curves of TX-114 **(A)** and GX-080 **(B)** in the absence and presence of PAP_{com} (140 and 280 μM). Medium: 50 mM NaCit buffer pH 5.20. The graph area corresponding to the lowest cloud points for TX-114 is zoomed in the inset of the Figure **(A)**. Each point is the mean of three independent

determinations. Standard deviations are in the range 0.02-0.07 °C, therefore the error bars resulted practically overlapped by the symbols.

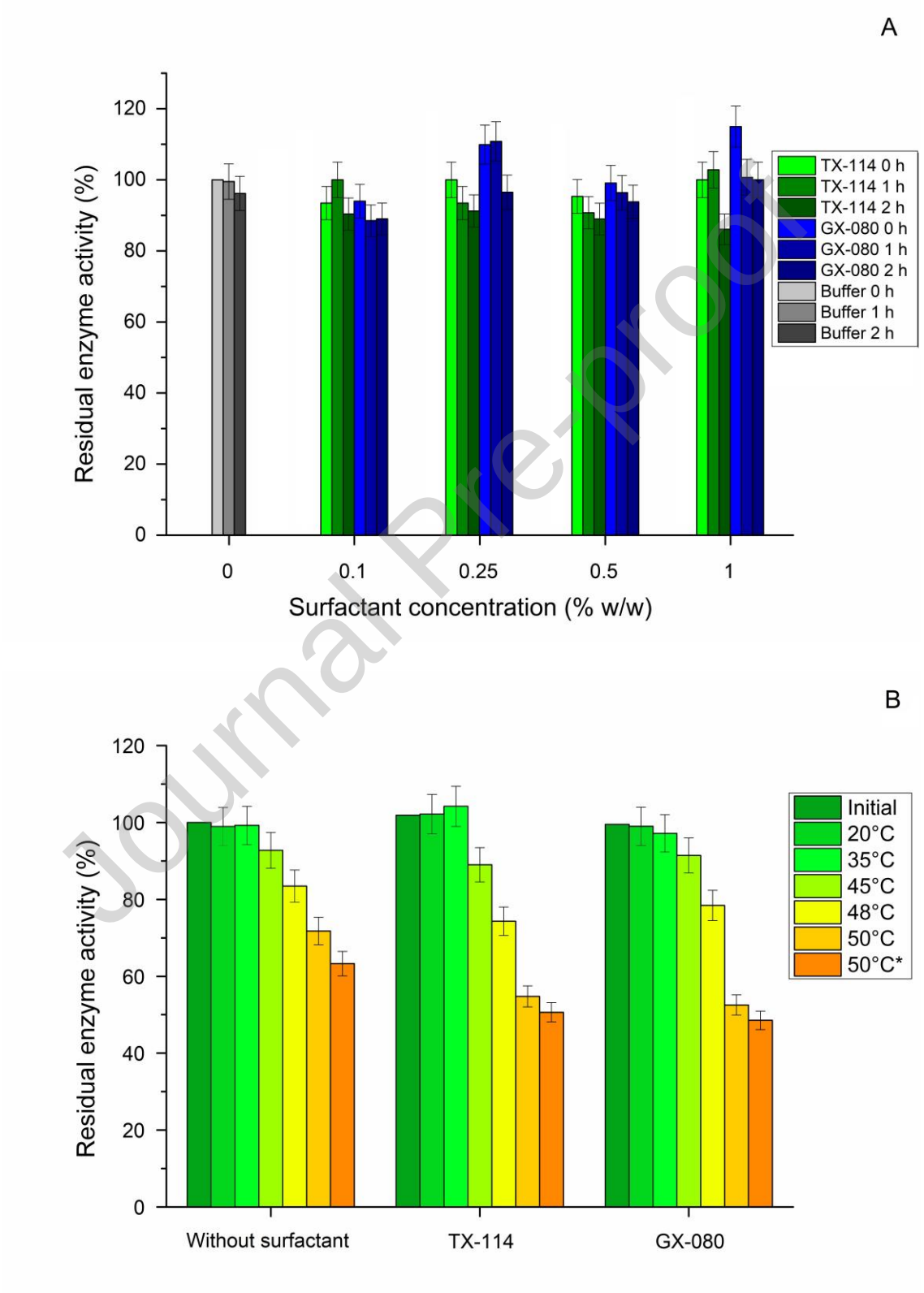


Figure 2. (A) Residual activity (%) of PAP after incubation in TX-114 and GX-080 for 1 and 2 h at different surfactant concentrations. Temperature 20 °C. Initial enzyme activity (zero time) in the absence of surfactant was considered 100%. **(B)** Effect of temperature on the residual activity (%) of PAP in the absence and presence of TX-114 and GX-080 (1% w/w) after 2.50 h incubation. The value of enzyme activity at the initial time (zero time) at 20°C is considered the initial condition. 50°C* represents the residual activity obtained after incubation at 50°C (2.50 h.) followed by incubation at 20°C (1 h).

