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## One-step recovery of latex papain from *Carica papaya* using Three Phase Partitioning and its use as milk-clotting and meat-tenderizing agent

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**Running title:** Three Phase Partitioning recovery of latex papain

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### Abstract

Three Phase Partitioning (TPP) system as an elegant non-chromatographic and bulk separation method was successfully applied for the extraction and recovery of papain from the latex of *Carica papaya*. The optimized parameters of TPP allowed achieving a purification fold of 11.45 and activity recovery of 134% with 40% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1.0:0.75 ratio of crude extract: t-BuOH at pH and temperature of 6.0 and 25°C, respectively. The recovered papain had a molecular weight of 23.2 kDa and revealed maximum activity at pH 6.0 and temperature of 50°C. The maximum values of *K<sub>m</sub>* and *V<sub>max</sub>* parameters were 10.83 mg.mL<sup>-1</sup> and 33.33 U.mL<sup>-1</sup>, respectively. The protease with 4 isoforms was stable at 40 – 80°C and a pH range of 6.0 – 7.5 against numerous metal ions and none of them inactivated its activity. Moreover, 10 mM Ca<sup>2+</sup> improved 2-folds the activity and half-life of the protease at temperatures from 30 – 50°C. The milk-clotting activity tests revealed high stability of the protease at storage, namely at -20°C compared to 4°C and 25°C for up than 5 weeks. As a meat tenderizing agent, it showed promising role under different treatments by improving texture. The findings indicated that, one-step TPP system is a simple, quick, economical and very attractive process for fast recovery of latex papain compared to other proposed protocols.

**Keywords:** Separation; Three Phase Partitioning; Plant proteases; Papain; Enzyme activity

## 1. Introduction

Downstream processing of proteins as well as other macromolecules of interest can represent more than 70% of the total production costs. Therefore, the development of efficient and economical downstream processing strategies for their recovery and purification, namely for enzymes, has become an important area in different fields of applied biotechnology [1, 2]. Among the proposed bulk separations, aqueous systems has the potential for high-throughput purifications to solve the drawbacks of chromatographic techniques described as expensive, time consuming, involving number of steps and furthermore their scale up is difficult [3, 4]. These systems based namely on precipitation, allow to concentrate and purify the macromolecules by attaining the desirable degree of purification. Moreover, the ease of modification in properties of the aqueous solution such as pH or temperature, or amounts of salts and solvents, allow fast precipitation and recovery of the targeted proteins [3, 5].

Three Phase Partitioning (TPP) is one of the aqueous separation systems that were efficiently applied for the recovery of enzymes from various sources (fluids, tissues and cells) such as plants, animals and microorganisms including atypical environments and fermentation broths [5-7]. TPP is a modest, fast and relatively emerging bioseparation tool first described by Lovrien's group [8], and nowadays intensively used to purify several target macromolecules with wide applications as it becomes a versatile and early-exploratory strategy [5, 7, 9-11]. From the large literature, about 70% of the studies that used TPP have been focused on the recovery of proteins and enzymes, while the other 30% concerned oils, lipids, small-molecule organic compounds, DNA and carbohydrates [5]. TPP was described as a three-stage recovery batch method, which is a hybrid of alcohol precipitations and salting-out for extracting, dewatering, purifying and concentrating the proteins of interest for either small or large manufacturing operations [5]. Its principle consists in mixing the crude slurry (protein extract) with solid salt, mostly ammonium sulfate  $[(\text{NH}_4)_2\text{SO}_4]$  and an organic solvent, usually t-BuOH (*tert*-butanol) to obtain three phases. Thus, the process is based on the ability of the salt to separate a t-BuOH – water mixture, an otherwise miscible solution, into two phases, an upper organic phase and a lower aqueous phase [12]. In the presence of proteins, an intermediate protein layer is formed from which the target macromolecule precipitate and can be easily extracted. The process of TPP involves collective operation of principles including salting out, isoionic precipitation, cosolvent precipitation, osmolytic, kosmotropic precipitation, protein hydration shifts and conformation tightening, which all play in a sophisticated interaction manner, important role to recover the target protein [5-7, 9, 10].

TPP was used to separate several enzyme classes [5, 9, 10] as its efficiency is evident from the reported increase in the total activity [9, 11, 13-15]. Proteases, such as ficain [11], zingibain [16, 17], cucumisin [13], papain from dried papaya peels [18], alkaline proteases from farmed giant catfish [19], proteinase and calotropain from *Calotropis procera* latex [12, 20] and many others were successfully purified. Proteases constitute the most important classes of enzymes used in food industry, contributing to about 60% of total industrial market [21]. Among their sources, latex of *Carica papaya* contains four main cysteine proteases that are papain, chymopapain, glycyldoypeptidase, and caricain endopeptidases [18, 22].

Papain, a cysteine protease of 212 amino acids, has a strong proteolytic capacity, hydrolyzing a board of proteins into small peptides and amino acids. It is widely used in several industries such as food applications for meat tenderization, cheese-making, brewing/wine industry, baking industry as well as for the production of protein hydrolysates [23-28]. The purification of papain from papaya latex has been traditionally achieved by precipitation methods [22]; however, the purified enzyme still remains contaminated with other molecules [29]. As alternative purification approaches, chromatographic techniques including ion exchange, covalent, or affinity chromatography were used [30-32]. Most of them as stated above have multiple steps, need large process times as well as high operation costs. Because of potential and applicability of papain in food industry and other biotechnology sectors, we proposed in this report TPP process as a one-step alternative tool for quick and efficient extraction, concentration and recovery of this protease. Thus, the optimal conditions after optimizing the main TPP parameters ((NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>; ratio of crude extract: t-BuOH; pH and temperature) to achieve a maximum purity and yield of papain were investigated, followed by a comparison with previously reported methods. An overall biochemical characterization of the recovered latex papain was performed and its potential use as meat-tenderizing or milk-clotting agent was further investigated.

## **2. Materials and Methods**

### **2.1. Substrates and Chemicals**

Unless specified, all the substrates, chemicals, and reagents used in this study were of analytical grade or highest available purity and were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All solutions were prepared with double-distilled water.

## 2.2. Latex sampling

Fresh latex was collected from *C. papaya* trees grown in Mwanza, Tanzania. After washing the fruits with distilled water, several vertical incisions were made along the surface with a knife to a depth of 2–3 mm (**Fig. 1**). The exuded latex was collected into a glass beaker set on ice. After collection, the latex was fractionated into volumes of 50 mL, stored at  $-18^{\circ}\text{C}$  and transported in the same conditions until use.

## 2.3. Crude extract preparation

Thawed latex was mixed with fresh distilled water at a ratio of 1:0.5 (v/v) using a vortex. The insoluble material was removed by centrifugation at 2500 rpm for 10 min at  $4^{\circ}\text{C}$ . The clarified supernatant was saturated at ambient temperature with 20% ammonium sulfate; the mixture was then centrifuged for 10 min at 2500 rpm at  $4^{\circ}\text{C}$  to collect the supernatant, which was concentrated using ammonium sulfate to 60% saturation. The pellet was dissolved with fresh distilled water (**Fig. 1**). The volume of fresh distilled water is the initial volume of latex + distilled water used at the beginning multiply by 1.2. The crude extract obtained was dialyzed overnight (membrane molecular weight cut off: 14 kDa) against 30 mM, pH 6.5 phosphate buffer containing 2mM L-Cysteine at  $4^{\circ}\text{C}$ . After dialysis, the crude papain extract sample was subjected to three phase partitioning (TPP) purification system (**Fig. 1**).

## 2.4. Three phase partitioning experiments of papain

TPP experiments were carried according to the guidelines reviewed by Gagaoua [5] following the detailed protocol used for latex ficin [11]. First, according to the preliminary assays, the crude extract of *C. papaya* was concentrated at  $25^{\circ}\text{C}$  with 40% ammonium sulfate, followed by addition of an equal volume of t-BuOH. The mixture was vortexed gently and then allowed to stand for an approximate of 45 min at room temperature. Afterwards, the mixture was centrifuged at 4000 rpm for 10 min at  $4^{\circ}\text{C}$  to facilitate the separation of the phases (**Fig. 1**). The upper t-BuOH phase was removed by a Pasteur pipette. The lower aqueous and interfacial phases were taken carefully for enzyme activity and protein content analyses. The interfacial precipitate was fully dissolved in a low volume of 50 mM, pH 7.5 phosphate buffer by gentle vortex and dialyzed (**Fig. 1**).

For the optimization of the parameters affecting the TPP process, effects of ammonium sulfate (AS) salt concentrations (20, 30, 40, 50, 60, 70 and 80%) (w/v) at the constant crude extract: t-BuOH ratio (1.0:1.0) was investigated. Then, various : t-BuOH ratios (crude extract: t-

BuOH; 1.0:0.5, 1.0:0.75, 1.0:1.0, 1.0:1.25, 1.0:1.5, 1.0:2.0) were employed with constant 40% AS saturation at 25°C. After, the t-BuOH and salt, different pH values effects on the medium were investigated. The crude extract was saturated with 40% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and pH was adjusted to 3, 4, 5, 6, 7, and 8 with fresh 0.5 M HCl or 0.5 M NaOH, then 1.0:0.75 t-BuOH was added and the best pH value on the recovery of papain was determined. The last investigated factor was temperature that we varied from 15°C to 40°C to determine the conditions leading to obtain the high partitioning behavior of the system using the optimized conditions of 40% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1.0:0.75 t-BuOH ratio and medium pH of 6.0 (**Fig. 1**).

After optimization of those parameters, the aqueous phase containing the highest papain activity was collected and dialyzed overnight against phosphate buffer (50 mM, pH 7.0). The recovered papain was stored at -20°C until use for further characterization to determine its general biochemical properties.

## 2.5. Protein content determination

Protein concentrations were determined with the dye binding method of Bradford [33] using bovine serum albumin as a standard (Bio-Rad Protein Assay kit, Bio-Rad, France).

