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To cite this version:

Mohammed Gagaoua, Nawel Boucherba, Amel Bouanane-Darenfed, Ferhat Ziane, Sabrina Nait-Rabah, et al.. Three-phase partitioning as an efficient method for the purification and recovery of ficin from Mediterranean fig (Ficus carica L.) latex. Separation and Purification Technology, 2014, 132, pp.461-467. 10.1016/j.seppur.2014.05.050 . hal-04156547

HAL Id: hal-04156547
https://hal.inrae.fr/hal-04156547

Submitted on 21 Sep 2023

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Three-phase partitioning as an efficient method for the Purification and Recovery of Ficin from Mediterranean fig (*Ficus carica* L.) latex

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Abstract

Three phase partitioning or TPP consists of proteins' precipitation due to simultaneous presence of ammonium sulfate and *t-butanol*. The technique has been successfully used for the partial purification of ficin (EC 3.4.22.3) from *Ficus carica* latex. In this study, ficin of Mediterranean *Ficus carica* was for the first time partially purified and recovered in a single step using TPP. Optimal purification parameters of the system were 40% ammonium sulfate saturation (w/v) with 1.0:0.75 (v/v) ratio of crude extract:*t-butanol* at pH 7.0, which gave 6.04 purification fold with 167% recovery of ficin. The enzyme was found to be exclusively partitioned in the interfacial precipitate phase. The Tricine SDS–polyacrylamide gel electrophoresis analysis of the enzyme showed comparatively purification and protein molecular weight of the enzyme was nearly found to be 23.4 kDa. Characterization of enzyme showed that optimal pH of purified enzyme was 6.5 and optimal temperature was 60°C. The enzyme was found to be stable at 40 – 70°C for 1 hour. This study showed that TPP is a simple, economical and quick method for ficin recovering from *Ficus carica* latex trees and can be performed in a purification process to be used successfully in food industries.

Keywords: Fig (*Ficus carica* L.) latex; Ficin; Recovery; Purification; Three phase partitioning (TPP).

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1. Introduction

Numerous purification protocols have been described in the last decades but none of them can be generally applied. Most of these separation techniques are expensive, time consuming, involving number of steps and furthermore the scale up of these methods is difficult. Considering these problems, there is a prominent need to perform simple, more efficient and economical purification strategies for separation and purification of target proteins, especially enzymes. Three-phase partitioning (TPP), a technique first described by Lovrien's group [1], was intensively used to purify several target proteins [2-6].

TPP is a relatively novel method, which uses tertiary butanol (t-BuOH) and ammonium sulfate to form a two-phase system in which macromolecules precipitate at the interface, hence forming the third phase. TPP involves several techniques such as salting out, isoionic precipitation and co-solvent precipitation of proteins. It is easily scalable and can be used directly with crude suspensions [7]. 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 the proteins but also concentrate them into one of the phases [4, 8, 9]. The present study describe, for the first time, the purification and recovery of ficin (also spelled as ficain, EC 3.4.22.3) from a crude extract of Mediterranean latex fig trees (*Ficus carica*) using the TPP system by optimizing some parameters such as concentration of *t*-butanol, pH and ammonium sulfate ($(NH_4)_2SO_4$) amount. *Ficus carica*, a deciduous tree belonging to the Moraceae family, is one of the earliest cultivated fruit trees and an important crop worldwide for both dry and fresh consumption. The latex of this plant constitutes an important source of many proteolytic components known under the general term of ficin, which belongs to the cysteine proteases of the papain family. They can be used in food industry as meat tenderizers [10, 11] and in cheese production [12]. They are also used, in their purified forms, as proteolytic agents in many laboratory procedures [13].

2. Materials and Methods

2.1. Materials

Tert-Butanol, ammonium sulfate, Coomassie Brilliant Blue R-250, acrylamide, bisacrylamide, sodium dodecyl sulfate (SDS), dithiothreitol (DTT), 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). Bradford reagent and molecular markers for electrophoresis were purchased from Bio-Rad

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Laboratories (Hercules, CA, USA). Trichloroacetic acid (TCA), hydrochloric acid and tris-(hydroxymethyl)-aminomethane were from Merck (Germany). 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. Collection of latex

Fresh fig latex was collected by breaking the immature green fruits of *Ficus carica* L. tree growing in Kabylia, a Mediterranean region (Beni-Djellil, Bejaia), in a clean tube containing 0.05% NaN_3. All the latex samples used in this study were collected early in the morning at the end of August. The latex fluid was transported to the laboratory in adequate conditions and immediately stored at – 20°C until use.

