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ABSTRACT

The present work describes for the first time an elegant non-chromatographic method, the three phase partitioning for the purification and recovery of zingibain, a milk-clotting enzyme, from *Zingiber officinale* rhizomes. Factors affecting partitioning efficiency such as $(NH_4)_2SO_4$ saturation, crude extract to *t-butanol* ratio and pH on zingibain partitioning were investigated. Optimal purification parameters were 50% $(NH_4)_2SO_4$ saturation with 1.0:1.0 ratio of crude extract:*t-butanol* at pH 7.0, which gave 14.91 purification fold with 215% recovery of zingibain. The enzyme was found to be exclusively partitioned in the aqueous phase. The enzyme showed a prominent single band on SDS-PAGE. It is a monomeric protein of 33.8 kDa and its isoelectric point is 4.38. The enzyme exhibited maximal proteolytic activity at a temperature of 60 °C and pH 7.0. It was found to be stable at 40–65 °C during 2 h. The enzyme was found to be highly stable against numerous metal ions and its activity was enhanced by Ca^{2+}, K^+ and Na^+. It was completely inhibited by heavy metal ions such as Cu^{2+} and Hg^{2+} and partially by Cd^+. Zingibain milk-clotting activity (MCA) was found to be highly stable when stored under freezing (−20 °C) for 30 days compared at 4 °C.

1. Introduction

Proteolytic enzymes or proteases, which hold the first place in the world market of enzymes, play an important role in food biotechnology. In food industries, proteases are frequently used in different productions, as well as in cheesemaking. For millennia milk-clotting enzymes, which are preparations of proteases, have been used in cheesemaking and they seem to be the oldest known application of enzymes [1]. The rennet, obtained from the abomasum of slaughtered young ruminants especially that of the calf, was extensively used as a traditional milk-clotting source. In fact, its use is limited for numerous reasons such as religion, diet (vegetarianism) or the very laborious and expensive enzyme supply [2]. Because of the limited availability of calf rennet, there is a prominent need for other alternatives. The bulk of these enzymes come from microbial sources, but vegetable enzymes, extracted or purified from higher plant organs, have been also investigated [1,2].

Among rennet substitutes, several proteases from many plants such as fruits (e.g., *Actinidia chinensis* [3] and *Cucumis melo* [4]), latex (e.g., *Ficus carica* [5] and *Calotropis procera* [6]), flowers (e.g., *Cynara cardunculus* [7] and *Centaurea calcitrapa* [8]), seeds (e.g., *Albizia lebbeck* [9] and *Solanum dubium*) [10]), leaves (e.g., *Lactuca sativa* [11]) and roots (e.g., *Zingiber officinale* [12]), have already been used. Their use as milk coagulants is of great interest since they are natural enzymes and can also be used for producing cheeses aimed to lacto-vegetarian consumers. In addition, cysteine proteases such as papain from *Carica papaya* [13], ficain from *F. carica* [14], bromelain from *Ananas comosus* [15], actinidain from *A. chinensis* [3] and zingibain from *Z. officinale* [16] received a considerable commercial importance due to their activity properties over a wide range of temperature and pH.

Amongst the coagulant proteases that have been reported to produce satisfactory final products in dairy technology, zingibain, the protease present in rhizomes of *Z. officinale* Roscoe [17] deserves special attention. Ginger, is an underground rhizome plant that belongs to the family of *Zingiberaceae*, which is widely used all over the world [18]. Ginger rhizome proteases (GP) or zingibain (EC 3.4.22.67), were first isolated in two forms termed ginger protease I (GP-I) and ginger protease II (GP-II) by [19]. Later on, Ohtsuki et al. [20] separated GP into three fractions by isoelectrofocusing, following which the 3-D structure of GP-II was elucidated by X-ray crystallography [21]. They were shown to have high activity against protein substrates such as gelatin, casein, bovine serum albumin and collagen [22,23]. They are inactivated by sulfhydryl reagents

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[24] and thus are grouped with other plant cysteine proteases, such as papain, ficain, bromelain and actinidain belonging to the papain superfamily.

While the potential exists for using zingibain more extensively in food processing (milk-clotting or meat tenderizing) and other biotechnological processes, fast and efficient purification methods, is scarce. In zingibain extraction and purification processes, most of the techniques involve a combination of two or more steps [12,16,20,24–26]. These purification protocols are too complicated to be used as simple and effective industrial methods. Also, separations by chromatographic techniques are expensive, time consuming, involving number of steps and furthermore the scale up is difficult. Thus, an alternative method for zingibain purification is required to solve the aforementioned drawbacks.

Three-phase partitioning (TPP) has been reported as an effective alternative method for recovery, concentration and purification of numerous industrial enzymes. TPP, a technique first described by Lovrien's group [27], was intensively used to purify several target proteins [28–31]. This one-step enzyme purification approach is carried out by mixing a salt, ammonium sulfate $(NH_4)_2SO_4$, and an organic solvent, *tertiary* butanol (*t*-BuOH) to obtain organic phase, interfacial precipitate phase and aqueous phase [31]. This elegant non-chromatographic process employs collective operation of principles involved in numerous techniques like (i) salting out, (ii) isoionic precipitation, (iii) co-solvent precipitation, (iv) osmolytic, and (v) kosmotropic precipitation of proteins. It is easily scalable and can be used directly with crude extract suspensions [32]. By this method, the desired enzymes or proteins are selectively partitioned to one phase while contaminants such as pigments and lipids to the other one. It does not only purify proteins but also concentrate them into one of the phases [29–31].

The present study describes for the first time, the purification and recovery of zingibain from a crude extract of *Z. officinale* rhizomes using the TPP system. Hence, the main focus of the present study was to achieve a maximum purity and yield of zingibain using this one-step purification approach. The influence of various parameters such as concentration of *t*-butanol, pH and $(NH_4)_2SO_4$ saturation on purification of zingibain has been studied. Characterization and stability of the purified milk-clotting enzyme after storage at 4 °C and under freezing (−20 °C) for 1 month were also investigated.