## 2.6. Enzymatic activity

Papain activity was measured under room temperature (RT) following the modified method of [34] in 50 mM phosphate buffer (pH 7.5) containing 38 mM EDTA and 34 mM cysteine, using casein as substrate. Briefly, 800 µL of 1% (w/v) aqueous solution of casein was added to 100 µL of recovered fraction of papain (with 20 BSA equivalent /mL) diluted in 700 µL of buffer. The reaction mixture was then incubated for 10 min. Subsequently, hydrolysis casein was stopped by addition of 1.6 ml of 10% trichloroacetic acid solution. The mixture was incubated at RT for 30 min, centrifuged at 2500 rpm for 10 min and the absorbance of the supernatant measured at 280 nm. One unit of activity is defined as the amount of enzyme that increases the absorbance by 0.001 min<sup>-1</sup>.

## 2.7. Purity, molecular weight and identity of the recovered papain

To assess the content and purity of the different fractions of TPP process, tricine SDS-PAGE (1-dimensional electrophoresis (1DE)) was carried out according to Gagaoua et al. [11] using 12% separating and 4% stacking gels. Briefly, the protein extract fractions were mixed at a ratio of 1.0:1.0 with 100 mM Tris-HCl (pH 6.8), 1% (w/v) SDS, 0.75% (w/v) DTT, 0.02% (w/v)

Coomassie Brilliant Blue R250 and 20% (w/v) glycerol and heated for 10 min in a bath water at 75°C. Ten micrograms of proteins were loaded and then subjected to separate for 3 h at 130 V. After separation, the gel was stained overnight with staining solution containing 4.9 mM Coomassie Brilliant Blue G-250 in 50% (v/v) ethanol and 7.5% (v/v) acetic acid. Un-Scan-It gel 6.5 analysis program (Silk Scientific, Orem, UT) was used to estimate the molecular weight of the protein bands. Another identical gel run in the same conditions was used to transfer the proteins onto polyvinylidene difluoride (PVDF) membranes (Millipore, MA, USA) and blocked using Tris-buffered saline with Tween 20 containing 5% skim milk. A polyclonal antibody (rabbit) specific to papain is used to recognize the protein. A second anti-papain antibody (goat) conjugated with horse-radish peroxidase (HRP) is added which binds to any fraction of papain [35]. The membranes were incubated with the chemiluminescence SuperSignal West Dura (Pierce) for 10 min, and the autoradiograms were scanned and treated as blue colored gels.

## **2.8. Two-dimensional electrophoresis and pI determination**

Isoelectric focusing (IEF) was carried out following the protocol described by Picard *et al.* [36] using 18 cm-long IPG (immobilized pH gradient) strips with a pH range of 3 – 7. The proteins were separated in the second dimension according to the following conditions: 200 V for 1 h; 500 V for 1 h; 1000 V for 3 h, increasing to 8000 V over 9 h; 8000 V continuously until 73,500 Vh. After migration, the gel was not stained and used as for 1DE for immunoblotting.

## **2.9. Effect of temperature and pH on partitioned papain activity and stability**

The temperature profile of papain was determined by performing the standard assay procedure at different temperatures of 20, 30, 40, 50, 60, 70, 80, and 90°C [13]. The relative activities as percentages were expressed as the ratio of papain activity obtained at a certain temperature to the maximum activity obtained at the given temperature range.

The effect of pH was evaluated from pH 2.5 to 7.5 [37] using the following buffers: 50 mM citrate buffer (pH 3.0 - 6.0) and 50 mM phosphate buffer (pH 6.5 - 7.5). Activity of the papain was determined as mentioned above. Furthermore, the thermal and pH stability of the enzyme was determined by measuring the residual activity of papain under the same conditions after 1h incubation.

The residual activity of the protease up to 3h after 30 min intervals for temperature (from 30 to 70°C, with 5°C intervals) and pH (from 3.5 to 7.0, with 0.5 pH units' interval) were further



determined. After desired incubation periods, enzyme aliquots were withdrawn and assayed at optimal assay conditions to determine the residual enzyme activity.

### **2.10. Effect of inhibitors and metals on papain activity**

The effect of various metal ions (KCl, NaCl, CaCl<sub>2</sub>, MgCl<sub>2</sub>, CuSO<sub>4</sub>, ZnSO<sub>4</sub>, MnSO<sub>4</sub> and FeSO<sub>4</sub>) and enzyme inhibitors (EDTA, EGTA, PMSF, iodoacetamide, iodoacetamic acid and iode) on the partitioned papain activity were evaluated by its incubation with the metal ion (2 mM) or enzyme inhibitor (5 mM) at 20°C for 30 min, followed by determining the remaining activity using casein assay as described above. The enzyme activity assayed in the absence of metal ions or inhibitor was considered as control and defined as 100% [13].

### **2.11. Half-life of the recovered papain**

The half-life of the recovered enzyme was determined in the presence of calcium at different concentrations [13]. Briefly, the residual activity was assessed over a total period of 3h min using concentration of 1.0, 2.5, 5 and 10 mM of Ca<sup>2+</sup>, and then the mixture sample was incubated at 30 until 70°C with 5°C intervals. The values were averages of three independent experiments.

### **2.12. Kinetic parameters of the recovered papain**

*K<sub>m</sub>* and *V<sub>max</sub>* of the recovered papain were determined by measuring the enzymatic activity at various concentrations of bovine casein (from 0 to 50 mg/mL) as a specific substrate [16]. The activity was measured under the standard assay conditions as discussed above. Michaelis-Menten and Lineweaver-Burk (Reciprocal) plots were acquired using Sigma Plot 12 software to determine *K<sub>m</sub>* and *V<sub>max</sub>*.

### **2.13. Storage stability**

Stability at storage at 25°C, 4°C and -20°C of the recovered papain for 36 days was assayed [13, 17]. The activity of the papain was determined as mentioned above.

### **2.14. Preliminary tests for potential industrial applications of the recovered papain**

#### **2.14.1. As a milk-clotting agent**

Milk clotting activity (MCA) was determined according to Gagaoua et al. [13, 17]. Briefly, the substrate was prepared by dissolving commercial bovine skimmed milk powder in 100 mL



of 10 mM CaCl<sub>2</sub> to a final concentration of 12% (w/v, pH 6.4). Then, 2 mL of the substrate was pre-incubated for 5 min at 37°C and 0.2 mL of the recovered papain was added. Test tubes were periodically rotated by hand until appearance of visible discrete particles. One milk-clotting unit was defined as the amount of enzyme that clots 10 mL of the substrate within 40 min (2400 s) at 37°C [13, 17]. The effect of pH, temperature and concentration of CaCl<sub>2</sub> on MCA of papain was further evaluated [13]. The temperature of milk was varied from 20 to 90°C; pH from pH 6.5 to 8.5, and the concentration of CaCl<sub>2</sub> from 0 to 50 mM.

#### 2.14.2. As a meat-tenderizer agent

*Longissimus thoracis* and *Semitendinosus* muscles were excised 48 h *post mortem* from 3 male dromedary [38] with approximate age of 5 and 6 years. For that, 10 pieces of 3 × 2 × 2 cm in size were cut from each muscle. The pieces of meat from each muscle were sealed in individual plastic boxes, labeled, and randomly used for different tenderizing treatments using the same concentration 4mg of the recovered papain (defined after preliminary tests). Five treatments were applied:

- i) Control: without any treatment (a control for each condition was applied);
- ii) Immersion: the meat pieces were immersed in 100 mL distilled water containing 4mg of the recovered papain;
- iii) Injection: the meat pieces were injected with 4mg of the recovered papain;
- iv) Pulverization: the meat pieces were pulverized with 4 mg of the recovered papain;
- v) Freeze/thaw cycle: the meat pieces were first pulverized with 4mg of purified papain and then subjected to one freeze/thaw cycle.

Each sample and for the whole treatments was first incubated 60 min at 37°C before storage for 24 at 4°C. Afterwards, the petrotest PNR 10 penetrometer was used to evaluate the final texture of each treated sample [39]. The instrument device was provided with a discerning body (2.5 g weight), which penetrates in free fall (perpendicularly to the muscular fibers) the sample under its own weight, during approximately 5 sec. The depth of penetration was measured in mm and expressed as unit of penetration (UP), where 1 UP = 0.1 mm. The measurements were repeated 5 times for each piece of muscle.

Water holding capacity (WHC) was evaluated according to the Grau-Hamm method with slight modifications [39]. Briefly, 300 ± 5 mg from each treated sample was weighed (G) and deposited on a previously desiccated and weighed (P) filter paper disk (Whatman No. #1). After that, the sample on the paper was placed between two Plexiglass plates and a weight of 2.25 kg

was applied. After 5 min, the areas of meat spot (M) and released juice (T) were drawn on clear plastic and the damp paper filter was weighed (D) after removing the compressed meat sample. The mean of three replicates was used. Water Holding Capacity was calculated as  $M \times 100 / T$  of the areas. The percentage of released water (PRW) was calculated as  $(D - P) \times 100 / G$ . The areas of meat spot (M) and released juice (T) were first scanned and measured using the open source ImageJ 1.48 software.

### 2.15. Statistical analysis

The XLSTAT 2018.2 (AddinSoft, Paris, France) software was used to analyze the data. Most of the determinations, unless stated, were performed in three independent replicates, and the control experiments were performed under the same conditions. The experimental results were expressed as the mean of the replicate determinations and standard errors. Statistical significance was evaluated using t-tests for two-sample comparison and one-way analysis of variance (ANOVA) followed by t-test. The results were considered statistically significant at a significant level of 5%.