2.2.2. Preparation of Crude Extract

The frozen latex was thawed at 4°C and diluted with distilled water (1.0:0.5), mixed well and then subjected to centrifugation at 5000 rpm for 15 min at 4ºC to remove gum and other debris. The insoluble material was discarded and the supernatant was filtered through Whatman paper No. 1. The clarified juice referred to as "crude extract" was dialyzed overnight (membrane molecular weight cut off: 14 kDa) against 50 mM, pH 7.0 phosphate buffer, at 4 °C. After dialysis, the crude extract sample was subjected to TPP purification system. A flow sheet of the various steps involved in the purification is shown in Fig. 1.

2.2.3. Three Phase Partitioning of Ficin

TPP experiments were carried out as described by Dennison and Lovrien [7] with slight modifications. First, the crude extract of *Ficus carica* latex (2ml containing 21.34 units and 15.78 mg protein) was saturated at 25°C with 30 % ammonium sulfate. The mixture was vortexed gently and then allowed to stand for 45 min at 25°C. Afterward the mixture was centrifuged at 3000 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 phosphate buffer.

The effect of salt concentrations (20, 30, 40, 50, 60, and 70 %) (w/v) on the crude enzyme extract for the TPP at the constant crude extract:*t-butanol* ratio (1.0:1.0) was investigated.

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After that, various t-butanol ratios (crude extract: *t-butanol*; 1.0:0.25, 1.0:0.5, 1.0:0.75, 1.0:1.0, 1.0:1.25, 1.0:1.5, 1.0:1.75, 1.0:2.0) were employed with a constant ammonium sulfate saturation at 40% at 25°C.

After, the t-butanol and ammonium sulphate, effects with different pH values of medium study were studied. Crude extract was saturated with 40% ammonium sulfate and pH was adjusted to 3, 4, 5, 6, 7, 8, 9 with the 0.5 M HCl or 0.5 M NaOH, then 1.0:0.75 *t-butanol* was added and the best pH value on the partitioning behaviour of ficin was investigated.

After optimisation of the parameters (40% ammonium sulfate, 1.0:0.75 ratio crude extract to *t-butanol* and pH 7.0), the interfacial precipitate phase always containing the higher ficin activity was collected, dissolved in 50 mM, pH 7.0 phosphate buffer, dialyzed overnight against the same buffer and concentrated by ultrafiltration using Amicon Ultra device with a 10 kDa molecular weight cut-off. The concentrated enzyme was stored at –20°C until use for further characterization studies in order to determine the general biochemical properties of the enzyme.

2.2.4. Protein Determination

The protein concentration was quantified by the dye binding method of Bradford [14] using the Bio-Rad Protein Assay (Bio-Rad). Bovine serum albumin (BSA) at a concentration of 1 mg ml^{-1} was used as standard.

2.2.5. Enzymatic activity measurements

Ficin activity was assayed at 37°C using bovine casein (1% w/v) as substrate in a 100 mM potassium phosphate buffer pH 7.0 containing 250 mM EDTA and 250 mM L-cysteine at enzyme concentrations of 0.25 mg/ml. The hydrolyzing activity of ficin was based on the method described by Kunitz with some modifications using denatured casein as a substrate [15]. In activity measurements, 0.12 ml enzyme was incubated at 37 ± 1°C for 20 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 0.001 min^{-1} under given assay conditions.

2.2.6. Effect of temperature on the activity and stability of partitioned ficin

The temperature profile of ficin was determined by performing the standard assay procedure at different temperatures (20, 30, 40, 50, 60, 70, 80, and 90°C). The relative activities as percentages

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were expressed as the ratio of ficin 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 phosphate buffer for 1h with continuous shaking. After desired incubation periods, enzyme aliquots were withdrawn and assayed at optimal assay conditions to determine the residual enzyme activity.

2.2.7. Effect of pH on Enzyme Activity

The effect of pH on the enzyme activity of ficin was evaluated from pH 3.0 to 9.0 as described by Devaraj *et al.* [16] using the following buffers: 50 mM citrate buffer (pH 3.0–6.0), 50 mM potassium phosphate buffer (pH 6.5–7.5), and 50 mM Tris-HCl buffer (pH 8.0–9.0). Activity of the ficin was determined as mentioned above.