2. Materials and methods

2.1. Materials

2.1.1. Ginger rhizomes

Fresh fully ripened ginger rhizomes (*Z. officinale* roscoe) were purchased from a local market in Khenchela region (North-East), Algeria.

2.1.2. Chemicals

Tert-butanol, ammonium sulfate, Coomassie Brilliant Blue R-250, Triton X-100, acrylamide, bisacrylamide, sodium dodecyl sulfate (SDS), dithiothreitol (DTT), ethylene diamine tetraacetic acid (EDTA), L-cysteine, ammonium persulfate, *N,N,N′,N′*-tetramethylethylenediamine (TEMED), Casein from bovine milk, bovine serum albumin (BSA) and Tricine, were of pure grade and were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trichloroacetic acid (TCA), hydrochloric acid and tris-(hydroxymethyl)-aminomethane were from Merck (Germany). Molecular weight standards (precision plus protein dual color, #161-0374) and Bradford Reagent were purchased from Bio-Rad Laboratories Inc. (Hercules, CA, USA). Unless otherwise stated, all other chemicals and reagents were of the highest available purity and used as purchased. All solutions were prepared using deionized water and stored at the appropriate temperatures.

2.2. Methods

2.2.1. Crude extract preparation

Fresh ginger rhizomes (200 g) were washed and cut into fine pieces (~2.5 mm thickness) before homogenization in a blender with 450 mL of 50 mM sodium phosphate buffer (pH 7.0) containing 10 mM L-cysteine. The obtained homogenate was then left to stand under a continuous stirring (150 rpm) for 45 min at 4 °C before filtration through a double-layered cheesecloth. The insoluble residual debris was discarded and the supernatant was filtered through Whatman paper no. 1. The obtained ginger juice was spun in a centrifuge at 4000 rpm for 15 min at 4 °C to collect the supernatant, which was concentrated using ammonium sulfate up to 80% saturation. After an overnight dialysis (membrane molecular weight cut off (MWCO): 14 kDa) of the precipitate against two changes of 5 L of 50 mM sodium phosphate buffer, pH 7.0 at 4 °C, the crude clarified and dialyzed enzyme extract was subjected to TPP purification system.

2.2.2. Three phase partitioning of zingibain

TPP experiments were carried out as described by [32] with slight modifications as recently reported by Gagaoua et al. [30]. Briefly, the crude clarified and dialyzed *Z. officinale* enzyme extract was saturated at room temperature with 30% ammonium sulfate, followed by addition of an equal volume of *t-butanol*. The mixture was gently vortexed and then allowed to stand for 1 h at room temperature. Afterwards, the mixture was centrifuged at 4500 rpm for 10 min at +4 °C to facilitate the separation of the three phases. The upper *t-butanol* phase was removed by a Pasteur pipette. The lower aqueous phase and the interfacial phase were separated carefully and analyzed for enzyme activity and protein content. The interfacial precipitate was dissolved in 50 mM, pH 7.0 sodium phosphate buffer.

The effect of salt concentrations (30, 40, 50, 60, 70 and 80%) (w/v) on the crude enzyme extract for the TPP at the constant crude extract:*t-butanol* ratio (1.0:1.0) was investigated.

After that, various *t-butanol* ratios (crude extract: *t-butanol*; 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) were employed with a constant ammonium sulfate saturation at 50% at room temperature.

After, the *t-butanol* and ammonium sulfate, effects with different pH values of medium study were tested. Crude extract was saturated with 50% ammonium sulfate and pH was adjusted to 3, 4, 5, 6, 7, 8 and 9, then 1.0:0.1.0 *t-butanol* was added and the best pH value on the partitioning behavior of zingibain was investigated.

After optimization of the parameters, three repetitions were conducted to confirm the overall results using 50% ammonium sulfate, 1.0:1.0 ratio crude extract to *t-butanol* and a pH of 7.0. The aqueous phase always containing the higher zingibain activity was collected, dissolved in 50 mM, pH 7.0 sodium phosphate buffer, dialyzed overnight against the same buffer containing 100 mM EDTA and concentrated by ultrafiltration using Amicon Ultra device with a 10 kDa MWCO. The concentrated enzyme was stored at +4 °C or −20 °C until use for further characterization studies in order to determine the general biochemical properties.

2.2.3. Protein determination

The protein concentration was quantified by the dye binding method of Bradford [33] using the Bio-Rad Protein Assay (Bio-Rad Laboratories Inc.). The protein content was calculated from the bovine serum albumin (BSA) standard curve.

2.2.4. Enzymatic activity measurements

Zingibain activity was assayed at 40 °C using bovine casein (1% w/v) as substrate in a 50 mM sodium phosphate buffer pH 7.0 containing 250 mM EDTA and 1 mM DTT at enzyme concentrations of 0.20 mg/mL. The hydrolyzing activity of zingibain was based on the method described by Kunitz with some modifications using denatured casein as a substrate [34]. In activity measurements, 0.12 mL enzyme was incubated at 37 ± 1 °C for 15 min prior to the assay. The reaction was stopped by the addition of 1.8 mL of 5% trichloroacetic acid (TCA). For the blank, the substrate was added after the enzyme was first inactivated by TCA. The resulting precipitate was removed by centrifugation at 4500 rpm for 15 min, and the absorbance of TCA soluble peptides in the supernatant was measured at 280 nm. One unit of activity is defined as the amount of enzyme that increases the absorbance by 0.01 min^{-1} under given assay conditions.

2.2.5. Effect of temperature on the activity and stability of the partitioned zingibain

The temperature profile of zingibain was determined by performing the standard assay procedure at different temperatures (20, 30, 40, 50, 55, 60, 65, 70, 80, and 90 °C). The relative activities as percentages were expressed as the ratio of zingibain activity obtained at a certain temperature to the maximum activity obtained at the given temperature range.