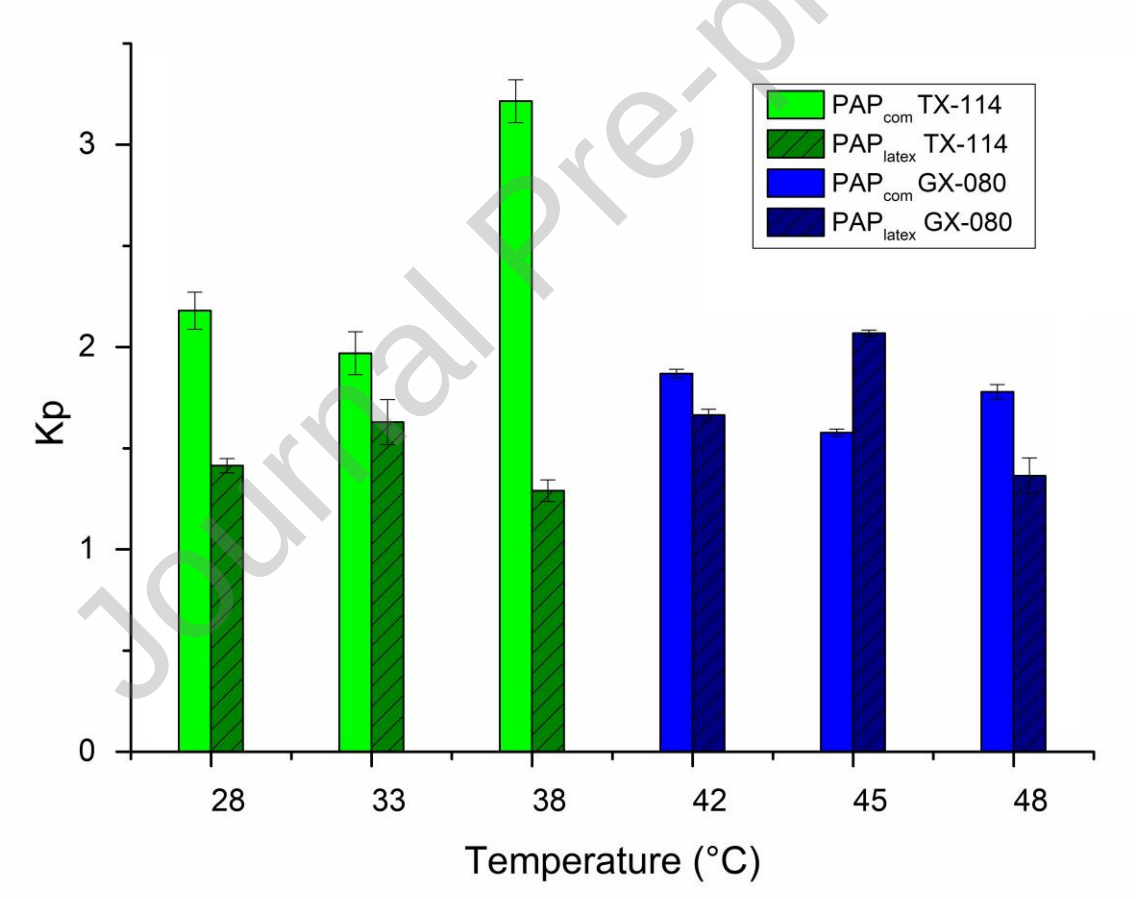
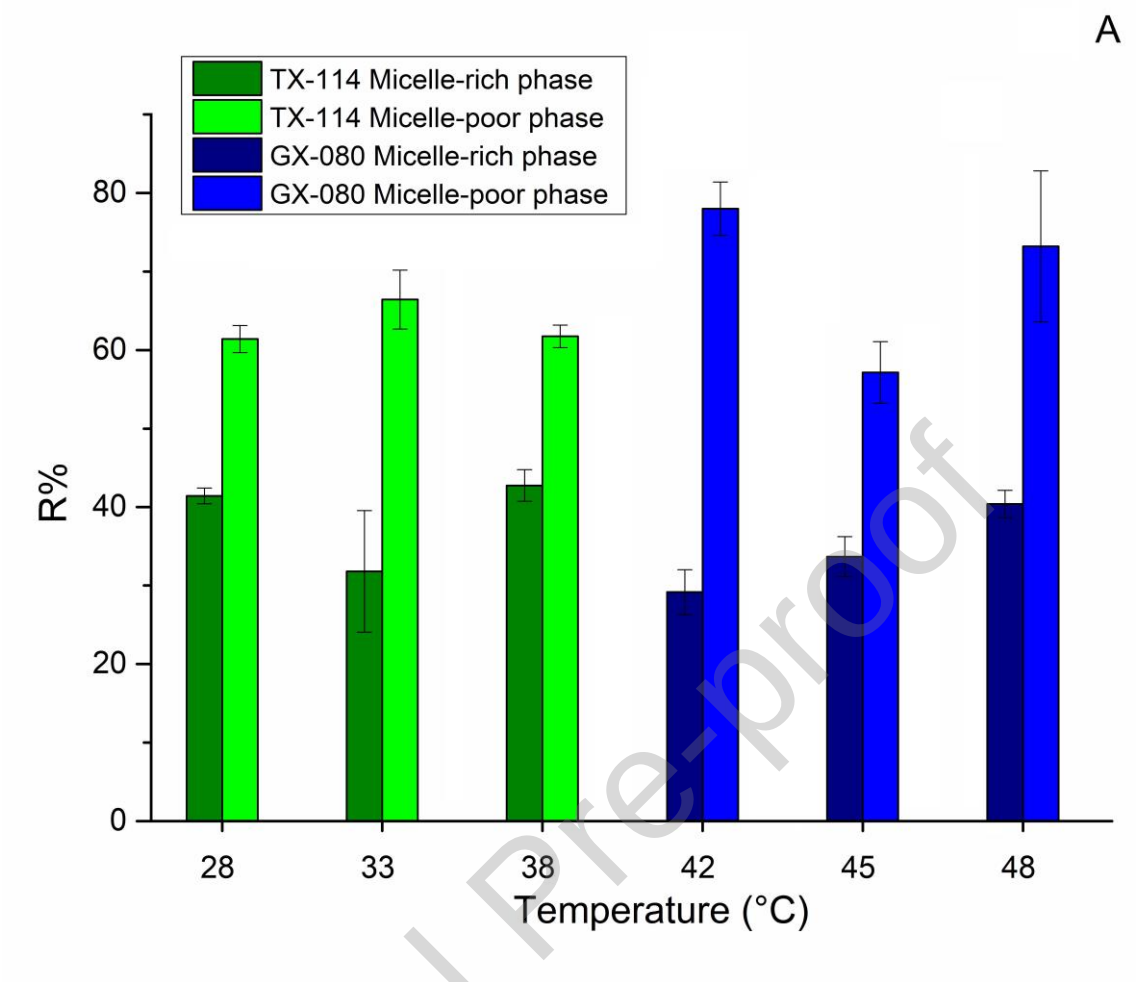


Figure 3. Partitioning behavior of latex and commercial PAP on TX-114 and GX-080 AMTPSs at different temperatures. Incubation time: 2.5 h.