2.2.8. Tricine SDS-Poly Acrylamide Gel Electrophoresis

Tricine SDS-PAGE with slight modifications was carried out according to Haider *et al.* [17] using 10% separating and 4% stacking gels. The samples were mixed at a ratio of 1:1 with the buffer sample containing 100mM 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. Fifteen micrograms of proteins were loaded and then subjected to separate in 25mM Tris, 25mM Tricine and 0.05% (w/v) SDS migration buffer for 4 h at 120V. After separation, the gel was stained overnight with staining solution (4.9 mM Coomassie Brilliant Blue R-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 molecular weight markers obtained from Bio-Rad (No. 161-0304), contained: Lysozyme (14.400), Soybean trypsin inhibitor (21.500), carbonic anhydrase (31.000), ovalbumin (45.000), bovine serum albumin (66.200) and Phosphorylase b (97.400) was used. The molecular mass of the protein bands was calculated using the Un-Scan-It gel 6.5 analysis program (Silk Scientific, Orem, UT).

3. Results and Discussions

Proteases from plant sources have received special attention from the pharmaceutical industry and by food biotechnology due to their properties of activity over a wide range of temperature and pHs. Proteases from plant latex were widely used to reduce meat toughness [10]. These proteases include papain from *Carica papaya* [18], ficin from *Ficus carica* [19] and many others (for review see [11]). They are also used, in their purified forms, as proteolytic agents in many laboratory procedures. For

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example, two partially purified ficin forms were observed to activate human coagulation factor X [13]. When compared to other cysteine proteases like papain and bromelain, ficin was shown to specifically cleave mouse IgG1, giving better yields and immunoreactivity [20]. Considering their potential uses in industry, it becomes desirable the developing of simple and efficient methods for the recovery and purification of these enzymes.

3.1. Three-phase partitioning of Ficin from *Ficus carica* latex

The latex of *Ficus carica* L. constitutes an important source of many proteolytic components known under the general term of ficin or recently ficain (EC 3.4.22.3). This enzyme is similar to papain in structure and it contains sulfhydryl group as the main involving part for the proteolytic activity [21, 22]. To this date, most of the work has been carried out on the enzyme prepared from the latex of *Ficus glabrata* (the commercially available dried latex) and a very few studies have been done on the ficin isolated from *Ficus carica* latex. This study aimed the purification of ficin using a rapid and efficient method, given that all known methods are expensive and time-consuming. In our study, the enzyme was purified for the first time to homogeneity from immature green fruits of the Mediterranean *Ficus carica* fig using three phase partitioning system. TPP is a simple, economical and large-scale applicable but an elegant non chromatographic process used for purification and concentration of proteins. Separation of the enzyme from complex media during the partitioning was operated by multiple effects, such as salt concentration, solvent ratio and pH [7]. Therefore, the effect of various parameters on TPP was investigated. For this purpose, different partitioning experiments at various saturations of ammonium sulfate $(NH_4)_2SO_4$ (20%, 30%, 40%, 50%, 60% and 70%, w/v), crude extract to *t-butanol* ratios (1.0:0.25, 1.0:0.5, 1.0:0.75, 1.0:1.0, 1.0:1.25, 1.0:1.5, 1.0:1.75 and 1.0:2.0, v/v) and pHs (3.0, 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0) were performed. The starting protein concentration (containing 10.65 units/ml of ficin activity) was 7.89 mg/ml. The effects of these parameters on the degree of purification and on the activity recovery of ficin from the crude extract are presented in Figs. 2-4, respectively. As presented in the figures, all these process parameters are very important and effective on the partitioning of the ficin from *Ficus carica* fig latex.

The efficiency of the salting out of proteins will first depend on $(NH_4)_2SO_4$ and second on the net charge of the proteins. Ammonium sulfate saturation is of a critical importance and must be optimized so that adequate quantities of ficin are extracted with minimal interference from contaminating material coming from complex media. Generally, one starts with a minimum salt concentration of 20% (w/v) and optimizes this so as to obtain the maximum amount of the desired