The thermal stability of the enzyme was determined by measuring the residual activity of the enzyme exposed at the same temperatures in sodium phosphate buffer for 2 h with continuous shaking. After desired incubation periods, enzyme aliquots were withdrawn and assayed at optimal assay conditions to determine the residual enzyme activity.

2.2.6. Effect of pH on zingibain activity

The effect of pH on the enzyme activity of zingibain was evaluated from pH 3.0–9.0 using the following buffers: 50 mM citrate buffer (pH 3.0–5.5), 50 mM sodium phosphate buffer (pH 6.0–7.5), and 50 mM Tris–HCl buffer (pH 8.0–9.0). Proteolytic activity of the zingibain was determined as described above.

2.2.7. Effect of metal ions on zingibain activity

The effect of various metal ions ($CdCl_2$, KCl, NaCl, $CuSO_4$, $ZnSO_4$, $HgCl_2$, $CoSO_4$, $MnSO_4$, $Fe(SO_4)_3$, $CaCl_2$ and $MgCl_2$) on the partitioned zingibain activity were evaluated by its incubation with the metal ion (2 mM) at 40 °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 was considered as control and defined as 100%. All solutions were prepared with double-deionized water.

2.2.8. Isoelectric point (pI)

The isoelectric point (p*I*) of a protein is the pH where the net charge on the protein is zero. The p*I* of the zingibain was determined by exploiting the property of protein precipitation when the pH of a mixture is adjusted. Herein, we used the method first described by Nath and Dutta [35] to determine the isoelectric point of zingibain in a series of glycine–HCl buffer, acetate buffer, phosphate buffer and glycine–NaOH buffer prepared at intervals of 0.1 on the pH scale. The pH of the solution giving maximum turbidity after enzyme incubation for 5 min indicates the isoelectric pH of the given enzyme fraction.

2.2.9. Electrophoretical analysis (tricine SDS-PAGE)

Tricine SDS-PAGE with slight modifications was carried out according to [36] as recently described by [30] using 12% separating and 4% stacking gels. The samples were mixed at a ratio of 1:1 with the buffer sample containing 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 (4.9 mM Coomassie Brilliant Blue G-250) in 50% (v/v) ethanol, and 7.5% (v/v) acetic acid. Protein patterns were then visualized after destaining the gel until a clear background was achieved. A mixture of protein with a known molecular weight standard (precision plus protein dual color, #161-0374) obtained from Bio-Rad Laboratories, Hercules, CA, was used. The molecular weight of the protein bands was calculated using the Un-Scan-It gel 6.5 analysis program (Silk Scientific, Orem, UT).

2.2.10. Substrate-PAGE or zymography

Zymography (activity staining) was performed to detect the band with protease activity of zingibain on the purified protein only according to [37] with slight modifications using the above SDS-PAGE conditions. After electrophoresis, the proteins in the gel were renaturated by treatment in 2.5% (v/v) Triton X-100 for 30 min, and then washed twice with deionized water. The gel was immersed in 50 mL of 2% casein in 50 mM Tris–HCl buffer (pH 7.0) for 30 min at room temperature, and then incubated at 30 °C for 2 h. After that, the gel was stained and destained as previously described. The appearance of clear colorless area in the gel reveals the proteolytic activity of the protease.

2.2.11. Milk-clotting activity

Milk clotting activity (MCA) was determined according to [38] with slight modification. Briefly, the substrate was prepared by dissolving commercial bovine skimmed milk powder in 100 mL of 10 mM $CaCl_2$ to a final concentration of 12% (w/v, pH 6.4). The substrate (2 mL) was pre-incubated for 5 min at 37 °C and 0.2 mL of zingibain was added. Test tubes were periodically rotated by hand until appearance of visible discrete particles was discernible.

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. The following formula was used:

$$\mathrm{MCA(U/ml)} = \frac{2400 * V}{t * v} \quad (1)$$

where "*V*" is the volume of milk (mL), "*v*" the volume of enzyme (mL) and "*t*" the clotting time in seconds.

2.2.12. Storage stability

Stability at storage at 4 °C and −20 °C of the enzyme for 1 month was assayed and the results were expressed as MCA units (U/mL).

3. Results and discussion

3.1. Three-phase partitioning of zingibain from *Z. officinale* rhizomes

Separation of the protease from complex media during the partitioning was operated by multiple effects, such as salt concentration, solvent ratio and pH [30,31]. Therefore, the effect of various parameters on TPP was investigated. For this purpose, different partitioning experiments at various saturations of ammonium sulfate, $(NH_4)_2SO_4$, crude extract to *t-butanol* ratios and pHs were performed. The starting protein concentration (containing 30.84 units/mL of ginger protease activity) was 165.3 mg/mL. The effects of these parameters on the degree of purification and on the activity recovery of zingibain from the crude extract have been depicted in Figs. 1–3, respectively. The results showed that, all these three process parameters are very important and effective on the partitioning of zingibain from *Z. officinale* rhizomes.

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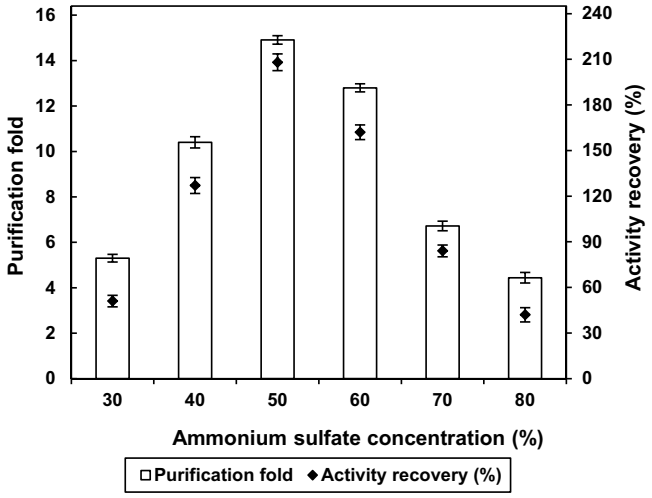

Fig. 1. Effect of varying saturations of ammonium sulfate ($(NH_4)_2SO_4$) on the degree of purification and activity recovery of *Z. officinale* zingibain. The crude extract (2 mL containing 61.68 U) was brought to different levels of saturation and *t-butanol* was added in the ratio 1.0:1.0 (v/v) with respect to the volumes of the aqueous saturated extract.