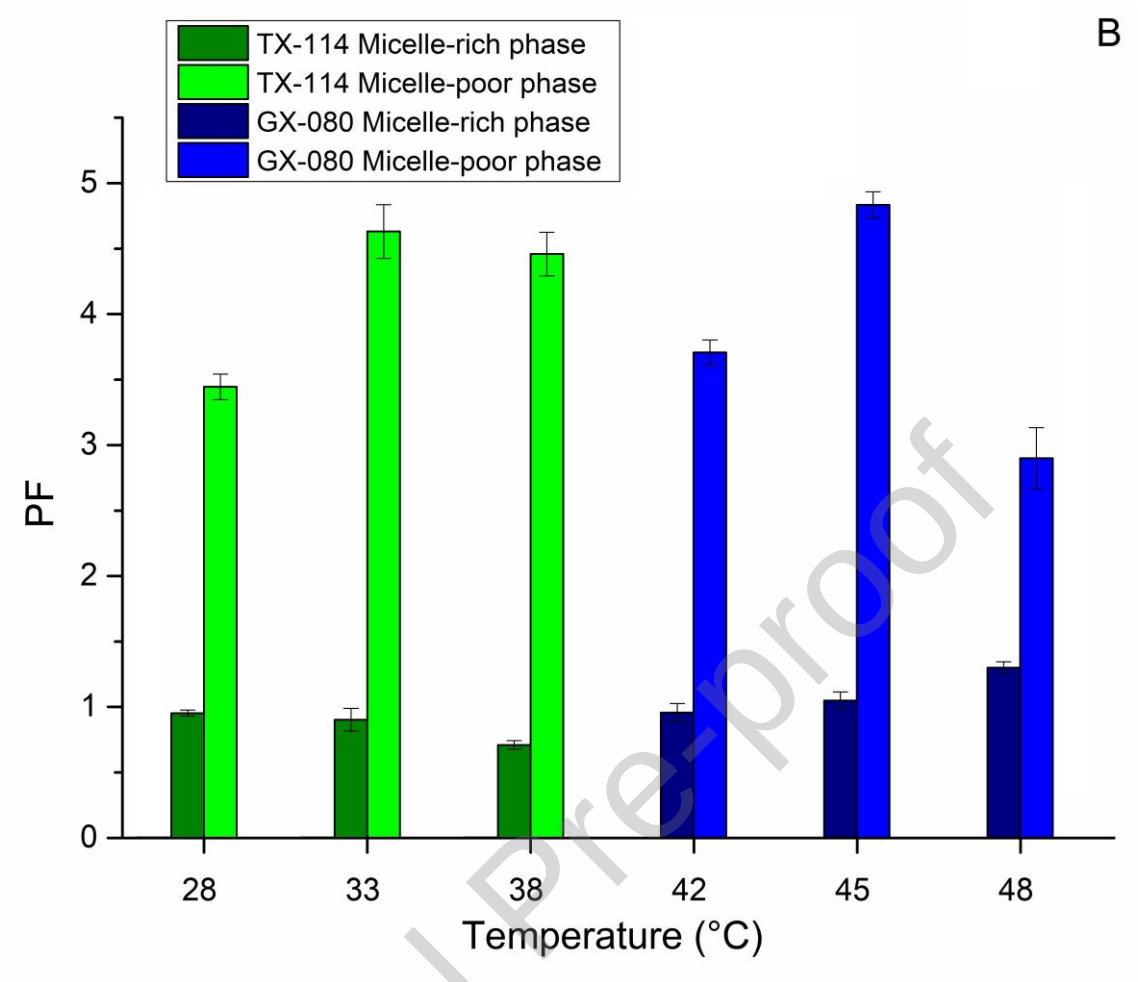


Figure 4. **(A)** Recovery percentage (R%) and **(B)** Purification factor (FP) for PAP from papaya latex on TX-114 and GX-080 AMTPS. Incubation time: 2.5 h.