## 3. Results and discussion

### 3.1. Three phase partitioning

Proteases are the primary active agents in several industrial sectors thanks to their activity properties over a wide range of temperature and pH. Considering their potential industrial uses, it becomes desirable the developing of simple and efficient methods for their recovery and purification. As it is stated above, TPP is quietly appropriate for this aim because it is economic, fast and simple to apply in order to achieve high yield recovery of the protease in only one step. This report is the first to use TPP for the recovery and purification of papain from latex of papaya. The main focus of its use was to achieve a maximum purity and yield of latex papain. Therefore, the effects of various parameters on TPP were investigated. The starting protein amount of the crude extract and its enzymatic activity were 9.6 mg/mL and 52.1 U/mL, respectively (**Table 1**). From this same slurry, different partitioning experiments at various **i**) saturations (w/v) of  $(\text{NH}_4)_2\text{SO}_4$ : 20%, 30%, 40%, 50%, 60%, 70% and 80%; **ii**) crude extract to t-BuOH ratios (v/v): 1.0:0.5, 1.0:0.75, 1.0:1.0, 1.0:1.25, 1.0:1.5, and 1.0:2.0; **iii**) pHs: 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0; and **iv**) temperatures: 15°C, 20°C, 25°C, 30°C, 35°C and 40°C, were optimized to get the highest purification fold and recovery yield (**Fig. 2**). In all the TPP experiments of this trial, papain was found to exclusively partition in the aqueous phase (**Fig.**

**3A**). In this bottom phase, high enzymatic activity recovery and purity as assessed by SDS-PAGE were found (**Fig. 3B**). The influence of these process parameters on the degree of purification and on the activity recovery of papain is shown in **Fig. 2**.

### **3.1.1. Effect of $(\text{NH}_4)_2\text{SO}_4$ saturation**

The maximum purification fold of 11.92-fold along with 134.4% recovery of papain activity in the aqueous phase was obtained with 40%  $(\text{NH}_4)_2\text{SO}_4$  saturation (**Fig. 2A**). Below and beyond this salt concentration, a decrease in percentage recoveries of the latex papaya protease was observed. This would be explained by the irreversible denaturation of the protein [17, 40, 41]. In this regard,  $(\text{NH}_4)_2\text{SO}_4$  saturation is of critical importance and plays a major role in TPP, as it is responsible for protein–protein interaction and precipitation. Therefore, 40%  $(\text{NH}_4)_2\text{SO}_4$  concentration was selected as the best amount for the optimization of the other factors. This salt concentration was sufficient enough to concentrate the protease with the desired purity. We can suggest that at this lower saturation,  $(\text{NH}_4)_2\text{SO}_4$  was unable to change the hydrophobic surface of papain. By increasing salt concentration, both the surface and interfacial tensions of the TPP system increased following the interfacial behavior of proteins in TPP [5, 42]. It well known that in TPP process, salting-out of a protein by  $\text{SO}_4$  can be viewed in different ways: **i**) ionic strength effects, **ii**) kosmotropy, **iii**) dehydration (osmotic stressor), **iv**) exclusion crowding and **v**) the binding of  $\text{SO}_4^{2-}$  to the cationic sites of the targeted protein [5]. The findings for this first parameter agree to the known proteases properties to precipitate at 40 – 60%  $(\text{NH}_4)_2\text{SO}_4$  saturation. For example, in an earlier preliminary study, most proteases from papaya extract of dried peels were found to precipitate in the aqueous phase of a two-step TPP process [18].

### **3.1.2. Effect of crude extract to t-BuOH**

The relative amount of t-BuOH within the protein extract is further important in TPP. The saturation of  $(\text{NH}_4)_2\text{SO}_4$  was fixed to 40% and the ratio of crude extract to t-BuOH varied from 1.0:0.5 to 1.0:2.0 (**Fig. 2B**). The best purification fold of 11.87-fold along with 132.3% recovery of papain activity were obtained with 1.0:0.75 (v/v) ratios. Previously, Gagaoua [5] reviewed that the optimum ratio of t-BuOH arises as a result of two conditions. If the quantity of t-BuOH is lower, it does not sufficiently synergize with the salt [5, 11, 12]; however, if it is higher, it is likely to cause a saturation of the medium and hence strongly decrease the activity of the targeted protease [6]. The reason of this decrease in production yield would be due to an increase in the viscosity of the medium. Meanwhile, owing to the larger molecular size and branched structure of t-BuOH, no permeation inside the folded proteins can occur, and we

suppose that no direct denaturation effect can be observed. It is worthwhile to note that most studies reported an increase in the activities of the recovered enzymes in t-BuOH/water mixtures [8], a peculiarity of t-BuOH that is confirmed by several reports (for review: [5-7, 9, 10, 12]). For instance, we believe this is related to the kosmotropic nature of this C4 nonionic alcohol, which stabilizes intramolecular interactions in proteins and thus offers milder process of dehydration when compared with small molecules of either methanol, iso propanol or ethanol [13, 16] that can easily penetrate, and thus degrade, many proteins [6, 41].

### 3.1.3. Effect of pH

The pH of the medium is very important for best recovery and activity of the targeted protease. Indeed, it would affect the protein concentration in a pH-dependent net charge manner due to electrostatic interactions between phases and charged residues of the protein [5]. The recovered papain was separated selectively in the bottom phase at pH 6.0 as the best condition, hence allowing the highest purification fold of 11.55 with 133% activity recovery. The TPP behavior for pH is illustrated in **Fig. 2C**. The raised recovery at this pH could be a result of a better conformational stability of papain towards t-BuOH and salt at that pH. Otherwise, the recovery is very low at pHs 3.0, 4.0 or 7.0 and 8.0, but interesting as well at pH 5.0. According to Pike and Dennison [43], the solubility of proteins was maximized when TPP was carried out at the *pI* of the protein. According to Gagaoua [5], when the pH of the system is much lower than the *pI* of the protein, there is protein target accumulation in the interfacial phase; and when the pH of the system is much higher than the *pI* of the protein, there is protein target accumulation in the aqueous phase. The 2-dimensional electrophoresis analysis (**Fig. 3**) supports these statement as the *pI* of the recovered papains are mainly around 4.3, in accordance with the recovery in the aqueous phase of several cysteine proteases such as zingibain [17], calotropain [20] or papain of dried papaya leaves [18].

### 3.1.4. Effect of temperature

The effect of temperature as it influences the protease configuration and overall stability [16] was further investigated at the above optimized parameters of 40%  $(\text{NH}_4)_2\text{SO}_4$ , ratio of 1:0.75 (v/v) of crude extract to t-BuOH and pH 6.0. The recovery of papain increased with an increase in temperature from 15 to 25°C, and then it decreased (**Fig. 2D**). At temperatures above 25°C, there may not be significant kosmotropic and crowding effects, which resulted in decreased recovery and purity of papain. Thus, the highest purification fold of 11.74 and activity recovery of 134% were obtained at 25°C. Accordingly, Nadar *et al.* [10], highlighted that at 25 °C, the

conformation of enzyme changed into highly active form, explaining the good partitioning obtained at this temperature. From the large literature, it seems also that working at lower or at room temperatures, the recovery yield or activity may be quietly similar, which is postulated to be due to the low movement of particle solvent [5, 6]. In agreement to our findings, an earlier study by Jian *et al.* [44] investigated the effect of temperature on the yield and purification fold of papain separated by metal affinity partitioning in aqueous two-phase systems at 25, 35, and 45°C. They observed that the yield and purification recovery of papain were almost the same at the three temperatures, hence indicating less variation whatever the temperature. One can relate this resistance to temperature and enhancement of papain activity, to its three-dimensional structure as it displays a globular protein with three disulfide bonds and two catalytic L- and R-domains (**Fig. 4**). From this graph, Fernández-Lucas and co-workers pointed out the interesting Trp177 that is involved in the generation of the nucleophilic character of Cys25/His159 ion pair [23], reported so far to play important role in its stability.

### 3.1.5. Overall evaluation of the TPP recovery profile

The partitioning and concentration of *C. papaya* latex papain with one-step TPP is given in the overall purification profile of **Table 1**. As presented above, the partitioned papain has tendency to concentrate in the bottom phase of TPP system using optimal parameters of 40%  $(\text{NH}_4)_2\text{SO}_4$ , 1.0:0.75 ratios of the crude extract to t-BuOH at pH 6.0 and 25°C. The highest recovery and purification fold of 134% and 11.45, respectively, were achieved. The specific activity of the recovered fraction that was used to determine the biochemical characteristics of papain as well as its ability of use by industry has a specific activity of 62.11 U/mg.

In **Table 2**, we compared the one-step TPP protocol described in this trial to other protocols from the large literature aimed to purify papain from different parts of *C. papaya* plant. Overall, the protocol we developed gave almost the best results in terms of recovery and purification folds. For example, He and co-workers purified papain from unripe green papaya fruits by reversed phase expanded bed adsorption chromatography and obtained a papain fraction of 23.4 kDa with 74.98% of recovery and 7.04 purification fold [45]. Purwanto *et al.* [46] used ion exchange chromatography after 60%  $(\text{NH}_4)_2\text{SO}_4$  saturation, and they found a very weak purification recovery of 10.7. Further studies applied aqueous two-phase system, and reported overall recovery ranging from 72 to 88% [47, 48]. Our findings, comforted the previous TPP based separations highlighting high superiorities of this system in comparison to conventional

techniques; where selective partition and concentration of desired protease into one phase, less money and steps, and work at room temperature, are the main peculiarities and advantages [5].