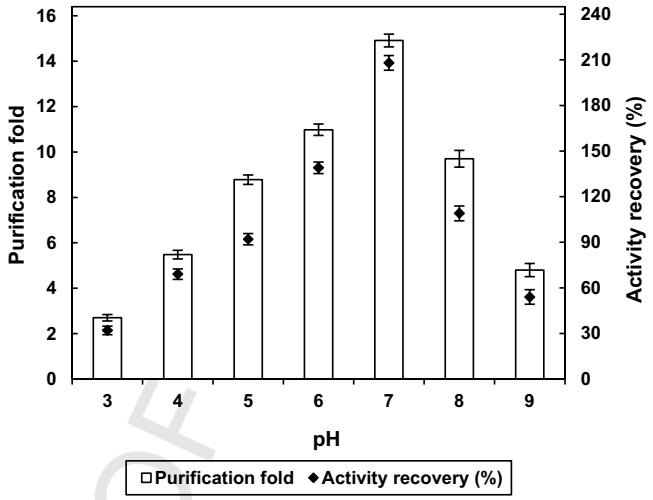

Fig. 3. Influence of pH on the degree of purification and activity recovery of *Z. officinale* zingibain. Ammonium sulfate (50%, w/v) was added to the crude extract (2 mL containing 61.68 U). The pH of the medium was adjusted to different pH values. This was followed by addition of *t-butanol* in a ratio of 1.0:1.0 (v/v).

3.1.1. *Effect of ammonium sulfate saturation on zingibain partitioning*

Salting out can be used to separate proteins based on their solubility in the presence of high salt concentration [39]. Ammonium sulfate, $(NH_4)_2SO_4$, is the most salt used for protein salting out because of its high solubility. Additionally, NH_4^+ and SO_4^{2-} are at the ends of their respective Hofmeister series and have been shown to stabilize protein structure. $(NH_4)_2SO_4$ saturation is of critical importance in TPP and must be optimized to get the maximum recovery. The efficiency of the salting out will first depend on $(NH_4)_2SO_4$ and second on the net charge. Herein, the experiment was carried out by keeping the experimental parameters as follows: ammonium sulfate loading 30% w/v, crude extract: *t-butanol* ratio 1:1, time 1 h, temperature 25 °C and pH 7. Ammonium sulfate concentration was varied from 30% to 80% w/v of the crude extract concentration and results were shown in Fig. 1. Indeed, the maximum fold purification of 14.91-fold along with 208% recovery of ginger protease activity in the aqueous phase was obtained with 50% $(NH_4)_2SO_4$ saturation. Otherwise, at the lowest (30%) and at the highest (80%) salt concentrations, very low percentage recoveries were found and are respectively 51% and 42%. Beyond 50% there is a decrease in the % recovery of the ginger protease. Hence, 50% w/v $(NH_4)_2SO_4$ concentration was selected as the optimum value for the next set of experiments. This salt concentration was sufficient enough to concentrate the protease, a finding in agreement to its known property to precipitate at 50–60% $(NH_4)_2SO_4$ saturation [16,40,41]. Generally, in TPP process, the researchers start minimum salt concentration of 20% (w/v) to optimize the partitioning conditions [32]. In this study we also started at minimum salt saturations for the beginning and the increasing of salt concentrations resulted with the increasing of recovery and purification fold in the aqueous phase until 50% (w/v) saturations. Higher salt concentrations leads to the reduction of recovery and purification fold, which may be due to the irreversible denaturation of protein as reported in earlier studies [32,42,43]. Fig. 1 also showed that TPP strongly depends on saturation of $(NH_4)_2SO_4$. During the TPP process, activation of enzyme is also possible. This may be due to the phenomenon that the increased flexibility of enzyme can lead to higher catalytical activity in TPP process [30,31,44,45]. A remarkable increase in the catalytic activity and yield of pectinase [46], ficin [30] and invertase [28] obtained from TPP has been reported. Sulfate ion and *t-butanol* are known to be excellent protein structure markers or kosmotropes [31,32]. Kosmotropes, inversely to chaotropic agents, cause water molecules to favorably interact, which also stabilizes intramolecular interactions in the proteins [47]. Ammonium sulfate is the traditional kosmotropic salt for the salting out of protein from an aqueous solution. The principle of SO_4^{2-} ion for salting out protein has been viewed in five different ways namely, (1) ionic strength effects, (2) cavity surface tension enhancement osmotic stressor (dehydration), (3) kosmotropy, (4) exclusion crowding agent and (5) the binding of SO_4^{2-} to cationic sites of protein [31].

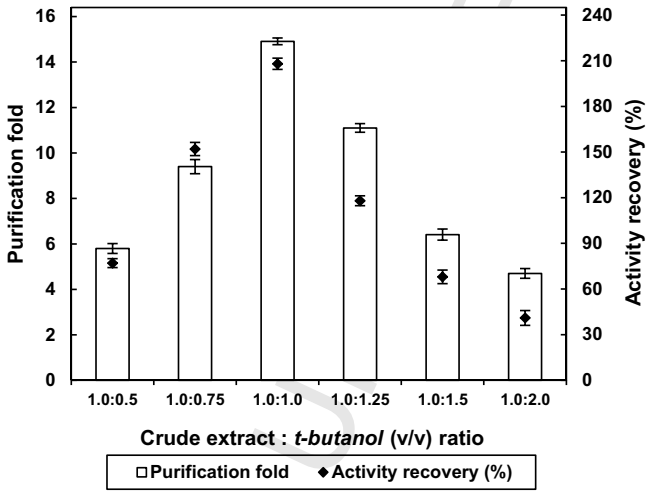

Fig. 2. Optimization of crude extract to *t*-butanol ratio for the recovery of *Z. officinale* zingibain. Various amount of *t-butanol* was added to crude extract, 2 mL containing 61.68 U and saturated with 50% ammonium sulfate.