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protein in the interfacial precipitate [7]. The influence of $(NH_4)_2SO_4$ saturation was studied by maintaining the ratio of crude extract to *t-butanol* ratio constant (1.0:1.0) and varying the saturation of $(NH_4)_2SO_4$ from 20% to 70% (w/v) (Fig. 2). As shown in Fig. 2, the maximum fold purification of 6.4-fold along with 158% recovery of ficin activity in the interfacial precipitate was obtained with 40% $(NH_4)_2SO_4$ saturation. This salt saturation was sufficient enough to concentrate the ficin in this phase, a finding in agreement to its known property as a soluble protein against ammonium sulfate [23]. Sometimes TPP leads to simultaneous activation of the enzyme and this may be the result of increased flexibility in the enzyme molecule [6]. A remarkable increase in the catalytic activity and yield of pectinase [24] and invertase [2, 25] obtained from TPP has been reported. With an increase in concentration of ammonium sulfate, the degree of purification (purification fold) decreased significantly above 40% (w/v), while the activity recovery initially increased from 61% at 20% $(NH_4)_2SO_4$ to 158% at 40% $(NH_4)_2SO_4$ and then decreased to 38% at 70% $(NH_4)_2SO_4$ (Fig. 2). Sulfate ion and *t-butanol* are known to be excellent protein structure markers (kosmotropes) [7]. The principle of sulfate ion for salting out protein has been viewed in five different ways namely, i) ionic strength effects, ii) kosmotropy, iii) cavity surface tension enhancement osmotic stressor (dehydration), iv) exclusion crowding agent, and v) the binding of SO_4^{2-} to cationic sites of protein [6]. At concentrations of $(NH_4)_2SO_4$ greater than 40%, the activity yield decreased significantly due to the precipitation of protein and may possibly cause its irreversible denaturation. Higher concentrations of $(NH_4)_2SO_4$ (>40%) causes a reduction in the selectivity of extraction and also degree of purification. Therefore, ammonium sulfate saturation was used as 40% (w/v) for further partitioning experiments.

After the selection of $(NH_4)_2SO_4$ saturation, the ratio of the volume of crude extract to *t-butanol* which is also very important in TPP was optimized. *t-Butanol* was chosen as the organic co-solvent for partitioning of ficin in TPP as it has been generally reported to give best results [1, 3, 4, 7, 9]. Due to its size and branched structure, *t-butanol* does not easily permeate inside the folded protein molecules and hence does not cause denaturation. In order to determine the best ratio, the ammonium sulfate saturation was fixed to 40% (v/v) and the crude extract:*t-butanol* ratio was varied from 1.0:0.25 to 1.0:2.0 (v/v). The highest ficin recovery (172%) and purification fold (6.7) were obtained from the interfacial precipitate phase of the TPP system when the crude extract to *t-butanol* ratio was 1.0:0.75 at 40% $(NH_4)_2SO_4$ saturation (Fig. 3). From these findings, the ratio of crude extract to *t-butanol* of 1.0:0.75 was selected for investigating the effect of pH value on the TPP system. This optimum ratio presumably arises as a result of two factors. If the amount of *t-butanol* is lower, it does not adequately synergize with ammonium sulfate. If it is higher, it is likely to cause protein

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denaturation [3, 6]. In this study, we have sometimes met some difficulties in the reproducibility of the enzyme recovery using the same experimental conditions. Numerous explications can be provided. The first one concern the exclusive partitioning of our enzyme in the interfacial phase where sometimes we assist to a little loss of the protein in the bottom phase at the recuperation step, hence a little loss of the specific activity. The second one, the differences in the enzyme activities can be due to the fantastic characteristic of the TPP system by its enhancing activity. This curious feature of this elegant non chromatographic process believed to be unexpected in protein isolations, was reported to occur in numerous studies ([26, 27] as discussed above. Also, probably conformation changes occurred in the mixtures but with little differences in the rate and extent of the partitioning process in spite of the rigorous applied conditions. This point of view needs more investigations for better knowledge. Nevertheless, this phenomenon was already reported in previous studies. For example, when pure Proteinase K was subjected to TPP and its structure analyzed by X-ray diffraction at 1.5 A° resolution [26], a higher overall temperature factor was observed, as a result of which side chains of several amino acid residues in the binding site were found to adopt more than one conformation. This led us to stress forward that little differences in the excited state of the ficin at the moment of the separation were occurred. Another explication is probably the ficin autolysis/activity enhancing fold. The enhancing activity of the TPP system is the most favored in our case to prevent the enzyme digestion. Otherwise, the ability of TPP to refold proteins suggests that the higher specific activity often observed after TPP using the same conditions (for example see [2]) may be partly due to renaturation of partially denatured ficins.