### 3.2. Characterization of the purified latex papain

#### 3.2.1. Purity of the recovered latex papain and steps of TPP system by electrophoresis

The analysis of the different fractions of TPP system by an adapted Tricine SDS-PAGE confirmed that the one-step TPP allows an efficient removal of contaminating proteins or other molecules in the interfacial and upper phases, whereas the papain is mainly concentrated in the aqueous phase (**Fig. 3B**). The papain fraction was homogenous and had a molecular weight of 23.2 kDa. This is in agreement with the molecular weight reported for papain in several studies [29, 49-51]. Some of those studies are further summarized in **Table 2** in comparison to different plant parts of papaya as well as methods of purification. In comparison to the large literature, the estimated molecular weight of the recovered papain falls in the range of 19 – 29 kDa, which is the reported range of values for many papain-like proteases [52]. In comparison to cysteine proteases from latex plants, **Table 3** listed in a non-exhaustive manner some characteristics of the studied proteases including their molecular weight. On another hand, the concentrated TPP fraction was further run on 1-dimensional gel to be revealed by western-blotting using an antibody raised against papain (**Fig. 3C**), therefore confirming its identity. However, the same fraction separated using 2-dimensional gel (**Fig. 3D**) revealed four main different isoforms that had *pI* ranging from 5.0 to 6.0, with high abundance of one spot close to pH 5.0 than 6.0. Our study is the first to highlight this polymorphism, and further studies are worthy to be done to better characterize each protein isoform spot. Meanwhile, the isoforms would be also contaminating glycyl endopeptidase as it was earlier reported for chymopapain [53, 54]

#### 3.2.2. Effect of temperature and pH on the activity and stability of the recovered papain

The effect of temperature on papain activity and stability was studied in the temperature range of 20 – 90°C (**Fig. 5A**). The partitioned papain was very active over a broad temperature range, with an optimum temperature of 40 – 80°C and maximum activity at 60°C. The enzyme was active at 80°C. The purified papain was also stable under a wide range of temperature and it retained more than 60% of its activity in the temperature range from 30 to 70°C when it was incubated for 1 h and it retains 70% of its initial activity at 40°C. However, it loses the activity when incubated at temperature more than 70°C for 1 h. The results obtained showed that the recovered latex protease is more stable at the usual industrial temperatures. The findings are in



line with previous reports [29]. Furthermore, calcium at different concentration (1 mM, 2.5 mM, 5mM and 10 mM of  $\text{Ca}^{2+}$ ) enhanced the activity and the half-life of the latex protease whatever the temperature from 30 – 55°C but not above 55°C (**Fig. 6**). Similarly, the analysis of temperature effect in serial time by taking samples each 30 min up to 3h and for temperatures ranging from 30 to 70°C, with 5°C elevation temperature (**Fig. 7A**) confirms the broad range of temperature stability of the recovered latex papain. In agreement to our results, thermal denaturation studies of papain by both differential scanning calorimetry and spectrophotometric methods indicated that papain starts losing its activity from 55°C [55]. Moreover, most of the listed cysteine proteases in **Table 3** had optimal temperatures ranging from 40 to 60°C and stability plateau at 40 to 70°C

The effects of pH are given in **Fig. 5B**. An increase in the activity of papain was observed by increasing pH from 3.0 to 6.0. The protease had a neutral optimum pH; and seems that it is more stable at pH 6.5 to 7.5. Note that there was retention of activity after 1 h incubation at that pH range. The pH profile obtained in this experiment agrees with those of previous studies [46] which mainly described an optimum pH of 6.0 when papain was purified by ion exchange chromatography. An optimum pH in the range of 5 – 8 was also reported [49, 51]. Further comparisons to most studied cysteine proteases including papain-like are summarized in **Table 3**. Most of the enzymes were found to be likely stable at the neutral and basic pH than acidic. The broad pH stability found for papain is an important factor for industrial application of enzymes. For example, it can be easily applied for meat tenderizing or further industrial applications detailed above. As for temperature, the recovered papain by TPP is highly stable when it is incubated for 3h at pHs  $\geq 4.5$  (**Fig. 7B**). The high stability of the papain against pH and temperature together makes this protease potentially beneficial in industrial applications.

### 3.2.3. Effect of various effectors on partitioned papain activity

Various metal ions ( $\text{KCl}$ ,  $\text{NaCl}$ ,  $\text{CaCl}_2$ ,  $\text{MgCl}_2$ ,  $\text{CuSO}_4$ ,  $\text{ZnSO}_4$ ,  $\text{MnSO}_4$  and  $\text{Fe SO}_4$ ) at 2 mM concentration inhibited the enzyme activity for more than 20% (**Fig. 5C**), indicating that we have to use ion chelators such as EDTA to maintain or improve the proteolytic activity of papain. Among the inhibitors we investigated, iodoacetamide and iodoacetic acid, which are cysteine protease inhibitors, inhibited more than 75% of the proteolytic activity of the recovered papain, while PMSF, EGTA and iodide had no effect on the protease (**Fig. 5D**). EDTA, a metalloprotease inhibitor, enhanced as expected the proteolytic activity of the protease. These



findings for the effectors investigated in this study are mostly in line to the data described in the literature for papain purification [18, 29, 45-48, 51, 56], which are summarized in **Table 2**.

#### **3.2.4. Kinetic parameters**

In order to determine the kinetic constants ( $V_{max}$  and  $K_m$ ) of the partitioned latex papain, the variation of enzymatic activity with the initial casein concentration was investigated (**Fig. 8**). The initial casein concentration was changed between 0 and 50mg/ml. The  $V_{max}$  and  $K_m$  values were calculated from Lineweaver–Burk plot as  $33.33U\ min^{-1}$  and  $10.83mg\ ml^{-1}$ , respectively. The values are similar to those of cysteine or serine proteases recovered by one-step TPP such as ficain [11], zingibain [17] and cucumisin [13]. Furthermore, the Lineweaver-Burk plot indicated that the enzyme follows simple Michaelis-Menten kinetics.

#### **3.2.5. Storage stability at different temperatures**

The recovered papain was fractionated into different aliquots in Eppendorf tubes and stored at three temperatures ( $-20^{\circ}C$ ,  $+4^{\circ}C$  and  $25^{\circ}C$ ) up to 36 days to evaluate the residual activity based on milk-clotting activity (MCA) along this period (**Fig. 9**). A strong MCA was observed whatever the storage temperature during the first 10 days, where it stabilizes at  $-20^{\circ}C$  around 10 000 U/mL but it decreases drastically for  $25^{\circ}C$  and continue in the same value as for  $-20^{\circ}C$  for the  $+4^{\circ}C$  until 16 days. At 18 days, the protease lost 95% of its activity at  $25^{\circ}C$ , when this same lost is achieved at 28 days for  $+4^{\circ}C$ . The best condition of storage for the recovered partitioned latex papain is  $-20^{\circ}C$ . This MCA activity profile over days is similar to that we recently reported for cucumisin [13]. Overall, these findings suggest that the partitioned papain might be preserved at  $25^{\circ}C$  for a short period of 10 days, whereas a slightly longer life of 14 days or 36 days may be obtained by storage in a fridge at  $+4^{\circ}C$  or under freezing at  $-20^{\circ}C$ , respectively. Previous studies reported the potential use of additive stabilizers such as cysteine, ascorbic acid, sodium ascorbate, erythorbic acid, sodium erythorbate and many others to preserve the proteolytic activity of industrial enzymes including papain [17, 57-60].

#### **3.3. Milk-coagulation ability of papain for dairy industry**

In order to determine the optimal conditions of the recovered latex papain by TPP system for milk coagulation and thus for cheese-making, it is important to determine the effect of usual physic-chemical parameters such as temperature, pH and  $CaCl_2$  used by dairy industry. This investigation was done in the present study based on the determination of MCA.

The effect of pH of milk on the activity of papain has been assessed at pH ranging from 6.5 to 8.5. The choice of this pH interval is based on the fact that, in pH lower than 5.5, the coagulation can become an acidic coagulation due to acid pH (<4.6). According to the results presented in **Fig. 10A**, we observe a reduction of MCA as pH of milk increases. This activity decreases until only 20% of activity at pH 8.0 was retained. The increase of the pH up to 7.0 can provoke the inactivation of papain. The same effect of pH on papain activity has been previously reported [61], suggesting more MCA activity at neutral pH. In an earlier study on ficain, we observed that this cysteine protease from fig latex had an interesting MCA at pH 6.5 [11].

The optimal temperature was determined by MCA of papain on milk at temperatures varying from 20 to 90°C. The results are given in **Fig. 10B**. We note that more the temperature of milk increases, more its coagulation is fast. This profile is similar to that generally reported for plant proteases that were purified from *Balanites aegyptiaca* fruit pulp [62], thistle of *Cynara cardunculus* L. and artichoke [63, 64]. Besides, recently and using cucumisin recovered by TPP system from, we reported a strong increase in the MCA by increasing temperature until 70°C, then the protease loose progressively its activity beyond 75°C [13]. These results confirm those of previous studies, which reported the thermostable peculiarity of plant proteases [65-68].

The addition of the calcium chloride to milk, currently used in dairy industry, has as effect the reducing of coagulation time and increasing firmness of the coagulum [64]. The effect of CaCl<sub>2</sub> on MCA of the purified papain is shown in **Fig. 10C**, where it can be seen that addition of CaCl<sub>2</sub> strongly decreased the milk-clotting time. Optimal concentration for MCA was from 40 to 50 mM CaCl<sub>2</sub>. A similar behavior was previously observed [13].

### 3.4. Meat tenderization ability

The effects of various meat tenderizing treatments on penetration depth (which gives us information on texture or tenderness of meat pieces) and water holding capacity (WHC) of *Longissimus thoracis* (LT) and *Semitendinissus* (ST) are given in **Fig 11**. We observe that papain improved from 1 to 2 mm of penetration depth, consequently the tenderness of meat cuts. The reduction of meat toughness can be explained by the high activity of the papain. In line to these findings, an earlier study reported that papain is a powerful protease, that efficiently catalyzes the breaking of the peptidic bonds in the protein molecules as substrates and their degradation products to amino acids [69]. According to Ashie and co-workers [26], the enzymatic hydrolysis of meat proteins is generally known to increase the solubilisation of free

amino groups and hydroxyproline to a loss of the integrity of the muscle and therefore, an increase in meat tenderness. These authors, reported also that injection papain reduce shear force of the muscle [26]. Since 80's century, plant cysteine proteases were proposed to allow the breakdown of myofibrillar proteins and the disruption of the muscular fibril structure [70].