3.1.2. *Effect of the ratio of crude extract to t-butanol on zingibain partitioning*

After the selection of $(NH_4)_2SO_4$ saturation, the ratio of the volume of crude extract to *t-butanol* which is a very important factor that governs TPP was optimized. *t-Butanol* was chosen as the organic co-solvent for partitioning of zingibain in TPP as all the reports published on TPP have used *t-butanol* as the organic solvent, which has been generally reported to give best results [29,30,32,48]. *t-Butanol* is miscible in water, but after the addition

of enough salt, like $(NH_4)_2SO_4$, the solution gets separated into phases. Due to its size and branched structure, *t-butanol* does not easily permeate inside the folded protein molecules and hence does not cause denaturation. Also at 20–30 °C temperature, *t-butanol* imparts significant kosmotropic and crowding effects and enhances TPP [31]. Different crude extract:*t-butanol* ratio effect for the partitioning of zingibain at the constant saturation of $(NH_4)_2SO_4$ (50%, w/v) is shown in Fig. 2. It can be seen from Fig. 2 that the highest recovery (208%) was obtained at the ratio of 1.0:1.0 and activity recovery decreased with subsequent increase in crude extract to *t-butanol* ratio. At 1.0:0.5 and 1.0:0.75 ratios, there is less % recovery which may be due to smaller amount of *t-butanol* does not adequately synergize with $(NH_4)_2SO_4$ [31,32]. If it is higher than 1.0:1.0, it is likely to cause protein denaturation as reported in many earlier studies [30,31,49,50]. Otherwise, many previous studies and in agreement to our findings, a ration of 1.0:1.0, crude extract to *t-butanol* was reported to give maximum partitioning [44,51–53]. From these findings, the ratio of crude extract to *t-butanol* of 1.0:1.0 was selected for investigating the effect of pH value on the TPP system.

3.1.3. Effect of pH on zingibain partitioning

The pH of the reaction mixture was also reported to be a very important parameter in TPP as pH causes ionization of amino acids present in the protein [29–31,44]. It was reported that TPP systems usually sharply change around the isoelectric point (p*I*) of the proteins and it is efficiently depend on this parameter. The distribution and partitioning of proteins in TPP systems change with pH due to electrostatic interactions between phases and charged proteins. Hence, the effect of crude extract with different pH values ranging from 3 to 9 (i.e. acidic as well as basic solutions) on the partitioning of zingibain was investigated. After 1 h of incubation, the three phases were formed and collected separately. Zingibain had partitioned selectively to the aqueous phase. As depicted in Fig. 3, 50% (w/v) $(NH_4)_2SO_4$ saturation with 1.0:1.0 ratio of crude extract to *t-butanol* (v/v) at pH 7.0 gave a maximum 14.91-fold purification and 208% activity recovery of zingibain. It can be seen and stated from Fig. 3 that the purification efficiency was low in the acidic as well as basic range as compared to the neutral range. The possible reason for this type of behavior may be that in acidic pH there are more H^+ ions which compete over ginger protease interaction with the solvent molecules and this does not allow the enzyme to interact due to which there is a decrease in the recovery at acidic pH. In addition, it has been reported in earlier studies [32] that, a change in pH causes the net charge of the target protein and this influences its partitioning. This is a consequence of electrostatic reactions by the involvement of the sulfate anion by binding to the protein. When the pH value scale of the system is above isoelectric point (p*I*) of protein, hydrophilic amino acid residues are charged negatively and the protein will acquire net negative charge and will be propelled to bottom aqueous media. Therefore, proteins will stay at the lower aqueous phase. On the other hand, if system pH is below p*I* of target protein, it will be precipitated or accumulated at the interfacial phase. Dennison [31] reviewed recently that change in pH facilitates the change in net charge of target protein and influences partitioning of protein. Wang et al. [54] have reported enhanced partitioning of α-amylase inhibitors in bottom phase at a pH 5.25 and 6.0, which was above its p*I* 5.2. In our study the p*I* of zingibain was found to be 4.38. This is in concordance with the previous results reported by Hashim et al. [16]. Otherwise, when pH value of TPP medium is adjusted under p*I* value, amino acid residues are positively charged and protein molecule will be precipitated in the interfacial phase [32,54,55]. Thus, contaminant proteins were precipitated at the interfacial phase (as revealed by electrophoresis) or partitioned to upper *t-butanol* phase (ginger polyphenols and pigments). Hence, pH 7.0 was found to be optimum in this study for partitioning of *Z. officinale* zingibain.

3.1.4. TPP purification profile and recovery results

The overall purification profile of zingibain from *Z. officinale* rhizomes by TPP is summarized in Table 1. As understood from the obtained results, it can be said that ginger protease has tendency to concentrate in the aqueous phase of the TPP system that is related to its amino acids composition and structure. The TPP system consisting of 1.0:1.0 of slurry to *t-butanol* and 50% $(NH_4)_2SO_4$ resulted in the highest proteases recovery at 215%, and a 14.91-fold purification at the aqueous phase. TPP is known as a concentrating or dewatering step and some enzymes have enhanced catalytic activities in these conditions. This process system leads to simultaneous activation of enzyme which results to such an apparently observed yield value more than 100% [56]. This may be the result of increased flexibility in the enzyme molecule as discussed above. Several reports are available on the partitioning and purification of different enzymes from various sources with TPP such as pectinase [46], proteinase K [57], ficain [30] and trypsin inhibitor [29] and all of them were reported to be remarkably increased their activity and yield. For example, we have recently reported that when TPP was applied to a milk-clotting enzyme, ficain, a yield of approximately 167% with 6.04-fold purity was obtained [30]. Additionally, in another earlier study conducted by Dennison and Lovrien [32], they reported that a *Bacillus subtilis* protease, *Saccharomyces cerevisiae* invertase, and *Candida cylindracea* lipase gave a yield of 300, 100 and 900%, respectively. As shown in Table 1, zingibain was exclusively partitioned to the aqueous phase instead of the interfacial phase, as indicated by the very high recovery. Hence, this system was used to produce ginger proteases for application as milk-clotting enzymes.