The pH of the reaction mixture was also reported to be a very important parameter in TPP [4, 6, 28]. The efficiency of the salting out of proteins will depend on the net charge of the proteins present in the medium which is highly pH dependent. Also, distribution and partitioning of macromolecules in TPP systems change with pH due to electrostatic interactions between phases and charged proteins. Hence, the effect of pH on the partitioning of ficin was investigated. The ficin crude extract was saturated with 40% (w/v) of $(NH_4)_2SO_4$, and then the pH was adjusted. *t-Butanol* was added to this mixture in the ratio of 1.0:0.75 (v/v). After 45 min, the three phases were formed and collected separately. Ficin had partitioned selectively to the interfacial phase. 40% (w/v) $(NH_4)_2SO_4$ saturation with 1.0:0.75 ratio of crude extract to t-butanol (v/v) at pH 7.0 gave a maximum 6.74-fold purification and 174% activity recovery of ficin (Fig. 4). TPP systems usually sharply change around the isoionic point of enzymes because of the electrostatic component of the reactions when sulfate anion binding to cationic proteins is involved [7]. In TPP, if the system pH is above isoelectric point (pI) of protein, the protein will acquire net negative charge and will be propelled to bottom aqueous

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phase. On the other hand, if system pH is below pI of target protein, it will be precipitated or accumulated at the interfacial phase. Dennison [6] reviewed recently that change in pH facilitates the change in net charge of target protein and influences partitioning of protein. Effect of different pH on TPP of ficin (Fig. 4) indicates that, as pH increased from 3 to 7, fold purity of partitioned enzyme was increased along with increased % yield as pI of ficin is around 9.0. Wang *et al.* [28] have reported enhanced partitioning of α-amylase inhibitors in bottom phase at a pH 5.25 and 6.0, which was above its pI 5.2. Hence, pH 7.0 was found to be optimum in this study for partitioning of *Ficus carica* ficin.

The overall purification profile of ficin from *Ficus carica* latex by TPP is summarized in Table 1. As understood from the obtained results, it can be said that ficin has tendency to concentrate in the interfacial phase of the TPP system that is related to its structure. The TPP system consisting of 1.0:0.75 of sample to *t-butanol* and 40% $(NH_4)_2SO_4$ resulted in the highest proteases recovery at 167%, and a 6.04-fold purification at the interfacial precipitate. 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% [29]. This may be the result of increased flexibility in the enzyme molecule. A remarkable increase in the catalytic activity and yield of pectinase, proteinase K and trypsin inhibitor obtained from TPP has been reported [4, 24, 26, 30]. A TPP process has been gave the yield of 183% with purification fold of 9 for the interphase of tomato pectinase [24]. Singh *et al.* [26] have been reported an increase in the catalytic activity of proteinase K obtained from TPP. The specific activity of the enzyme obtained after TPP has been estimated as 210% of the initial enzyme activity. Wati *et al.* [4] used the TPP as a single step process to purify trypsin inhibitor which has been purified to 5-, 14- and 7-fold with 315%, 441% and 228% yield (for the bottom phases of TPP) for navy bean, red kidney and adzuki bean, respectively. Dennison and Lovrien [7], reported that a *Bacillus subtilis* protease, *Saccharomyces cerevisiae* invertase, and *Candida cylindracea* lipase gave a yield of 300, 100 and 900%, respectively, after TPP. Additionally, Kumar *et al.* [31] reported that when TPP was applied to laccase, a yield of approximately 161% with 27.8 fold purity was obtained. As shown in Table 1, ficin was exclusively partitioned to the interfacial precipitate instead of the aqueous bottom phase, as indicated by the very high recovery.

3.2. Characterization of the purified Ficin from *Ficus carica* L. latex

The good properties of an enzyme offer potential for use in production processes. The biochemical properties of ficin are very critical for evaluation of its potential use especially in food

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and medicine industry. Therefore, we have an attempt to characterize the partitioned *Ficus carica* ficin. We have also determined the properties of the enzyme regarding remaining activity, pH and thermal stability. The results are given below in proper sections and also discussed by comparing with other enzymes isolated from *Ficus* species.

