A MILK-CLOTTING PROTEASE FROM *MACLURA POMIFERA* (*OSAGE ORANGE*): THREE-PHASE PARTITIONING AND CHARACTERIZATION

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Abstract

Three phase partitioning (TPP) is used to recover and purify the Maclura pomifera proteases. Optimum partitioning parameters are determined as 40% (w/v) ammonium sulfate, 1:1 (v/v) crude extract:t-butanol ratio at pH 6.0 with 1 mg of protein extract. The protease is purified with the activity yield of 86% and purification fold of 2.85 by using optimized conditions in the middle phase of the TPP system. The molecular weight of protease is determined as 67.8 kDa. The maximum temperature and pH are found as 65 °C and pH 6.5, respectively. The partitioned protease exhibited excellent stability at 25-65 °C and pH 4-9. K_M and V_{max} values are calculated as 0.87 mg/mL and 8.82 U, respectively. Beside of its caseinolytic activity the protease also show best milk-clotting activity at pH 6.0. TPP partitioned protease with good biochemical characteristics might be the promising candidate for food industry, especially for dairy industry.

Key words: Maclura pomifera, Osage orange, Three-phase partitioning, TPP, Protease, Enzyme purification, Milk-clotting activity.

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INTRODUCTION

Proteases (EC 3.4.x.x) which are also known as proteolytic enzymes or proteinases catalyze the hydrolysis of peptide bonds. They are derived from plant, animal and microbial sources that find several applications especially in pharmaceutical, detergent, food, bakery, textile and leather industries for the removal of protein [1, 2]. In recent years due to their tolerance against to wide temperature and pH ranges plant proteases gain popularity for various industrial applications like cheese manufacturing. In dairy industry, for cheese making enzymatic coagulation of milk is needed. In this process, the enzyme hydrolyses the κ-casein at first and then coagulation of casein missels occur. Chymosin is a milk-clotting enzyme and obtained from the stomach of young calf. Although the chymosin is the best coagulant with high activity and thermal stability it is very expensive and also its production is limited [3-6]. Because of this fact, due to ethic and religious opinions about animals the searchers directed their research to new alternative enzymes. There is a demand to find a new low-cost plant sources for proteases to develop the taste and texture of cheese and to increase the cheese production yield. The use of plant proteases may also rises cheese acceptability by the lactovegeterian people to enhance their nutritional status [7, 8]. Several milk-clotting proteases were extracted from different plants like *Cucumis melo* [9], *Citrus auratium* L. flowers [3], *Ficus johannis* [10], *Wittania coagulans* fruit [7], kiwifruit [11], *Moringa oleifera* flowers [5], papaya [12], etc.

Maclura pomifera is a plant that belong to *Moraceae* family and named also as Osage orange Although its fruit is not edible for human, its extract have several biological activities such as antimicrobial, antidiabetic, estrogenic and cytotoxic activities. Therefore the fruit extract of *M. pomifera* was suggested as a new and good protease source [13-15].

Many papers are present for purification of proteases from various sources utilizing different conventional purification schemes including ammonium sulfate precipitation followed by chromatography: an alkophilic protease from Northwestern Himalayas [16], an alkaline protease from rainbow trout viscera [17], an alkaline serine protease from *Cucumis*

melo seeds [2], a milk-clotting metalloprotease from *Thermotomyces cypeatus* [18], a protease from *Citrus aurantium* flowers [3] and *Wrightia tinctoria* [6], kiwifruit [11], an aspartic protease from *Pichia pastoris* [19] and a cysteine protease from *Ficus johannis* [10], etc. Because of the drawbacks of these used conventional purification methods like time consuming, expensive and difficult to scale-up three-phase partitioning (TPP) is an alternative bioseparation technique in comparison to them. It is also very simple and effective for concentrating, recovering and purifying of proteins. TPP is both an upstream and downstream technique that uses collective operations of principles involved in several techniques such as salting-out, isoionic, co-solvent, osmolytic and kosmotropic precipitation of proteins [20-22]. TPP was used to purify several biological molecules from different sources like laccase from *Trametes hirsuta* [23], beta-amylase from *Abrus precatorius* [24], α-galactosidase from tomato [25] and watermelon [26], aloe polysaccharide from aloe pulp [27], levan from *Zymomonas mobilis* [28], etc. TPP technique is also efficiently used for the partitioning of different proteases. Rajagopolan and Sukumaran have concentrated and characterized a milk-clotting protease [6] by using TPP. Zingibain which is also have a milk-clotting activity was purified with this technique. The papaya peels [12], *Calotropis procera* latex [29] and fish viscera [30] sourced proteases were also successfully purified with TPP process.

In the present study, the partitioning, concentrating and recovery of proteases from the *M. pomifera* crude extract with TPP technique was described for the first time. Although it is not edible for human several phenolic compounds namely isoflavonoids, flavonols and xanthones were extracted and identified from many parts of *M. pomifera*. However, the proteins, enzymes and several components which have a potency for nutrition and health care need to worked out. The potential use of TPP partitioned *M. pomifera* protease as a new milk-clotting protease were also searched. The influence of ammonium sulfate concentration, enzyme: t-butanol ratio, pH and protein amount on partitioning of the protease has been investigated. The biochemical properties of the enzyme were evaluated to provide fundamental information needed for its applications especially in the food industry. The protease fraction obtained from TPP was also assessed for its milk-clotting potential.

MATERIALS AND METHODS

Materials

Casein and Folin & Ciocalteu's Phenol Reagent were purchased from Riedel de Haen, bovine serum albumin (Albumin Fraction V) was obtained from Bio-Rad Laboratories (Richmond, California). Coomassie Brilliant Blue G-250 and protein molecular weight markers were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Ammonium sulfate, tert-butanol and other chemicals and reagents were purchased from Merck (Darmstadt, Germany). *Maclura pomifera* was obtained from İzmir (Turkey).