3.2. Biochemical characterization of the purified zingibain

Proteolytic enzymes are responsible in many complex processes involved in the normal physiology of the cell as well as in abnormal pathophysiological conditions [58]. The good properties of a protease offer potential for use in industrial processes. Since the applications of zingibain in various fields, as milk-clotting enzyme or tenderizer agent, are broadening it is important to develop new efficient purification protocols. Also it is important to understand the nature and biochemical properties of these enzymes for their efficient and effective usage in production processes.

Table 1
Three-phase partitioning purification and recovery profile of Zingibain from fresh ginger (*Z. officinale* Roscoe) rhizomes.[a]

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
Crude extract	30.84	165.3	0.19	1	100
TPP aqueous phase	66.21	23.8	2.78	14.91	215
Interfacial phase of TPP	0.19	39.09	0.005	0.03	0.62

[a] The ammonium sulfate (50%, w/v) was added to the dialyzed crude extract of *Z. officinale* rhizomes and then pH was adjusted to pH 7.0. This was followed by addition of *t-butanol* in a ratio of 1.0:1.0 (v/v) (crude extract:*t-butanol*). 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.

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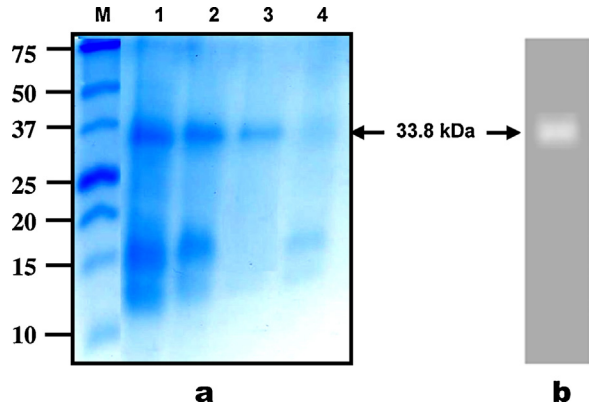

Fig. 4. Analysis of the purified zingibain from ginger (*Z. officinale*) rhizomes by (a) Tricine SDS-PAGE (12% separating polyacrylamide gel): lane M, molecular mass standards; lane 1, crude extract after ammonium sulfate saturation (80%); lane 2, dialyzed crude extract, lane 3, purified zingibain (aqueous phase) using TPP system after dialysis; lane 4, interfacial phase. (b) Activity staining (zymography) clear zone showed the hydrolysis of casein by the purified enzyme presented in the lane 3.

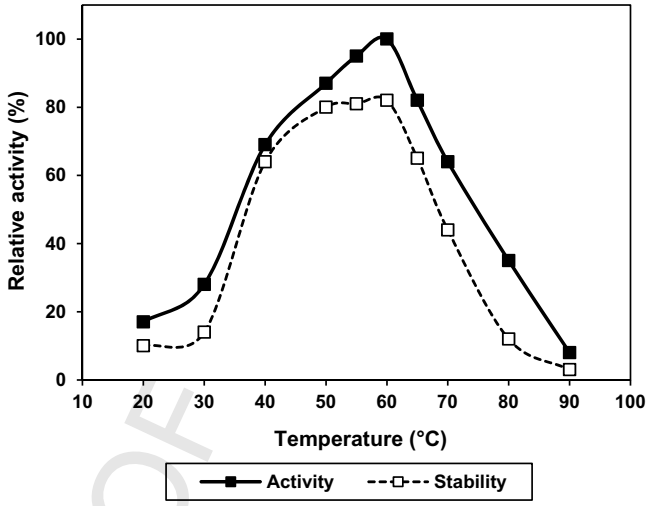

Fig. 5. Effect of temperature on the activity and the stability of *Z. officinale* zingibain. Activity was done at defined temperatures by using bovine casein. Thermal stability was done after 2 h incubation at the indicated temperatures.

3.2.1. SDS-PAGE and zymography analyses

Tricine SDS-PAGE analysis showed that the partitioned zingibain was highly purified as a single band, which suggested a monomeric nature of the enzyme (Fig. 4a). The molecular mass of the partitioned enzyme was estimated to be 33.8 kDa. Generally, the estimated molecular masses of ginger proteases falls in the range of 22–36 kDa (Table 2), which is also the reported range of values for many other cysteine proteases [59]. For example, Ichikawa et al. [19] purified two ginger proteases (GP-I and GP-II) which share a common molecular weight of 22.5 kDa. Otherwise, Ohtsuki et al. [20] separated ginger proteases into three fractions each with 29 kDa. A molecular weight of 34.8 kDa, very close to our finding, was reported by [26]. Moreover, recently a ginger protease purified using $(NH_4)_2SO_4$ fractionation, ion exchange and size exclusion chromatography techniques, was reported to migrate with an apparent molecular weight of 36 kDa [16]. On another hand, analysis of the interfacial phase showed clearly two protein bands with low molecular weight of approximately ~13.4 and ~16.3 kDa. These two proteins, with no proteolytic activity, are likely to correspond to two subunits as reported by [60] or other contaminants.