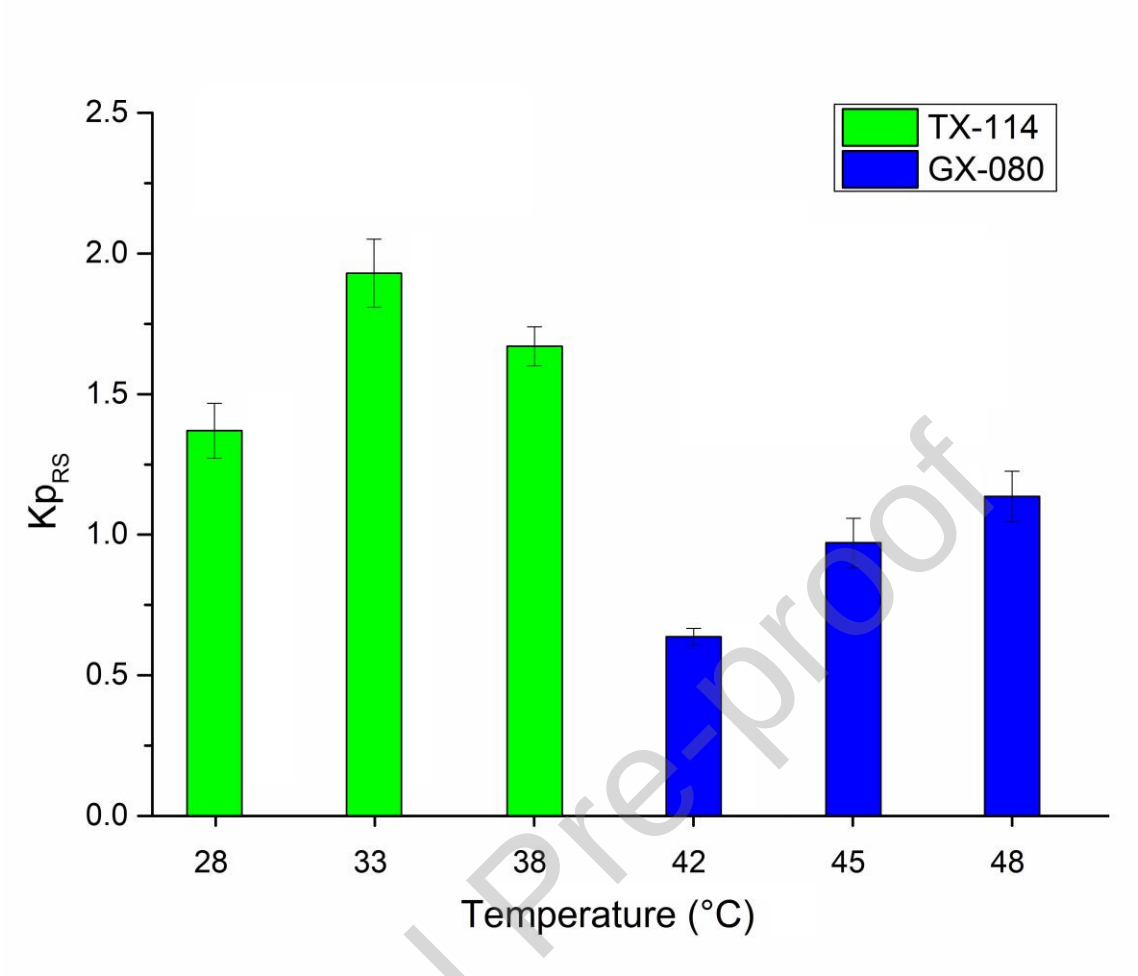


Figure 5. Partition behavior of reducing sugars present in papaya latex on TX-14 and GX-80 AMTPS at different temperatures. Incubation time: 2.5 h.