Isolation and three-phase partitioning of protease from *Maclura pomifera*

The *M. pomifera* mature fruits were collected at November from Narlıdere (İzmir). The fruits were washed with distilled water and after the removing of the peels they were cut into slices. The flesh was homogenized with phosphate buffer (0.05 M, pH 7.5) and filtered. After stirring of the suspension for 30 min it was centrifuged at 11.200 rpm for 15 min at 4 ^oC. The clear supernatant indicated as "crude protease extract" and used for TPP of the enzyme. The protein amount, enzymatic and specific activity of the protease were determined as 2.2 mg/mL, 5.22 U/mL, 2.37 U/mg, respectively.

For TPP of *M. pomifera* protease, the desired amounts of solid ammonium sulfate was put into the tubes and then the protease extract (4 mL containing 5.22 U, 2.2 mg protein) was added. The mixture was vortexed kindly to dissolve the ammoniumsulfate. The medium pH was also set to different values by using HCl or NaOH. t-Butanol with different ratios were added, vortexed again and then kept for 1

h at 4 ºC for phase separation. To distinctly separate the formed phases the tubes were centrifuged (6000 rpm at 4 ºC for 5 min). The upper phase containing t-butanol was taken out with Pasteur pipette. The lower phase also gathered and the middle interfacial precipitate containing protease activity was dissolved in phosphate buffer (0.05 M, pH 7.5). The enzymatic activity and protein amounts of the both middle and lower phases were assayed.

In order to obtain good purification fold and yield, the TPP process is optimized. For this aim, the effect of ammonium sulfate concentration (20-70%, w/v), crude enzyme extract: t-butanol ratio (1:0.25, 1: 0.5, 1: 0.75, 1:1, 1:1.5 and 1:2, v/v), pH (3.0, 4.0, 5.0, 6.0, 7.0 and 8.0) and protein amount (0.1, 0.25, 0.5, 1 and 1.5 mg) were searched. The best condition for protease partitioning was determined. The middle phase of every TPP systems showed the highest protease activity. The activity of the crude protease extract initially added (5.22 U) was taken as 100%.

For all TPP systems a blank system (without enzyme extract) was prepared. The ANOVA (one way) was performed applying Microsoft Excel. Every TPP trials were run in duplicate and the difference in readings in duplicates was less than ±5%.

Analytical assays

Protease activity

The protease activity (caseinolytic activity) was assayed accordingly to the procedure of Takami et al. by some modifications with using casein (in 0.05 M phosphate buffer, pH 7.5) as substrate [31]. The quantity of liberated tyrosine was determined spectrophotometrically at 660 nm. One unit (1 U) of protease activity was described as the amount of enzyme required to liberate 1 µg/min tyrosine from casein per min under standard analysis conditions. The information given for protease activity assays are mean values of triplicate assays in which the standard deviations were always smaller than 5%.

Protein determination

The amount of protein was quantified by the dye-binding method [32]. The bovine serum albumin (BSA) was used as a protein standard. The data presented for protein determinations are mean values of triplicate assays in which the standard deviations were always smaller than 5%.

Milk-clotting activity (MCA) of Maclura Pomifera protease

MCA was determined by the procedure explained by Arima & Iwasaki [33] with several alterations. The commercial bovine skimmed milk as substrate was prepared in 0.01 M $CaCl_2$ solution. The substrate (2 mL) was first pre-incubated at 37 ºC for 5 min and then partitioned protease (0.2 mL) was added. The tubes were peridiodically rotated manually and time taken for micellar formation is recorded. One milk-clotting unit is described as the amount of enzyme which clots 10 mL of substrate within 40 min at 37 ºC. The effect of various pHs (5.0, 6.0, 6.5, 7.0 and 8.0) on MCA was also determined. The all trials for TPP of the enzyme were run in triplicate and the difference in readings in triplicates was less than ±5%.

Determination of total phenolic and flavonoid amount

The phenolic content of *M. pomifera* extract and the upper phase of TPP system were assayed by Folin-Ciocalteu method with slight modifications [34]. Briefly, 1 mL of Folin-Ciocalteu reagent (50%, v/v) and 3 mL of Na_2CO_3 (20%, w/v) were put on to the sample (0.2 mL) and after that the volume was made up to 20 mL with water. The mixture was let to stay at room temperature for 2 h in the dark and spectrophotometrically assayed at 760 nm. Gallic acid (0.05-0.5 mgL^{-1}) was used as standard.

The flavonoid amounts were determined with aluminium chloride colorimetric method [34]. After the adding 4 mL of distilled water to the 1 mL of sample it was mixed with 5% (w/v) $NaNO_2$ (0.3 mL) and stayed at room temperature for 5 min. Then, 10% (w/v) $AlCl_3$ (0.3 mL) was added to this solution, neutralized with 1 M NaOH (2 mL) and the last volume was made up to 10 mL with water. The absorbance was measured at 415 nm. Catechin (0.02-0.1 mgL^{-1}) was used as standard.

The both analysis were made in triplicate and the difference in readings in triplicates was less than ±5%.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS–PAGE was applied according to the Laemmli procedure with 12 % separating gel in Biorad Mini Protean II electrophoresis unit [35]. After separation, the it was stained overnight with Coomassie Brilliant Blue R-250 for 1 h and then destained by 40% (v/v) methanol and 10% (v/v) acetic acid for 2–3 h. The molecular weight standard consisting of standard proteins with molecular weights of 14.4 to 66 kDa was used.

Characterization of Maclura pomifera protease

Effect of temperature on the activity and stability of protease

The temperature effect on enzymatic activity of TPP partitioned protease is measured at temperature range of 25-80 ºC by performing standard activity assay method and the optimum temperature was determined. The relative activities as percentages are expressed as the ratio of the protease activity obtained at a definite temperature to the maximal activity achieved at the dedicated temperature range. For the thermostability of protease it was first incubated for 30 min at various temperatures (25-80 ºC). After that, the enzyme was withdrawn, cooled immediately to room temperature and then the residual enzyme activity (%) was measured with standard assay. Each trial for temperature-activity and temperature-stability are realized in triplicates.