From the other treatments investigated in this study using the recovered papain, we note that for LT muscle, freeze/thaw cycle is characterized by the deepest penetration followed by marinade, then injection and finally pulverization treatment (**Fig 11**). For ST muscle, the freeze/thaw cycle allowed to obtain the greatest on tenderness followed by pulverization, injection and marinade. Therefore, freeze/thaw cycle presents the most interesting treatment to improve tenderness using the recovered papain by TPP at a concentration of 4 mg. The best treatment found in this study was further reported by [71] to enhance tenderness using actinidin from kiwi juice. Further investigations using accurate methodologies such as transmission electron microscopy are under process to better characterize the effect of a fixed concentration of the latex papain recovered by TPP on the ultrastructural changes that occur on muscle using the different treatments described above.

#### **4. Conclusion**

In comparison to chromatographic and other aqueous system methods, TPP is very cheap, simple and efficient method to purify papain from papaya latex. Effect of various process parameters has been evaluated and latex-papain was recovered in the aqueous phase to 11.45-fold and 134% recovery activity by optimized one-step TPP system: 40%  $(\text{NH}_4)_2\text{SO}_4$ , 1.0:0.75 ratios of the crude extract to t-BuOH, pH 6.0 and 25°C. The characterization of the recovered papain showed interesting and better stability over a broad temperature and pH range, resistance to metal ions and inhibitors. The protease was further tested for its ability as milk-clotting and meat tenderizing agent. TPP could be attractive for the purification of latex papain in an easy way to be implemented in food industry or other industries.

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#### **Conflict of interest**

The authors confirm that this article content has no conflicts of interest.

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**Figure captions**

**Fig. 1.** Workflow highlighting the main steps applied for the recovery of papain from the crude papaya latex using *Three Phase Partitioning* system.

**Fig. 2.** Optimization of the parameters affecting *Three Phase Partitioning* process and their effect on purification fold and activity recovery of papain from the crude papaya latex. **A)**  $(\text{NH}_4)_2\text{SO}_4$  concentration; **B)** crude extract: t-BuOH ratio, **C)** pH effect at 40% (w/v)  $(\text{NH}_4)_2\text{SO}_4$  saturation and 1:0.75 crude extract: t-BuOH ratio; and **D)** effect of temperature variation from 15°C to 40°C. Data represents mean  $\pm$  standard error.

**Fig. 3.** Three Phase Partitioning (TPP) and molecular weight and purity analysis of the recovered papain from the latex of *Carica papaya*. **A)** A TPP result depicted by a picture of the glass test tube highlighting the final aspect of the crude latex extract partitioning into three distinct separated phases, showing the recovered papain in the aqueous phase. **B)** Tricine SDS-PAGE (10% separating polyacrylamide gel): lane MM, molecular mass standards; lane 1, dialyzed crude extract; lane 2, interfacial phase of TPP; lane 3, recovered papain (aqueous phase of TPP) with an apparent molecular mass of 23.2 kDa and lane 4, concentrated papain using Amicon Ultra device (10 kDa MWCO) after dialysis against 30 mM, pH 6.5 phosphate buffer containing 2mM L-Cysteine at 4°C. **C)** Western-Blotting, using an anti-papain antibody raised against the fraction of the lane 4. **D)** Two-dimensional electrophoresis analysis revealed by western-blotting of the concentrated fraction of papain precursor (from lane 4 of **B**). The arrows in the 2DE gel designate the different isoforms by *pI*.

**Fig. 4.** **A)** Three-dimensional structure of papain (PDB accession code 1PPN). The arrow indicates the situation of active site. **B)** Catalytic residues in the active site of papain. Cys<sub>25</sub> and His<sub>159</sub> are forming the catalytic ion pair. (The figure was reprinted with permission from Fernández-Lucas, J., Castañeda, D., & Hormigo, D. (2017). *New trends for a classical enzyme: Papain, a biotechnological success story in the food industry. Trends in Food Science & Technology*, 68, 91-101).

**Fig. 5.** Activity and stability of the TPP recovered papain from latex of *Carica papaya*. **A)** Effect of temperature; **B)** effect of pH; **C)** effect of metal ions at a concentration of 2 mM; and **D)** effect of some usual inhibitors at a concentration of 5 mM. Data represents mean  $\pm$  standard error.

**Fig. 6.** Half-life of the TPP recovered papain from latex of *Carica papaya* in the presence of calcium ( $\text{Ca}^{2+}$ ) at different concentrations at a range of temperatures from 30 to 70°C. The values are means of three independent experiments determined after 3h of incubation.

**Fig. 7.** Effect of **A)** temperature and **B)** pH on the TPP recovered papain stability at different time points.

**Fig. 8.** Summary of the Michaelis-Menten and Lineweaver-Burk plot kinetics of the TPP recovered papain from latex of *Carica papaya*.

**Fig. 9.** Effect of storage temperature on the proteolytic activity of the TPP recovered papain from latex of *Carica papaya*. Aliquots of the recovered protease, stored at  $-20^{\circ}\text{C}$  (red line),  $4^{\circ}\text{C}$  (black line) and  $25^{\circ}\text{C}$  (blue line), were removed at different periods and the milk-clotting activities were determined. Data represents mean  $\pm$  standard error of three independent experiments.

**Fig. 10.** Milk pH, temperature, and salt ( $\text{CaCl}_2$ ) concentration effects on milk-clotting activity (MCA) of the TPP recovered papain. Data represents mean  $\pm$  standard error.

**Fig. 11.** Penetration depth difference (bars in mm) and water-holding capacity (continuous lines in percentage) of the samples from camel meat of both *Longissimus thoracis* (LT) (in blue) and *Semitendinosus* (ST) (in red) muscles treated with the TPP recovered papain at a concentration of 4 mg/mL. The four treatments (pulverization, injection, marinade and freeze/thaw cycle) were compared to the control treated in the presence of water. Data represents mean  $\pm$  standard error of three independent experiments.



Fresh papaya latex from immature fruits of *Carica papaya L.*

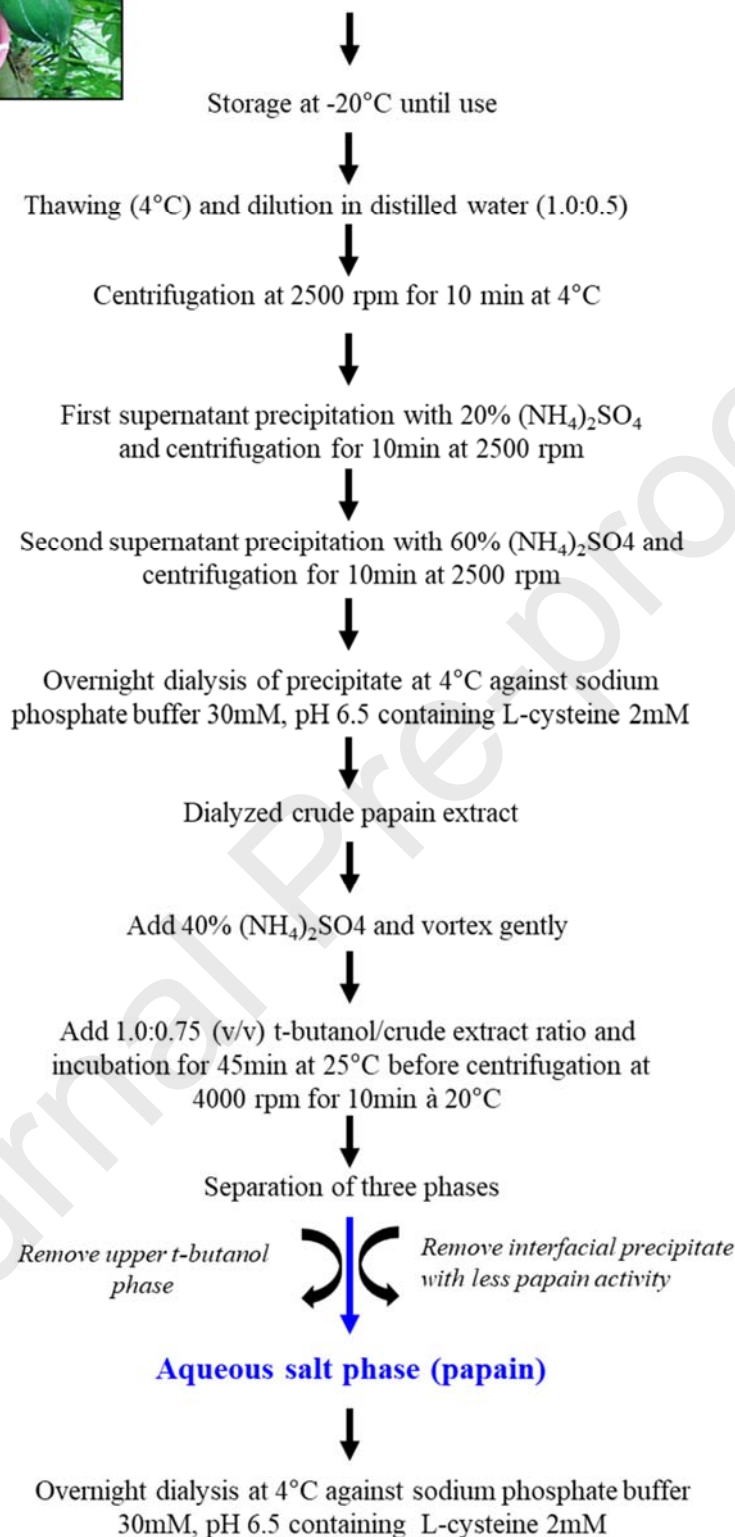


Fig. 1.