3.2.1. SDS-PAGE analysis

For good protein separation and high resolution, a Tricine SDS-PAGE analysis was judged more advantageous and hence used in this study to characterize the partitioned ficin. The enzyme was found to be nearly homogenous with a molecular weight corresponding to 23.4 kDa (Fig. 5). The molecular weights of ficin are varying according to their source and the applied method. Ficin with an apparent molecular weight of 26 kDa is obtained from the latex of *Ficus glabrata*, *F. anthelmintica* and *F. laurifolia* [32]. In agreement to our findings, Devaraj *et al.* [16] purified using cation-exchanger column chromatography, a single homogenous ficin from *Ficus carica* with an apparent molecular weight of 23.1 kDa ± 0.3 kDa. In addition, Zare *et al.* [33] purified recently a homogenous ficin from *Ficus carica* cv. Sabz which was found to share a molecular weight of 23 kDa. Generally, the estimated molecular masses of purified ficins falls in the range of 20 – 35 kDa, which is also the reported range of values for many other cysteine proteases [34]. Otherwise, the molecular weight of ficin fractions isolated from *Ficus glabrata* is in the range between 25 and 26 kDa [35-37]. From these findings, it appears that ficins may vary in their amino acid sequences, which can lead to conformational differences. Until now, most of the work has been carried out on the enzymes prepared from the latex of fig species, and very few studies have been done from the latex of specific fig cultivars. However, previous work showed that the number and relative amounts of the components of *F. carica* differed among the studied cultivars or varieties [23].

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

The effect of temperature on ficin activity and stability was studied in the temperature range of 20 – 90°C (Fig. 6). 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 [25, 31, 38]. The optimum temperature of the fractioned ficin was found to be 60°C (Fig. 6). 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 indicating optimal temperature range for ficin activity to be between 50 and 65°C [16, 39]. Thermal stability results indicated that the enzyme is stable between the temperatures' range of 40–70°C.

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3.2.3. Effect of pH on the activity of ficin

The purified ficin has pH activity as shown in the Fig. 7. The maximum activity was observed between pH 6.0-7.5 and showed optimum at pH 6.5. 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 Devaraj *et al.* [16], the results again confirmed that the enzyme is unstable at acidic pH and the enzyme is most stable at pH 7.0. Similar results have been reported earlier by Kramer and Whitaker [40] that the different ficin forms from *F. carica* are more active at the neutral pH range. The acid liable nature of ficin from *F. glabrata* was also reported by Eugland *et al.* [35]. Also, the optimal pH for ficin activity varies with the substrate, ranging from 5 (for gelatin and elastin) to 9.5 (for casein). With gelatin there is another optimum at pH 7.5, and casein has another optimum at pH 6.7 [21].

3.2.4. Comparison of purification protocols with literature

Table 2 shows purification protocols and the biochemical results of ficin from different *Ficus* species. As seen in Table 2, the purification of ficin from several *Ficus* species includes 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 high in previous studies, the present study showed the same degree of purification and activity yields using TPP as a single purification step.

4. Conclusion

TPP was effectively applied for the first time for the purification and recovery of ficin from Mediterranean *Ficus carica* latex. The enzyme was purified to 6.04-fold with 167 % recovery. TPP is an efficient method to separate proteins directly from crude enzyme extracts. The technique is of prime importance in protein concentration and purification, because it is fast, simple, scale-applicable and economic process. TPP system via *t-butanol* can be easily used at room temperature giving high recovery purifications. Using TPP system, ficin from *Ficus carica* was quickly purified to homogeneity with very high purity and activity. Using this technique, the enzyme was prevented from autolysis reported as a serious problem due to the different chromatographical steps used in the conventional methods.

Acknowledgements

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The authors would like to express their sincere thanks to INATAA institute, Constantine 1 University for financial support.

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Figure captions

Fig. 1. Flow sheet of the steps involved in the three phase partitioning (TPP) of ficin from fresh Mediterranean *Ficus carica* latex.

Fig. 2. Effect of varying saturations of ammonium sulfate on the degree of purification and activity recovery of *Ficus carica* ficin. The crude extract (2 ml containing 21.34U) 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 extract.

Fig. 3. Optimization of crude extract to *t-butanol* ratio for the recovery of *Ficus carica* ficin. Various amount of t-butanol was added to crude extract, 2 ml containing 21.34U and saturated with 40 % ammonium sulfate.

Fig. 4. Influence of pH on the degree of purification and activity recovery of *Ficus carica* ficin. Ammonium sulfate (40%, w/v) was added to the crude extract (2 ml containing 21.34U). The pH of the medium was adjusted to different pH values. This was followed by addition of *t-butanol* in a ratio of 1.0:0.75 (v/v).