Effect of pH on the activity and stability of protease

The pH effect on protease activity is determined by assaying the activity at various pHs using 0.05 M of the buffers: citrate/phosphate (pH 2.6-7.0), Tris-HCl (pH 7.0-9.0) and Glycine/NaOH (pH 8.5-10). The activity at pH optimum was accepted as control (100%) for the calculation of percent activity at each other pHs. The optimal pH is determined from the pH-activity graph. For pH stability, protease was incubated in 0.05 M of above buffers for 2 h at 4ºC and the residual activity (%) with respect to control is assayed. Every trial for pH-activity and pH-stability are carried out in triplicates.

Kinetic constants

The effect of casein concentration on protease activity was studied with the initial concentration of casein (1.0 to 10 mg/mL) in phosphate buffer (0.05 M, pH 7.5). The Michaelis–Menten constant (K_M) and the maximum velocity of reaction (V_{max}) were calculated from Lineweaver–Burk plot which is a plot of 1/V against 1/[S] for systems obeying the Michaelis–Menten equation. Each trial is run in triplicates.

RESULTS AND DISCUSSION

Three-phase partitioning of *Maclura Pomifera* protease

Proteases that are very important enzymes in the world market and industry especially in food industry which are widely used in cheese-making, milk-clotting and meat tenderizing. In comparison to microbial and animal sourced proteases the plant proteases have several superiority and potential in food industry. Therefore, concentrating and purifying of them with more attractive techniques like three-phase partitioning (TPP) is gained importance in recent years [21]. As known, in TPP system the upper organic phase contains non-polar compounds like pigments, lipids, enzyme inhibitors etc. The polar compounds such as saccharides are generally partitioned in the lower phase. The middle phase is composed of precipitated proteins and enzymes [22, 36]. In the present study, the *Maclura pomifera* protease is concentrated and purified with TPP for the first time. The enzyme is dominantly partitioned in the middle phase of the system. In order to improve performance of the TPP system for protease partitioning the efficiacy of several factors (ammonium sulfate saturation, crude enzyme extract to t-butanol ratio, pH and protein amount) are investigated.

Effect of ammonium sulfate concentration

In TPP systems ammonium sulfate which is a phase forming kosmotropic salt is generally used to purify and also concentrate the proteins in to one of the phase.

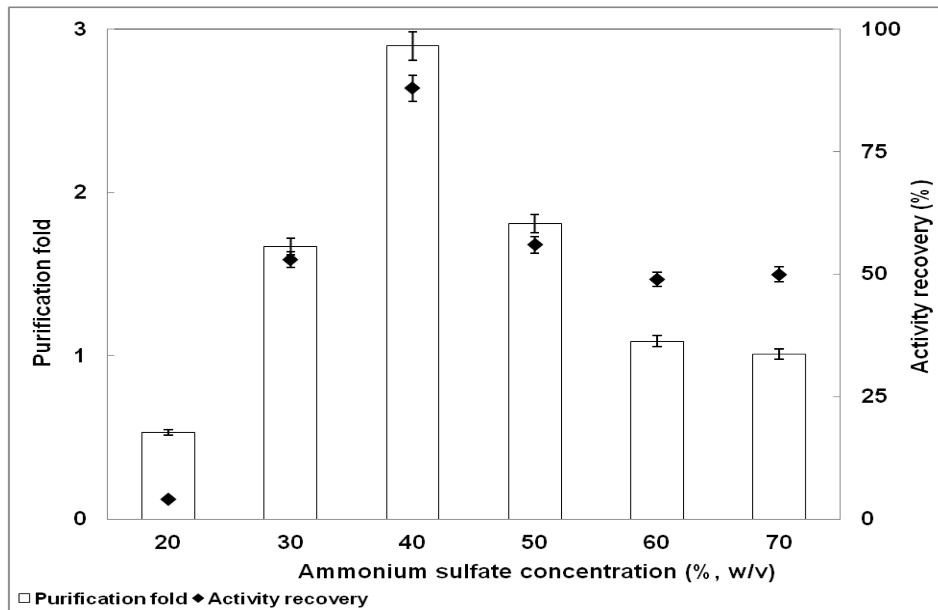

Figure 1. Effect of varying saturations of ammonium sulfate on the degree of purification and activity recovery of *Maclura pomifera* proteases (the crude extract (4 mL containing 5.22 U) was brought to different levels of saturation w.r.t. ammonium sulfate (20%, 30%, 40%, 50%, 60% and 70%) and t-butanol was added in the ratio of 1:1 (v/v) with respect to the volumes of the aqueous extract. The lower aqueous phases and interfacial precipitates were collected separately and analyzed).

It is properly used in protein purification protocols by salting-out effect becauseof its high solubility and ionic strength. Therefore, the ammonium sulfate saturation have an important role in TPP system as it is responsible for protein-protein interaction and precipitation [21, 37, 38]. The ammonium sulfate concentration effect on partitioning of protease is searched and the results are shown in Figure 1. The salt concentration is changed from 20 to 70% (w/v) by keeping crude extract : t-butanol ratio is constant (1.0:1.0, v/v). The purification fold and activity recovery in the middle phase are significantly improved up to 2.86-fold and 88%, respectively when the ammonium sulfate concentration is increased to 40% (w/v). At the lower concentrations of salt the proteins may have solubilized in the lower phase. When the ammonium sulfate concentration increased up to 70% (w/v) because of excessive dehydration proteins might be denaturated and so the purity and yield of the enzyme have a drop [22, 37]. The powerful salting-out effect may make less free water available to dissolve the protease and salt flocculates the enzyme an leads it into the middle phase [21, 27]. There are several studies that show efficiently partitioning of proteases in middle [6, 30, 39] and also in lower phase of the system [12, 40]. Rawdkuen, Vanabun & Benjakul have reported that the highest protease activity yield (163%) was achieved with 50% (w/v) salt saturation (30). A similar salt saturation (60%) is also reported as the best value for recovery of cucumisin from *Cucumis melo* var. reticulans juice [39] and for proteases from *Wrightia tinctoria* R. Br [6]. The researchers have also noticed that an increase in salt saturation generally lead to a decrease in purity. This might be the result of irreversible denaturation of the protein.