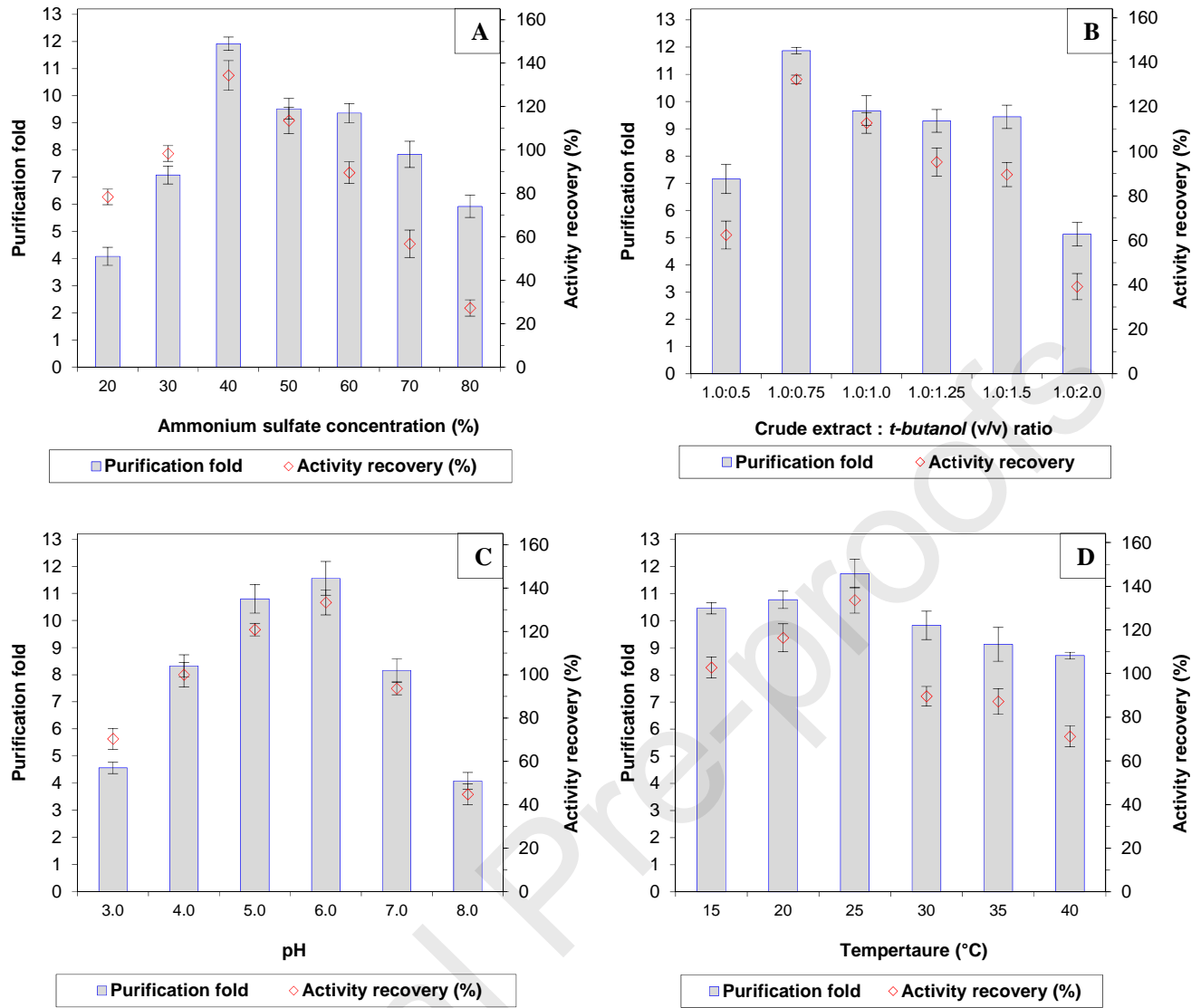


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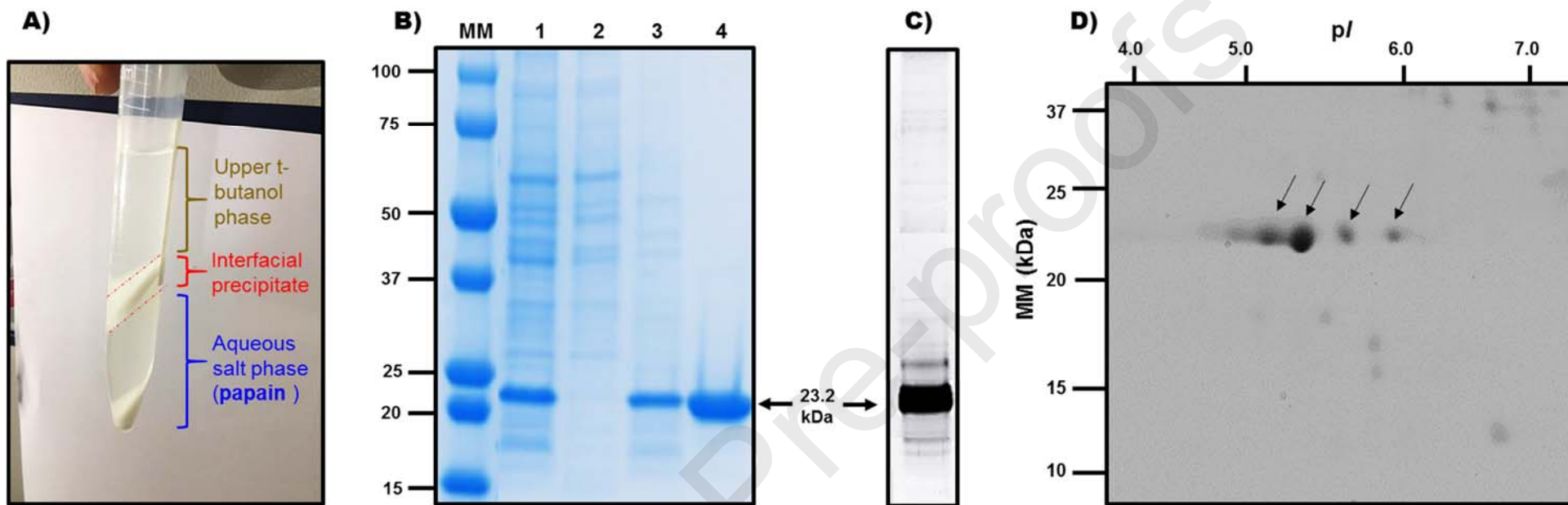
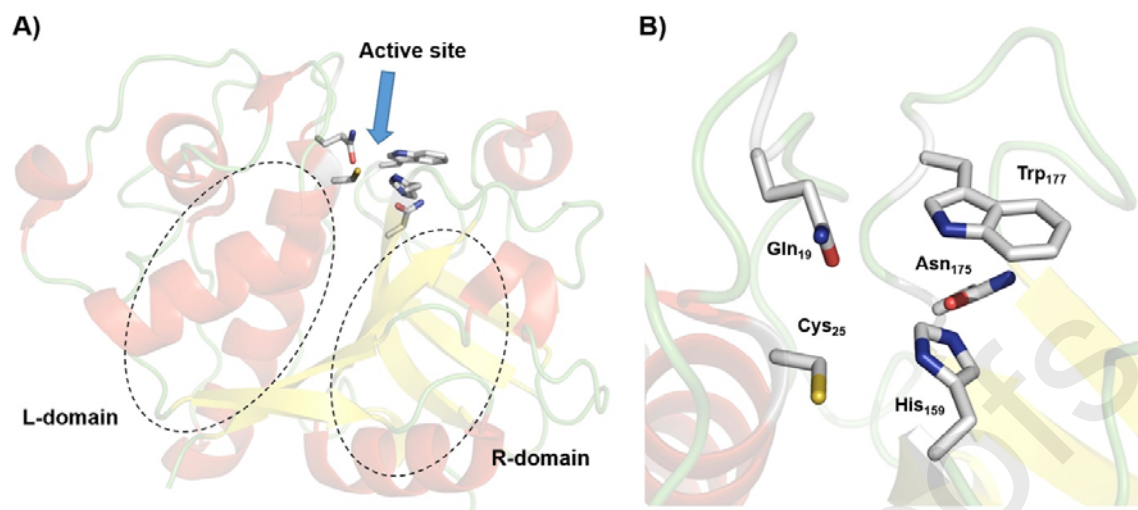


Fig. 3.