Separated proteins using SDS-PAGE were further subjected to determine activity staining and confirm the identity of the single band on substrate gel electrophoresis (Fig. 4b). Zymography analysis using casein as a substrate showed a unique and well-resolved protein band on SDS-PAGE which demonstrated the protease activity of the partitioned zingibain (Fig. 4b). Although the sample was treated with 1% SDS (w/v), the enzyme still displayed activity after electrophoresis migration, indicating its resistance to SDS. As reported by [61], SDS resistance is a property often associated with heat-stable proteases of thermo-stable archaea and bacteria. The binding of low concentrations of SDS to the protein usually masks the intrinsic charge and because of the bound SDS, the hydrogen atom of the sulfhydryl group of cysteine tends to be attracted. The same property was recently reported by Hashim et al. [16]. In addition, this noticeable property has also been reported in some other plant proteases especially cucumisin-like serine proteases [62].

3.2.2. Effect of temperature on the activity and stability of zingibain

The effect of temperature on zingibain activity and stability was studied in the temperature range of 20–90 °C (Fig. 5). It is well known that the reaction rate of enzymes increases with increasing temperature, but after a certain temperature activity, it decreases due to denaturation [16,30,41,44]. The optimum temperature of the partitioned zingibain was found to be 60 °C (Fig. 5). Our findings show that even at high temperatures the activity of the enzyme was not lost rapidly. The enzyme retained more than 50% of its initial activity at a broad temperature range of 40–70 °C. The results compare well with the previous studies (Table 2) indicating optimal

Table 2
Comparison of different processes from literature for ginger protease purification.

Purification method steps	MW (kDa)	Optimum pH	Optimum temperature (°C)	Thermal stability	Purification (fold)	Recovery (%)	Refs.
Ultrafiltration + DEAE Sephacel	31	6.2	40–60	40–65	–	–	[25]
Acetone extraction + Hi-Trap DEAE FF	45–66[a]	6.0	60	–	0.8	23.9	[12]
Acetone extraction + ammonium sulfate precipitation + DEAE-52 Sepharose + SP Sephadex 50	–	–	–	–	2.06	151	[40]
Acetone extraction + Sephadex G-25 + DEAE-Sepharose + Sephadex G-75	29	–	–	–	–	–	[20]
Acetone extraction + ammonium sulfate precipitation + Toyopearl DEAE-650	34.8	7.0	40	40–60	252	61	[26]
Acetone extraction + ammonium sulfate precipitation + Mono Q 5/50GL + Superdex 75	36	5.5	60	–	10.23	34.90	[16]
Three phase partitioning system	33.8	7.0	60	40–65	14.91	215	Present study

[a] Formation of dimers was reported.

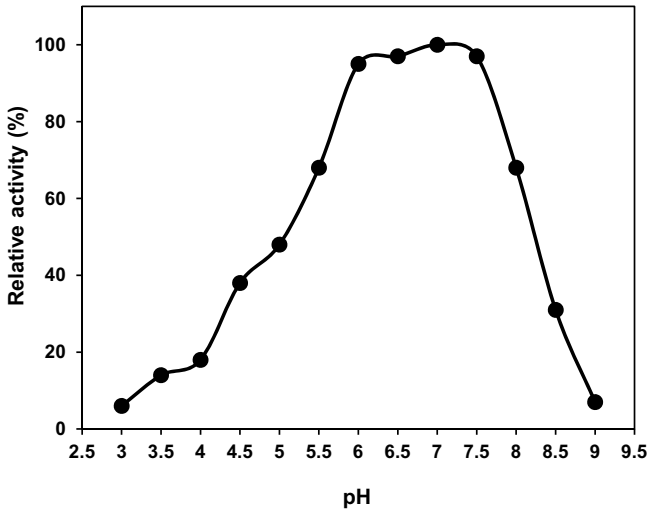

Fig. 6. Effect of pH on the activity of *Z. officinale* zingibain. Activity was done after incubation at pHs from 3.0 to 9.0 [(50 mM citrate buffer (pH 3.0–5.5), 50 mM sodium phosphate buffer (pH 6.0–7.5), and 50 mM Tris–HCl buffer (pH 8.0–9.0)] and activities were assayed using bovine casein.

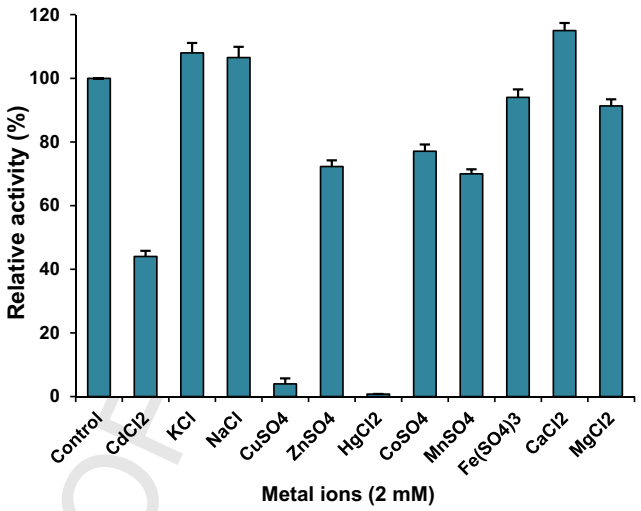

Fig. 7. Effect of metal ions on the activity of *Z. officinale* zingibain. Enzyme activity was evaluated by incubating purified zingibain in presence of various metal ions at 40 °C for 30 min using bovine casein as substrate. The enzyme activity assayed in the absence of any metal ion was considered as control and defined as 100%.

temperature range for ginger proteases activity to be between 40 and 60 °C [16,41]. Thermal stability results indicated that the enzyme is stable between the temperatures' range of 40–70 °C. The enzyme retained about 60%, 80%, 81%, 82% 65% and 44% of its initial activity after 2 h incubation at 40, 50, 55, 60, 65 and 70 °C, respectively (Fig. 5). These significant properties let the partitioned zingibain to be a good industrial enzyme candidate, which can be easily used in food industries, like milk coagulation or meat tenderizing.