Effect of enzyme to t-butanol ratio

t-Butanol which is a non-ionic kosmotrope that is taken to be the best solvent for three-phase partitioning of proteins. It is very soluble and miscible in water and can easily bind to the proteins. Owing to its size and branched structure it does not easily permeate inside folded protein molecules and does not lead denaturation. With its kosmotropic and crowding effects t-butanol increases TPP so the amount of t-butanol have to be optimized to get high yields [21, 22, 36]. So, the effect of the t-butanol amount on protease partitioning is

searched. The enzyme: t-butanol ratio is changed (1.0: 0.25, 1.0: 0.5, 1.0: 0.75, 1.0: 1.0, 1.0: 1.5 and 1.0: 2.0 (v/v)) by maintaining the salt concentration of 40% (w/v) and the outcomes are illustrated in Figure 2.

As shown in figure, highest extraction yield (88%) was obtained by using 1.0: 1.0 (v/v) ratio. If the ratio is lower than 1.0: 1.0 (v/v) t-butanol may not adequately synergize with the salt so the activity recovery is very low. The yield of extraction was decreased with the rise in this ratio. When the t-butanol ratio rises the viscosity of solution also increases which cause a reduce in molecular mobility that obstruct the interaction of biomolecules and also may cause protein denaturation [21, 22]. A previous study for the three-phase partitioning of zingibain is in agreement with our results that 1.0: 1.0 (v/v) ratio is sufficient to get best partitioning results (40). The ratios of 1.0:0.5 (v/v) [12, 30] and 1.0:1.25 (v/v) [39] were also selected by the researchers since they gave the highest recoveries.

Effect of pH

pH is an other significant factor that affect to the dispersion and partitioning of proteins. The main reason for the ionization of amino acid residues situated at the surface of protein is pH changes. Because of the electrostatic interactions among the charged protein and phases the protein partitioning is also influenced with pH [36]. A pH range between 3.0 and 8.0 is selected to search the impact of pH on the purification of *M. pomifera* protease and the results are shown in Figure. 3. The protease was partitioned to the interphase with giving 2.81 fold purification an 80% activity recovery of the enzyme at pH 6.0. When the pH of the medium increased from 3.0 to 6.0 the activity recovery (%) of protease gradually rised in the middle phase and then declined by increasing the pH. At pH 6.0, TPP of protease selectively exclude protease from aqueous phase to the middle phase. A significant decrement in enzymatic activity is observed especially at acidic pHs. The partitioning of an enzyme to the interphase or the lower phase relies on its isoelectric point (pI). If the system pH is below its pI the proteins are positively charged and may be partitioned in the middle phase. However above the pI, the protein carry negative charge and may be more soluble and left in the aqueous phase [21, 22].

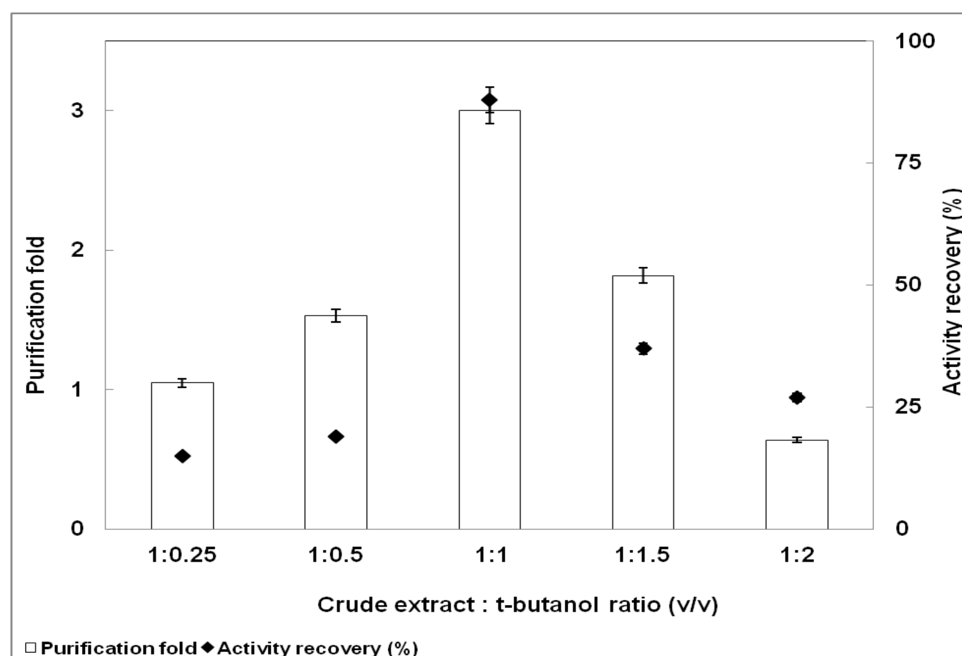

Figure 2. Optimization of crude extract to t-butanol ratio for the recovery of *Maclura pomifera* proteases (various amounts of t-butanol was added to crude extract (4 mL containing 5.22 U and saturated with 40% ammonium sulfate) in the following volumetric ratios viz. 1:0.25, 1:0.5, 1:0.75, 1:1, 1:1.5 and 1:2. The lower aqueous phases and interfacial precipitates were collected separately and analyzed).