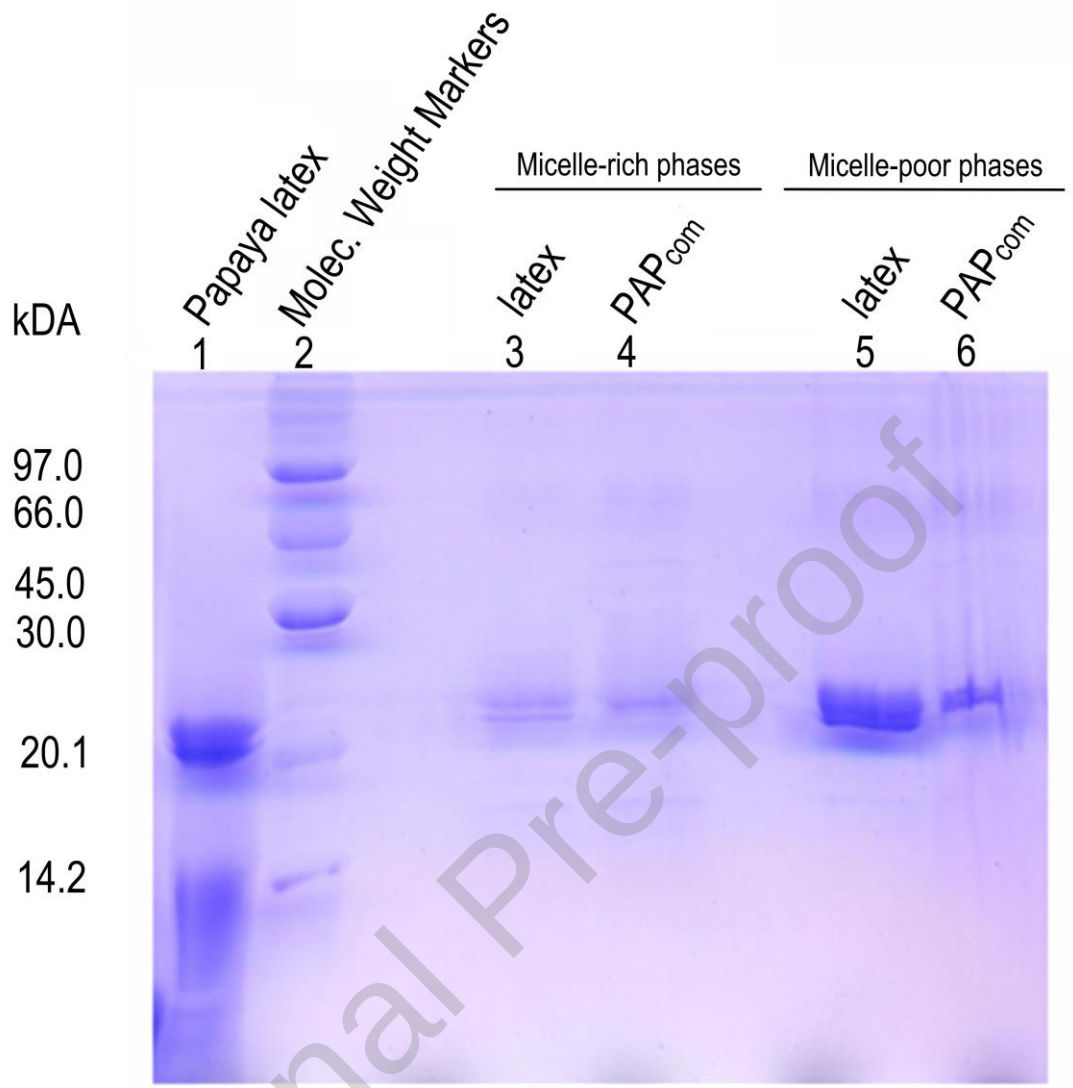


Figure 6. SDS-polyacrylamide gel electrophoresis. Lane 1: Papaya latex, lane 2: molecular weight markers, lanes 3 and 4: micellar phases from partitioning experiments with latex and commercial PAP, respectively, lanes 5 and 6: aqueous phases from partitioning experiments with latex and commercial PAP, respectively.

CAPTIONS OF SUPPLEMENTARY MATERIAL

Figure S1. (A) Schematic representation of micelle formation process and hydrophobic effect. **(B)** Chemical structures of Triton X-114 and Genapol X-080.

Figure S2. Temperature and time dependences of partition coefficient of commercial PAP on TX-114 and GX-080 AMTPS.

Figure S3. Comparison between reducing sugars (Kp_{RS}) and latex PAP ($Kp_{PAPlatex}$) partition coefficients and selectivity coefficients (β) values on TX-114 and GX-080 AMTPS at different incubation temperatures.