3.2.3. Effect of pH on the activity of zingibain

The pH activity profile showed that the purified zingibain is highly stable in a broad pH range (Fig. 6). The enzyme retained more than 65% of its activity over a wide range of pH from 5.5 to 8.0. Below pH 5.0 and above pH 8.0 the activity decreased rapidly. The maximum activity was observed between pH 6.0 and 7.5 and showed optimum at pH 7.0. These results show that the enzyme is more active in the neutral range as compared to the acidic range characterized by the very low activity. In agreement to numerous previous studies [12,25,26,41], the results confirmed once again that the enzyme is unstable at acidic pH and the enzyme is more stable at pH 6.0–7.0. This finding is in agreement with well known milk-clotting enzymes [11,15,30].

3.2.4. Effect of metal ions on the activity of zingibain

The effects of some metal ions, at a final concentration of 2 mM, on protease activity of zingibain were also investigated at pH 7.0 and 40 °C by the addition of the respective ions to the reaction mixture (Fig. 7). The enzyme was found to be highly stable against numerous metal ions and its activity was significantly enhanced by Ca^{2+}, K^{+} and Na^{+}. It was completely inhibited by heavy metal ions such as Cu^{2+} and Hg^{2+} and partially by Cd^{+}. Inhibition of proteases by Hg^{2+} is suggestive of the existence of amino acids containing group-SH at or near the active site as recently reported for this enzyme by Hashim et al. [16]. Similar findings were reported by previous studies [20,41]. In comparison, analog findings were reported by Demir et al. [63], where metal cations such as Hg^{2+} and Cu^{2+} caused substantial inhibition of a cysteine protease, capparain, extracted from capsules of *Capparis spinosa*.

3.2.5. Storage stability

After the enzyme preparations were stored at two different temperatures (4 °C and −20 °C) along one month, zingibain milk-clotting activity (MCA) was determined. As seen in Fig. 8, the purified zingibain showed higher milk clotting activity (339 U/mL) just after purification in agreement to the findings of Hashim et al. [16]. The enzyme retained about 82.6% of its MCA after being kept 6 days at 4 °C temperature; in contrast, it still retained 99% of its activity under freezing at −20 °C. Earlier studies suggested that ginger proteases stored at room temperature or 4 °C are adequate for domestic applications in spite of their short half-life [23,26,64]. A 95% loss of activity was observed for the protease stored at 4 °C for 30 days. This might be due to protein–protein interaction [65], to autolysis [30] or to molecular rearrangements in protein structure [66]. On the other hand, the zingibain stored at −20 °C lost only 3% of its MCA. This enhanced stability is probably a result of the prevention of autolysis by freezing. As appears from the below experimental results, a destabilization of the proteolytic activity has been obtained concomitantly with a substantial retainment of the storage stability of the enzyme, as measured by its milk-clotting capability, at reasonable storage conditions (e.g.

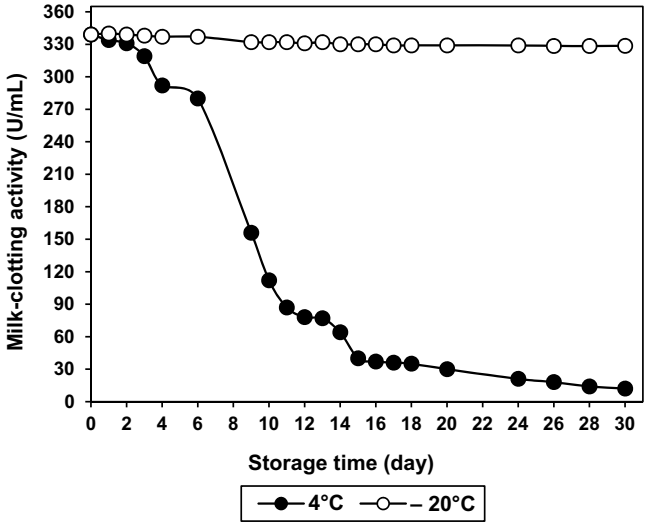

Fig. 8. Effect of storage temperature on milk-clotting activity of *Z. officinale* zingibain. Aliquots of the purified enzyme, stored at −20 °C (○) and 4 °C (●), were removed at different periods and milk-clotting activities were determined as described in Section 2. Each point was based on triplicate analysis.

refrigerator, about 4 °C). These results suggest that the partitioned zingibain might be preserved at 4 °C for a short storage of 6 days, whereas a longer life of more than 1 month may be obtained by storage under freezing at −20 °C. It is worth mentioning that previous studies used additive stabilizers like ascorbic acid to preserve ginger protease activity [26,41].

3.2.6. Comparison of purification protocols with literature

Table 2 shows purification protocols and the biochemical results of ginger proteases from *Z. officinale* rhizomes. As seen in Table 2, the reported purification protocols of zingibain from literature include at least two steps and one of them is certainly chromatography. These methods are cost effective and time consuming when compared to TPP. Though the overall purification is medium in previous studies, the present study showed better degree of purification and activity yields using TPP as a single purification step.

4. Conclusion

To the best of our knowledge, this is the first report for purification, recovery and characterization of rhizome (*Z. officinale*) zingibain by using three phase partitioning (TPP). In comparison to chromatographic methods, TPP it is very cheap, simple and efficient method. Effect of various process parameters has been evaluated and zingibain was purified to 14.91-fold with 215% activity recovery by optimized single step TPP system (50%, (w/v) $(NH_4)_2SO_4$ saturation, 1.0:1.0 (v/v) ratio of crude extract: *t*-butanol at pH 7.0). The enzyme was found to be exclusively partitioned in the aqueous phase. Using TPP system, ginger protease quickly purified to homogeneity with very high purity and activity, showing an effective milk-clotting activity compared to the classical purification protocols. It can be concluded that, as fast elegant non-chromatographic separation technique, TPP could be attractive for the purification of zingibain for its easy use in food industry as an efficient cheese-making or meat tenderizer agents.

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