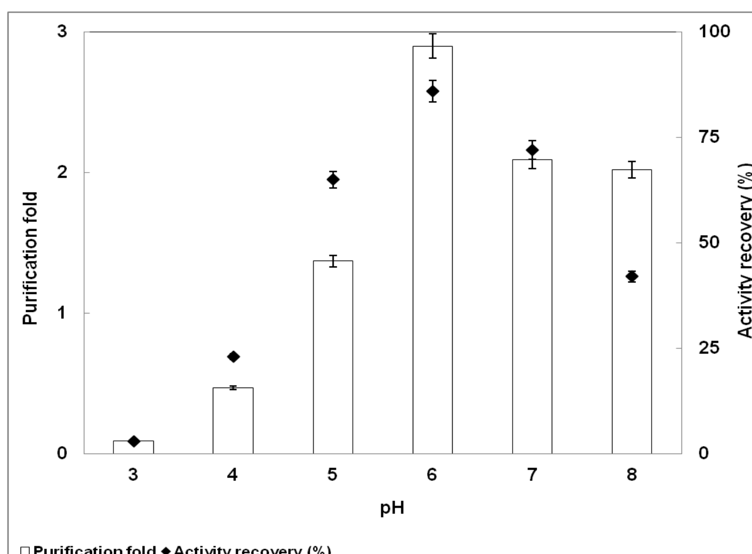

Figure 3. Influence of pH on the degree of purification and activity recovery of *Maclura pomifera* proteases (the ammonium sulfate (40%, w/v) was added to the crude extract of protease (4 mL containing 5.22 U). The pH of the medium was adjusted to different pH values. This was followed by addition of t-butanol in a ratio of 1:1 (crude extract to t-butanol). The lower aqueous phases and interfacial precipitates were collected separately and analyzed).

It is desirable to adjust the pH of the TPP system lesser than the pI of the protein to achieve maximum precipitation [36]. The similar observations are also noticed by Gagaoua et al., for three-phase partitioning of cucumisin. The cucumisin was selectively partitioned into the interphase at pH 8.0 with increased yield [39]. The best activity recovery and purification fold values for *M. pomifera* protease were provided at pH 6.0 and therefore the following studies were realized at this pH.

Effect of protein amount

To optimize the loaded protein amount during TPP is very essential that affect to the partitioning behavior and purification of protein [20]. Hence, the effect of loaded protein amount (0.1 mg to 1.5 mg) on partitioning of protease by keeping previously mentioned experimental conditions constant is studied (Figure 4).

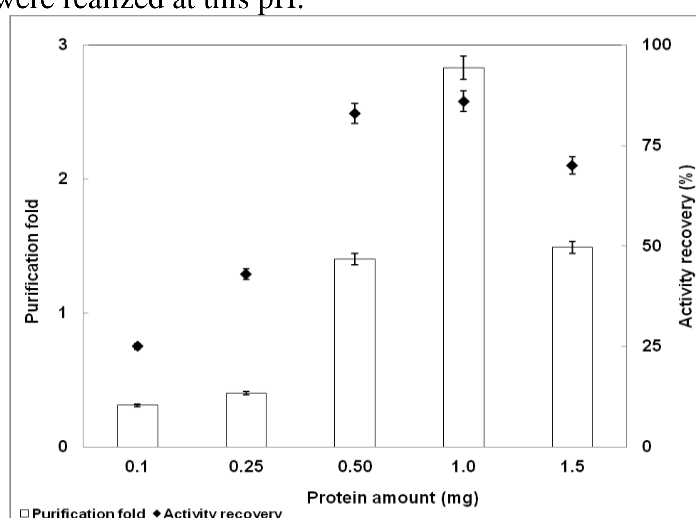

Figure 4. The effect of protein amount on the degree of purification and activity recovery of *Maclura pomifera* proteases (the crude extract containing 0.1, 0.25, 0.5, 1 and 1.5 mg protein was saturated with 40% (w/v) ammonium sulfate, t-butanol (1:1, v/v) was added, after the pH of the medium was adjusted to pH 6.0. The lower aqueous phases and interfacial precipitates were collected separately and analyzed).

Table 1. Overall purification of proteases from *Maclura pomifera* by three-phase partitioning.[a]

Step	Total activity (Unit)	Total protein (mg)	Specific activity (Unit/mg)	Purification fold	Activity yield (%)
Crude extract (85%, w/v, ammonium sulfate fraction)	2.37	1.00	2.37	1.00	100
TPP-middle phase	2.04	0.302	6.76	2.85	86
TPP-bottom phase	0.308	0.534	0.58	0.25	13

[a] The ammonium sulfate (40%, w/v) was added to the enzyme extract of *Maclura pomifera* protease (4 mL containing 5.22 U) , after then pH was adjusted to pH 6. Afterwards, t-butanol was added to the enzyme extract to the ratio of 1:1 (v/v) (crude extract: t-butanol). Three phases were spied on clearly. The upper phase was decanted and then the lower aqueous phase and interfacial precipitate were tested for enzyme activity and protein amount. Each experiment was carried out in triplicate and the difference in the readings was less than ±5%.

As shown in figure, the highest activity recovery and fold purification are obtained with 1.0 mg of protein. The activity recovery is decreased with the the increase in protein amount from 1.0 mg to 1.5 mg. This data might be based on the fact that an increment in protein quantity causes a decrease in distribution and partitioning capacity of the TPP system [20]. Thus the protein amount should be maintained.

The overall purification of M. pomifera protease

The overall purification of *M. pomifera* protease is given in Table 1. The optimum process parameters are found as 40% (w/v) ammonium sulfate saturation, 1.0:1.0 (v/v) protease : t-butanol, pH 6.0 and 1 mg of protein. The protease is concentrated and partitioned with the highest activity recovery and purification fold of 86% and 2.85, respectively. The results are also showed that under this optimized conditions protease has propencity to participitate and concentrate in the middle layer of the system. There are several studies that related to the purification of many proteases from several sources with TPP. Under optimized conditions these proteases were extracted, concentrated and purified by using TPP with various activity recovery and purification fold degrees like; zingibain from *Z. officinale* rhizomes at the aqueous bottom phase [40] with 215% recovery and 14.91-fold, cucumisin from *C. melo* juice in the interphase [39] with 156% activity recovery and 4.61-fold, a protease from the viscera of farmed giant catfish with 163% yield and 5-fold and from *W. tinctoria* with 9.24% recovery and 2.34-fold [6] in the middle phase and also a protease from papaya peels with 253.5% recovery and 15.8-fold in the lower phase [12] of TPP with the activity yield and purification fold of TPP. Some of these proteases also show milk-clotting activity beside of their caseinolytic activities [6, 38, 39] The results showed that TPP is an effective and useful method to obtain concentrated *M. pomifera* protease especially as a milk-clotting enzyme for cheese-making. TPP partitioned protease was stored for 3 months at 4°C and -20°C without a stabilizer to investigate the storage stability of the enzyme. The protease is maintained 86% and 96% of its original activity at the end of this period at 4 ^{o}C and -20°C, respectively.