**Fig. 4.**

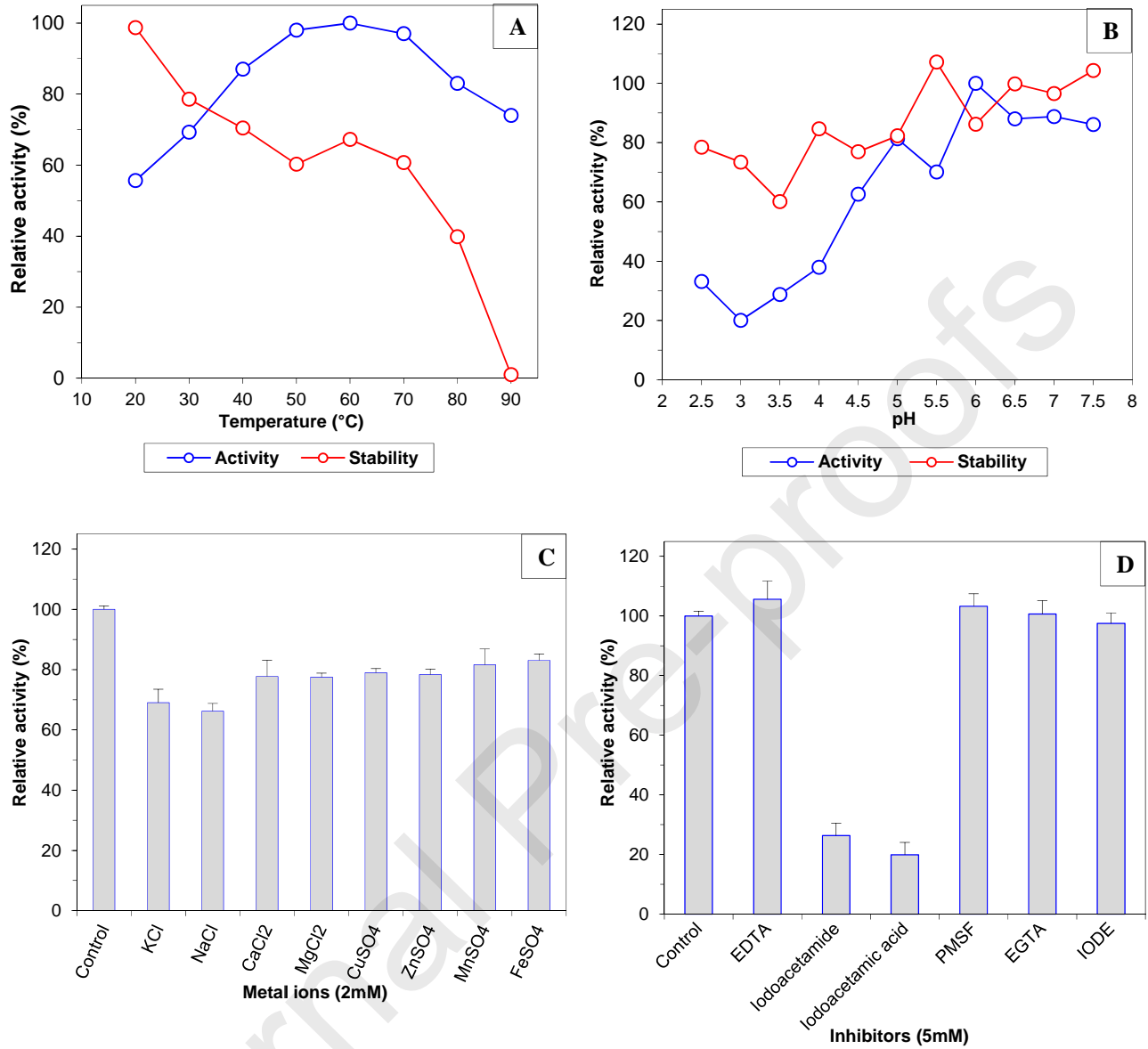


Fig. 5.

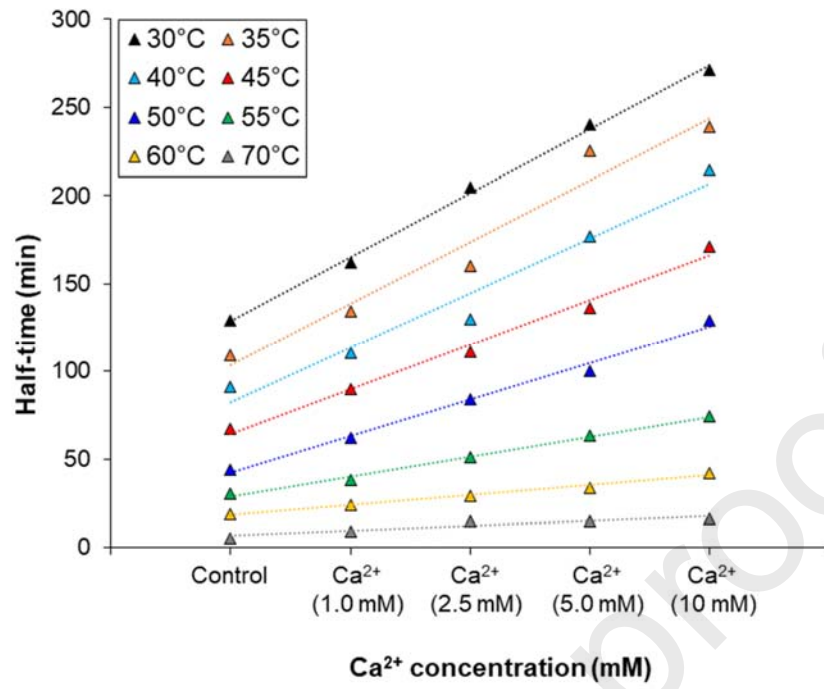


Fig. 6.

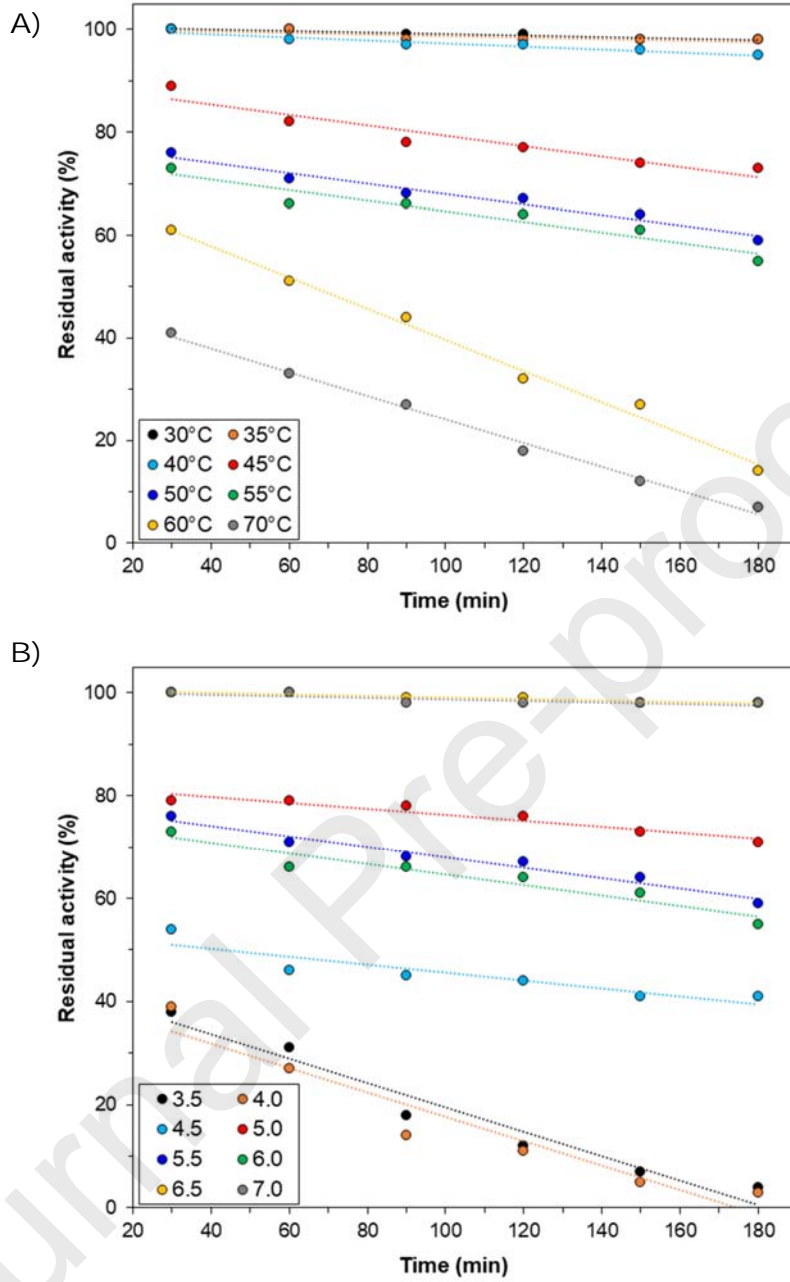


Fig. 7.

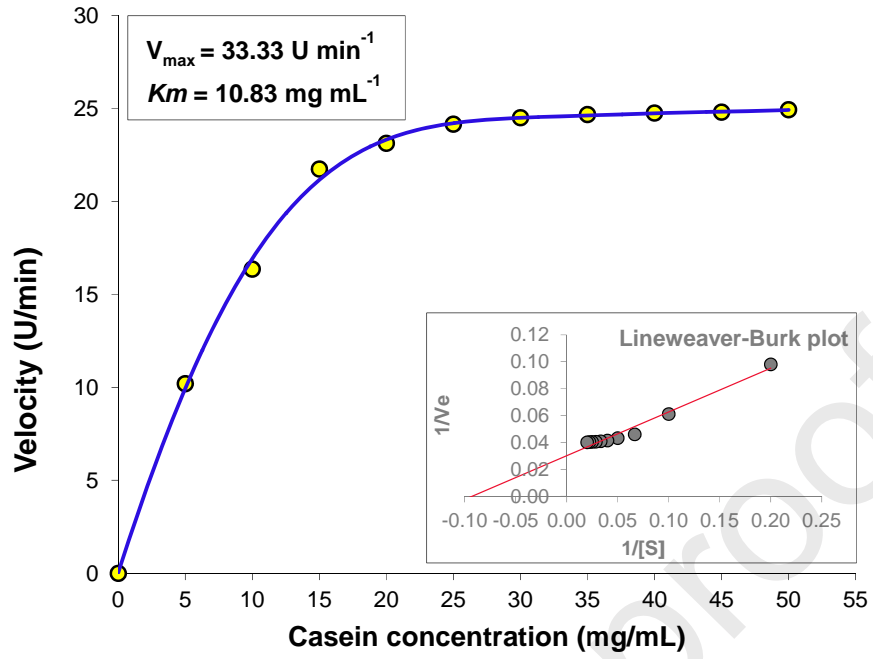


Fig. 8.