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Table 1: *Critical micelle concentration (CMC) for TX-114 and GX-080, in presence and absence of PAP*

	CMC (mM)	
[PAP] (μM)	TX-114	GX-080
0	0.1599 ± 0.002	0.1008 ± 0.013
20	0.1331 ± 0.007	0.0911 ± 0.009

Buffer: 50 mM NaCit pH 5.20.

Table 2: *Surfactant concentration and phase volume of TX-114 and GX-080 AMTPS at different temperatures.*

Temperature (°C)	Surfactant concentration (% w/w)			Phase volume (mL)	
	Total	Micelle-rich phase	Micelle-poor phase	Micelle-rich phase	Micelle-poor phase
			TX-114*		
28	4.00	14.14	0.15	1.10	2.90
33	4.00	17.14	0.12	0.90	3.05
38	4.00	21.16	0.10	0.75	3.30
			GX-080**		
42	4.00	15.91	0.09	1.00	3.05
45	4.00	18.61	0.06	0.85	3.15
48	4.00	21.17	0.04	0.75	3.25

Total system mass 4 g

*TX-114: micelle-rich phase, top phase; micelle-poor phase, bottom phase

**GX-080: micelle-rich phase, bottom phase; micelle-poor phase, top phase

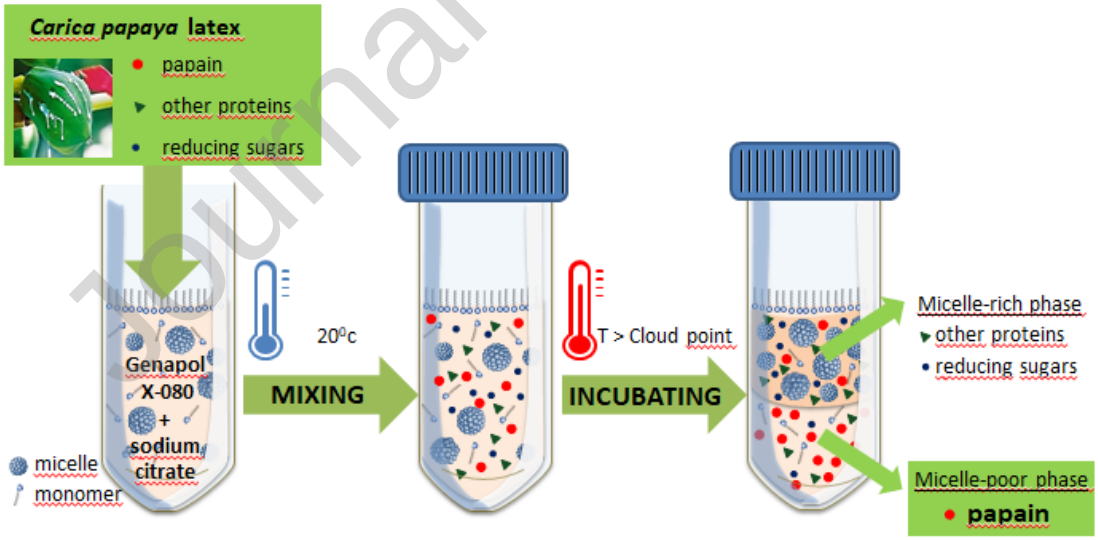
CRediT authorship contribution statement

Melisa Di Giacomo: Methodology, Formal analysis, **Fernando Bertoni:** Methodology, Formal analysis, Writing, Conceptualization, **María Victoria Rocha:** CMethodology, Formal Analysis, Resources**, Fernanda Rodriguez:** Conceptualization, Resources**, Bibiana Beatriz Nerl:** Conceptualization, Review, Resources, Supervision

Declaration of Competing Interest

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Graphical abstract



Highlights

- Cloud point extraction was used to recover papain from *Carica papaya* latex.
- Triton-114 and Genapol X-080 micellar systems were suitable to recover papain.
- Genapol X-080 systems achieved a papain yield of 78% and purification factor of 3.7.
- Papain exhibited thermal stability in surfactant solutions (0-1%) up 48 °C.