Biochemical characteristics of *M. pomifera* protease

SDS-PAGE

SDS-PAGE analysis is performed (Figure 5) to confirm the purity and analyze the molecular mass of *M.pomifera* protease partitioned in the TPP system. The profile exhibited three main clear band with the molecular mass of 17.8, 26.4 and 67.8 kDa. According to Mahajon & Badguar (2010) the molecular mass of proteases was changing between 20-135 kDa according to their type and source [4]. The plant serin proteases have shown molecular mass values ranged from 20 to 35 kDa [4, 10, 11, 19]. The aspartic proteases sourced from plants have a broad range of 28-65 kDa [4, 7].

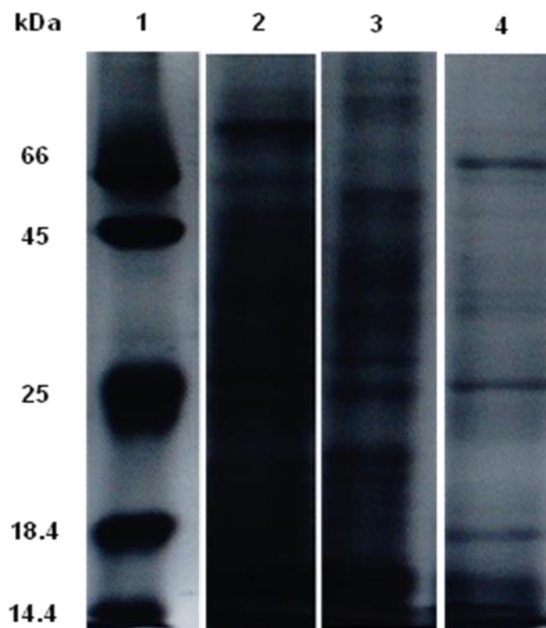

Figure 5. SDS–PAGE analysis of *Maclura pomifera* proteases (lane 1; molecular weight markers (14.4–66 kDa) (20 µg), lane 2; *Maclura pomifera* extract (20 µg), lane 3; TPP-bottom phase (20 µg) lane 4; TPP purified *Maclura pomifera* proteases (middle phase) (25 µg).

The molecular mass of a cysteine protease from *F. johannis* is found as 25 kDa [10]. The two protease (14 and 27 kDa) were also detected for the *Cynanchum otophyllum* protease extract [41]. The SDS-PAGE electroforegram of an aspartic protease from *W. coagulans* is showed a protein band as 31 kDa [7]. Rudenskaya et al. (1995) have found the presence of two protease in the fruits of *M. pomifera* as serin protease and cysteine protease with the molecular weight of 65 and 21 kDa, respectively. The results indicated that the serin protease was monomeric and also a glycoprotein [42]. The molecular weight of serine proteases are generally varying between 60-80 kDa [39]. The zingibain is also highly purified and gaved only a band with molecular mass of 33.8 kDa [40]. The results obtained from SDS-PAGE analysis for *M. pomifera* proteases are well agree with these plant proteases. The molecular mass of the protease is also in the range of these declared plant sourced proteases.

Effect of temperature on activity and stability of protease

Temperature is a parameter that affect to activity and stability of enzymes and also determine the effective and efficient use of enzyme. Temperature-activity and temperature-stability profiles are established between the range of 25-80 ºC using casein as substrate and summarized in Figure 6 and 7, respectively. The outcomes showed that the protease have a maximum activity at 65 ºC (Figure 6) and was highly active and stable at temperatures 25 to 65 ºC with retaining more than 50% of its activity (Figure 7). Above 65 ºC, a rapid enzyme inactivation was observed. This thermostability of the protease is very important criteria that determine the potential use of the enzyme in industrial processes [43]. This evidences are comparable to those of well known proteases. A similar optimal activity and thermostability results were exhibited by several proteases. The optimum temperature and thermostability range were found for zingibain as 60 ºC and 40 ºC-80 ºC [39], respectively. The optimal temperature for an aspartic protease from *W.coagulans* [7], a cysteine protease from *F. johannis*, a metaloprotease from *T. cylpeatus* were found as 65 ºC, 60 ºC and 45 ºC, respectively. Several plant proteases are glycosylated that this structure may increase the thermostability of them [44].