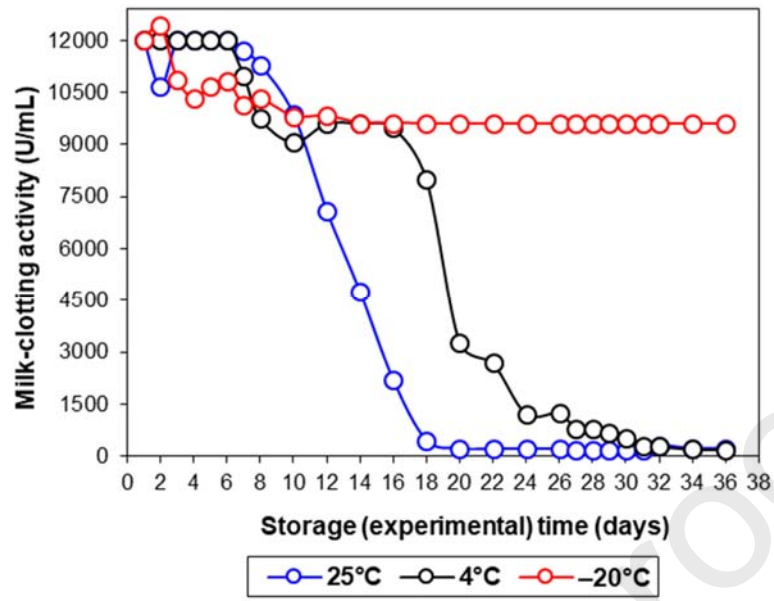


Fig. 9.

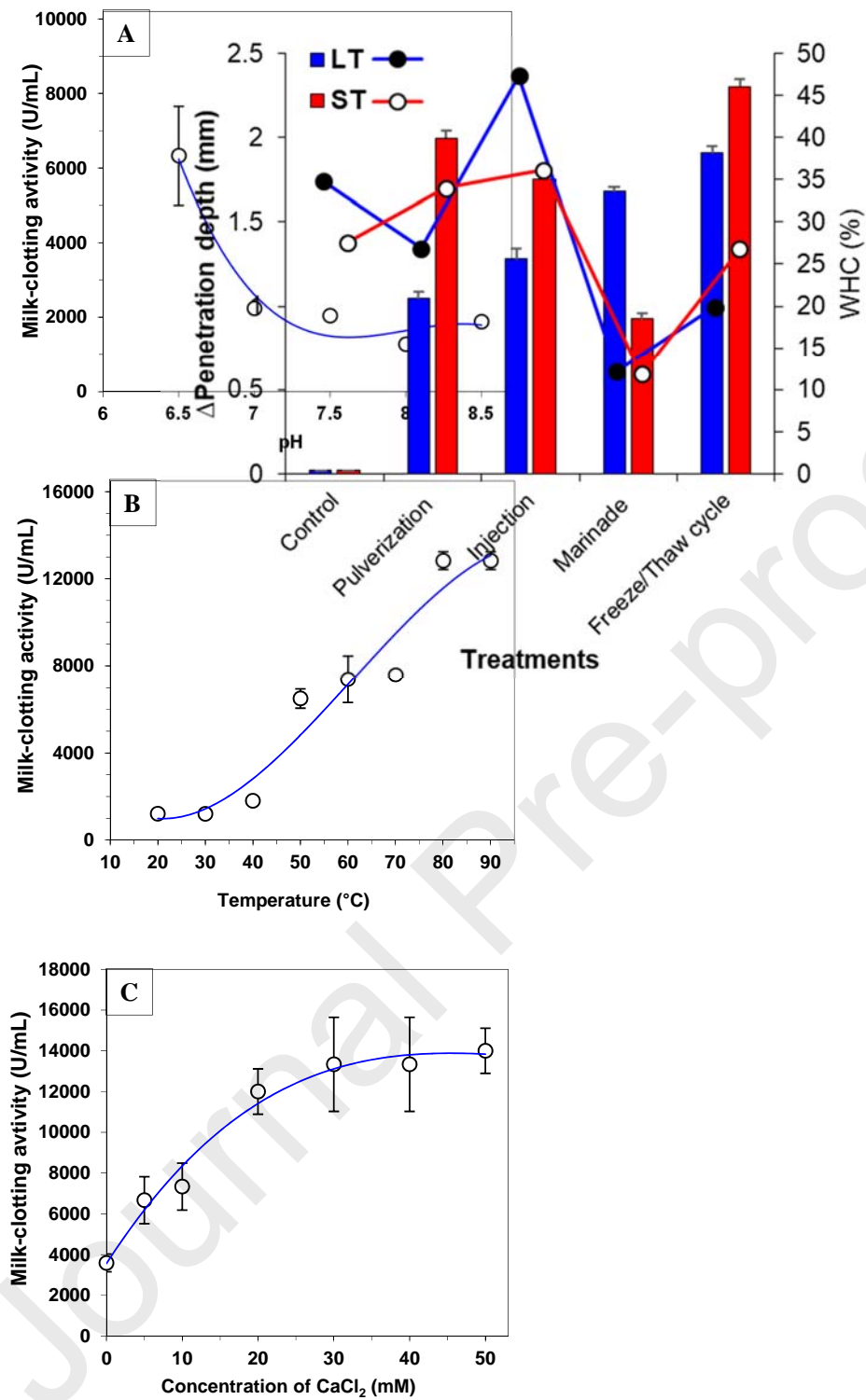


Fig. 10.



**Fig. 11.**

Journal Pre-proofs

**Table 1.** Recovery profile and overall purification of papain from crude *Carica papaya* latex extract by Three-phase partitioning.

Purification step <sup>1</sup>	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Recovery (%)
Crude extract	52.1	9.60	5.43	1.00	100
Aqueous phase of TPP (AP)	69.57	1.12	62.11	11.45	134
Interfacial phase of TPP (IP)	1.16	6.49	0.18	0.03	2.2

<sup>1</sup> The ammonium sulfate (40%, w/v) was added to the dialyzed crude extract of *C. papaya* latex and then pH was adjusted to 6.0. This was followed by the addition of t-BuOH in a ratio of 1.0:0.75 (v/v) (crude extract: t-BuOH) at an average temperature of 25°C. The three phases formed were collected separately. The upper phase was removed and then the lower aqueous phase and interfacial precipitate were tested for enzyme activity and protein amount. The aqueous phase containing the highest proteolytic activity was used for the overall characterization of papain.

**Table 2.** Comparison of different processes from literature for papain extraction and purification from various parts of papaya plant.

Used part	Purification methods	MW (kDa)	Recovery (%)	Purification fold	References
Unripe green papaya fruits	Reversed phase expanded bed adsorption chromatography	23.4	74.98	7.04	[45]
Dried papaya peels	Two-step three phase partitioning		253.5 89.4	15.8 10.1	[18]
	Precipitation of insoluble complexes of papain and poly vinyl sulfonate	23.4	86	2	[29]
	Aqueous two-phase system		88		[47]
	Two-step salt precipitation		49		
	Isolation and crystallization Gel filtration of Sephadex G-75 Chromatography	21			[56]
Latex	Column chromatography	23.4			[51]
	Ion exchange chromatography		10.7		[46]
	Fractionation using 60% ammonium sulphate and ion exchange chromatography		6.3		
	Aqueous two-phase systems		72	2.41	[48]
	<b>Three phase partitioning system</b>		<b>23.2</b>	<b>134</b>	<b>11.45</b>

**Table 3.** Brief overview of the characterized cysteine proteases from some latex of plants in comparison to the TPP recovered papain in this study.

Protease name	Plant source	MW (kDa)	pH optima	Temperature optima (°C)	pH stability	Temperature stability (°C)	References
<b>Papain</b>	<b><i>Carica papaya (latex)</i></b>	<b>23.2</b>	<b>6.0</b>	<b>60</b>	<b>6.0 – 7.5</b>	<b>40 – 80</b>	<b>Present study</b>
Papain	<i>Carica papaya</i>	23.4	8.75	-	5.0 – 8.0	-	[51]
Ficin	<i>Ficus carica</i>	23.4	6.5	60	6.0 – 7.0	40 – 70	[11]
Ficin S	<i>Ficus carica var. Horaishi</i>	26.0	8.0	60	2.0 – 8.0	60	[72]
Procerain	<i>Calotropis procera</i>	28.8	8.0	55	7.0 – 9.0	55 – 60	[73]
Procerain B	<i>Calotropis procera</i>	25.7	8.0	40	6.5 – 8.5	40 – 60	[74]
Araujain h I	<i>Araujia hortorum</i>	24.1	8.5	60	8.0 – 9.5	-	[75]
Araujain h II	<i>Araujia hortorum</i>	23.7	8.0	-	8.0 – 9.0	-	[76]
Araujain h III	<i>Araujia hortorum</i>	23.5	8.5	-	8.0 – 9.0	-	[76]
Ervatamin B	<i>Ervatamia coronaria</i>	26.0	6.0	55	6.0 – 5.0	50 – 55	[77]
Ervatamin A	<i>Ervatamia coronaria</i>	27.6	8.5	50	8.0 – 8.5	50 – 55	[78]
Funastrain c II	<i>Funastrum clausum</i>	23.6	9.5	-	9.0 – 10.0	-	[79]
Heynein	<i>Ervatamia heyneana</i>	23.0	8.0 – 8.5	52	2.5 – 11.5	10 – 60	[80]
Philibertain g I	<i>Philibertia gilliesii</i> Hook. et Arn	23.5	7.0 – 8.0	-	-	-	[81]
Philibertain g II	<i>Philibertia gilliesii</i> Hook. et Arn	23.9	9.0	-	7.0 – 10.0	25 – 45	[82]
Caricain	<i>Carica papaya</i>	23.2	7.0	50	5.5 – 9.5	40 – 55	[83]
Caricain II	<i>Carica papaya</i>	18.8	6.5	45	5.5 – 9.5	40 – 45	[83]

## Highlights

- First report on Three Phase Partitioning (TPP) of papain from *Carica papaya* latex
- Parameters affecting activity recovery and yield of latex papain have been determined
- Latex papain was recovered with 11.45 purification fold and 134% activity recovery
- Overall biochemical characterization of the recovered latex papain was performed
- TPP recovered papain has potential use as milk-clotting and meat tenderizing agent