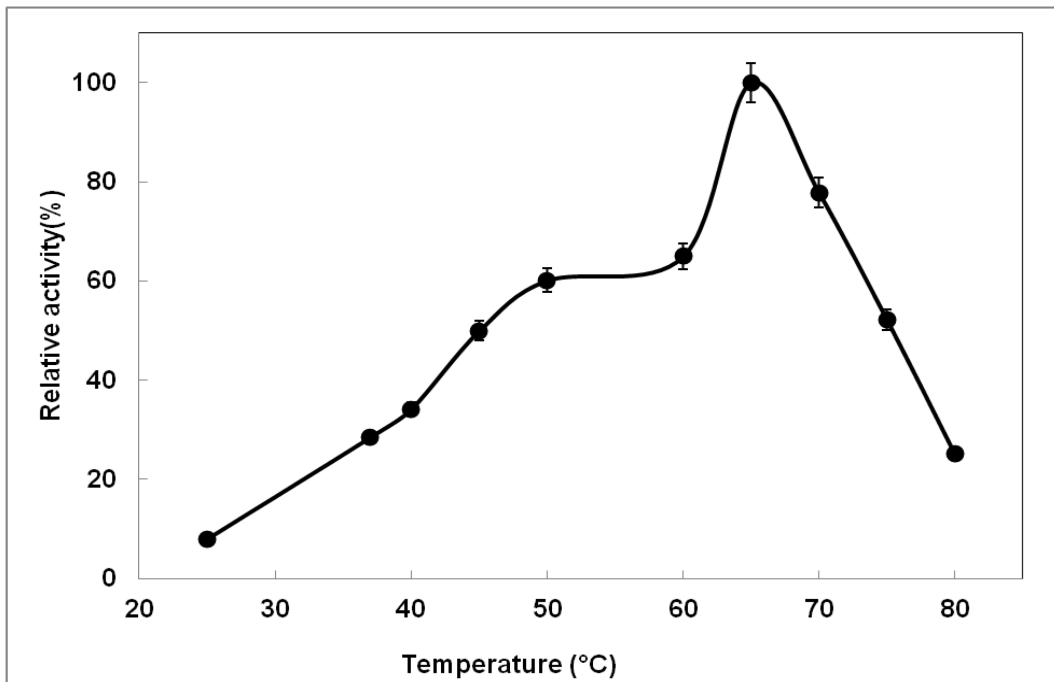

Figure 6. Effect of temperature on the activity of *Maclura pomifera* proteases.

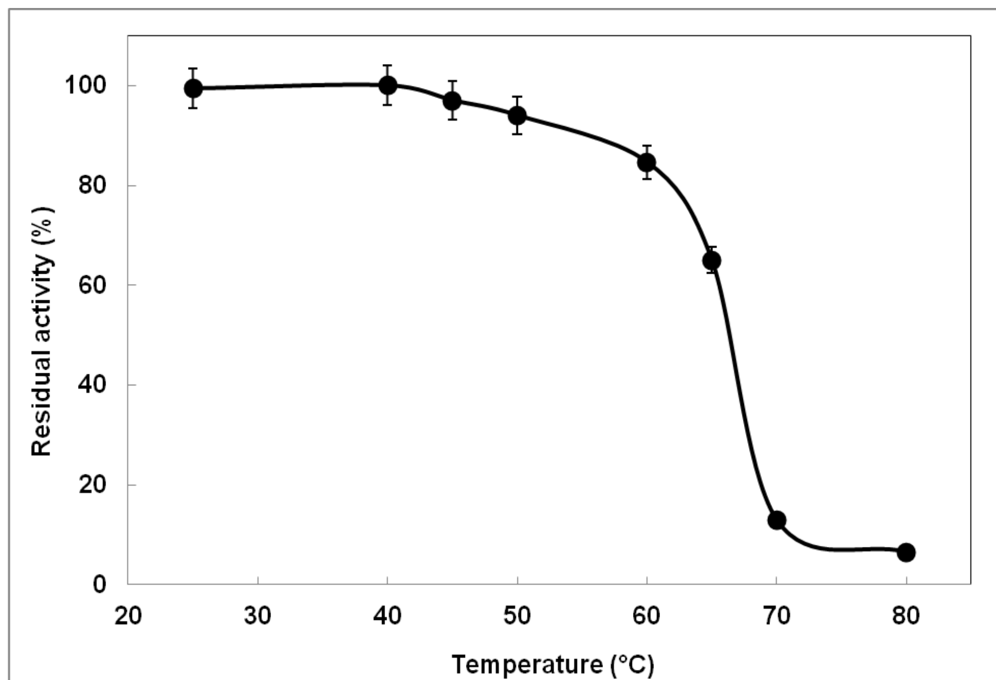

Figure 7. Thermal stability of *Maclura pomifera* proteases.

Effect of pH on activity and stability of protease

The pH effect on proteolytic activity of TPP partitioned protease is investigated at different pHs (3.0-10.0) and the optimal activity is obtained at pH 6.5 (Figure 8). The pH stability of the protease is demonstrated in Figure 9. As is seen from the figure 8 that the *M. pomifera* protease is much effective in neutral and alkaline pHs in comparison to the acidic pHs. This result is very important for milk coagulation which occur at pH 6.3-6.6 [45].

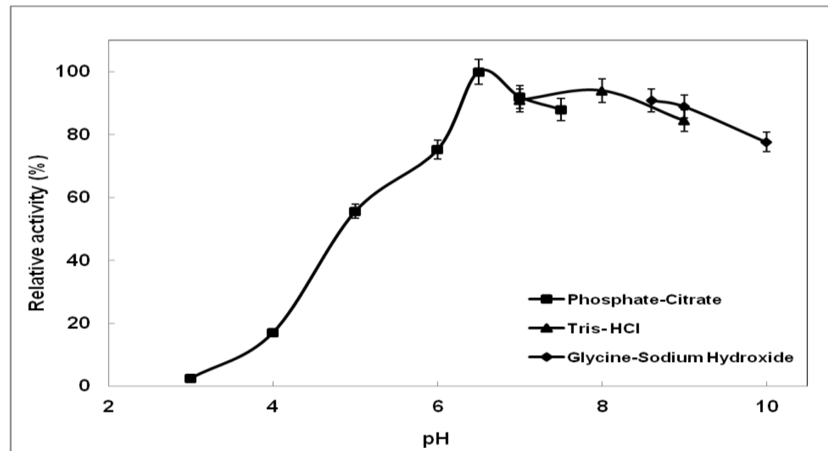

Figure 8. Effect of pH on the activity *Maclura pomifera* proteases.

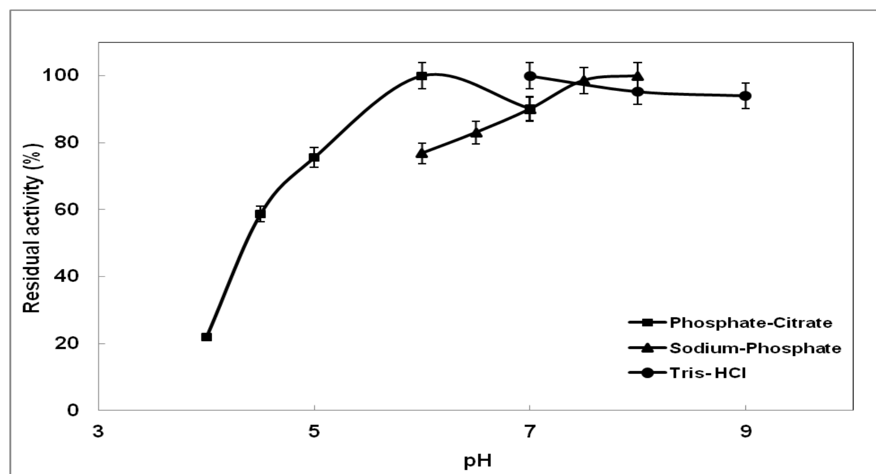

Figure 9. pH stability of *Maclura pomifera* proteases.

The pH-stability profile revealed that the protease showed a good activity in pH 5.0-9.0 range with retaining more than 80% of its activity. The results showed that the protease is also more stable in neutral and alkali range which is in agreement with previous reports. The pH stability of an enzyme is releated to its net charge at a particular pH. A capability to study in a wide pH range is significant criterion to determine the usage of an enzyme in various industrial processes. Various pH-activity and pH stability profiles for many proteases were reported by other researchers. The optimum pH and pH stability range were found for zingibain as pH 7.0 and pH 5.5.-8.0 [40], for a cysteine protease as pH 6.5 and pH 4.5.-9.5 [10], for fennel protease as pH 6.4 and pH 7.6 and pH 6.0-7.5 [8] for cucumisin as pH 9.0 and pH 7.0-11 [9], for a milk-clotting protease from *W. tinctoria* as pH 7.5 and pH 5.5-8.5 [6], for an alkaline protease as pH 7.0 and pH 6.0-11.0 [17], for a serine alkaline protease as pH 9.0 and pH 3-13 [46], for an aspartic protease pH 5.5 and pH 4.5-6.0 [7], for a metalloprotease as pH 5.0 and pH 4.0-6.0 [18].

Effect of casein concentration on protease activity

The *M. pomifera* protease exhibited a Michaelis- Menten type of kinetics with casein. K_M and V_{max} are determined as 0.87 mg/mL and 8.82 U, respectively from the L. Burk plot. The kinetic constants of an enzyme may vary depending on its source and used substrate. The K_M values for an alkaline serine protease [2], a cysteine protease [10], the cucumisin [39] and an aspartic protease [7] by using casein found as 2.5, 0.604, 2.24, 1.29 mgmL^{-1}, respectively. The estimated low K_M value indicated that the purified protease have an affinity toward the casein when compared with several plant proteases.

Milk-clotting activity of M. pomifera protease

In order to show the potential application of *M. pomifera* protease in food industry the milk-clotting activity (MCA) was searched in terms of pH (Table 2).

As is seen from the table, the milk-clotting times increased by increasing pH 6.0 to 7.0 whereas the MCA was decreased. The best clotting time with giving the best MCA was found at pH 6.0 as 420 seconds and 2.28 U/mL, respectively. The pH below 6.0 and above 7.0 the extract had the low clotting activities.This may be attributed to the interference from casein aggregation or irreversible changes in the conformation of casein [41]. There are several MCA results of different plant proteases which are related to used conditions and methods. The proteases sourced from *C. otophyllum Schneid* [41], *Citrus aurantium* [3], *Lagenaria siceraria seeds* [47] *Pichia pastoris* [19], kiwi fruit [11], cucumisin melo var. [39], *Moringa oleifera* flowers [5], zingibain [40], *Withania coagulans* fruit [7], fennel [8], *Ficus johannis* [10] etc.

Phenolic and flavonoid content of Maclura pomifera protease

TPP was mainly used to concentrate and purify the *M. pomifera* proteases. However, by using this system partitioning of phenolics and flavonoids were also achieved. The phenolic and flavonoid content of the crude enzyme, top phase and middle phase of TPP system are displayed in Table 3.

As indicated in the above table, they both were predominantly partitioned in the top phase of the system. There are various reports that include the isolation of phenolics from plants with conventional techniques such as solvent extraction, steam distillation and supercritical fluid extraction [13, 34, 48-50]. *M. pomifera* fruit is a plenty source for phenolic compounds especially in terms of isoflavones with antimicrobial and antioxidant activity [13, 51]. Generally plant phenolics and flavonoids have antioxidant activity and also contribute the quality and nutritional value in terms of modifying taste, aroma, colour and flavor besides supplying useful health effects [50].

Table 2. Milk-clotting activity of *Maclura pomifera* protease.

pH	Clotting-time (second)	MCA (U/mL)
6.0	420	2.28
6.5	480	2.0
7.0	540	1.78

Table 3. Phenolic and flavanoid content of TPP partitioned protease.

	Crude extract (mg)	Top phase (mg)	Yield (top phase) (%)	Middle phase (mg)	Yield (middle phase) (%)
Phenolics	0.63	0.57	91	0.06	10
Flavonoids	0.801	0.52	65	0.28	35

CONCLUSION

In dairy industry for cheese making, both caseinolytic activity and milk-clotting activity is required. Therefore, in current study a milk-clotting protease from *Maclura pomifera* was concentrated and purified for the first time with three-phase partitioning (TPP) and characterized. The enzyme was efficiently partitioned in the middle phase of TPP. As a non-chromatographic method TPP is simple, cost-effective and quick procedure that gave good recovery and purification fold. The TPP fractionated *M. pomifera* protease have a potential to coagulate the milk in cheese-making. However there is a need further biochemical and sensory studies to show its milk-clotting potential, suitability and acceptability in food industry.

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