Fig. 5. Tricine SDS-PAGE analysis using a 10% separating polyacrylamide gel. M: molecular mass standards; lane 1: crude extract after dialysis; lane 2: purified ficin using TPP system after dialysis; lane 3: concentrated ficin using Amicon Ultra device (10 kDa MWCO).

Fig. 6. Effect of temperature on the activity and the stability of *Ficus carica* ficin. Activity was done at defined temperatures by using bovine casein. Thermal stability was done after 1h incubation at the indicated temperatures.

Fig. 7. Effect of pH on the activity of *Ficus carica* ficin. Activity was done after incubation at indicated pHs and activities were assayed using bovine casein.

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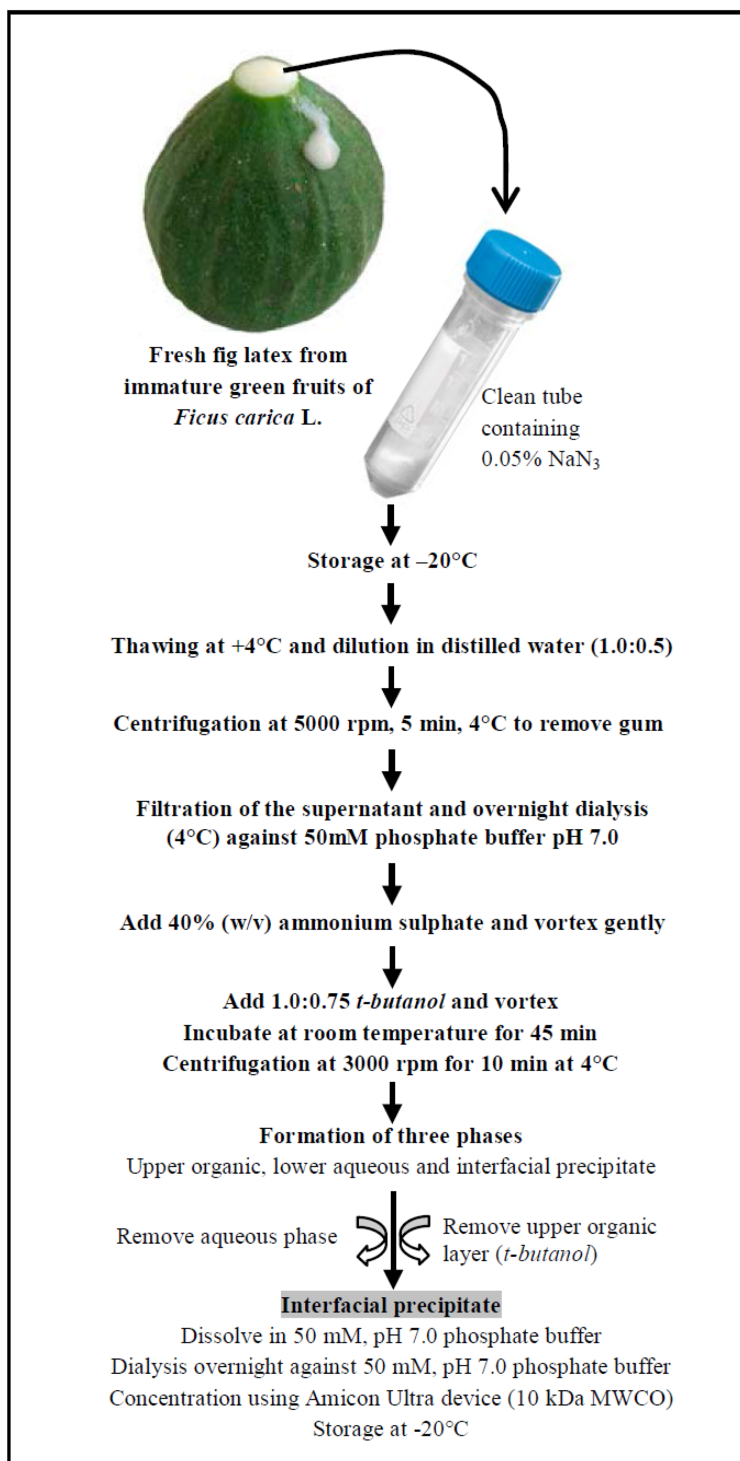

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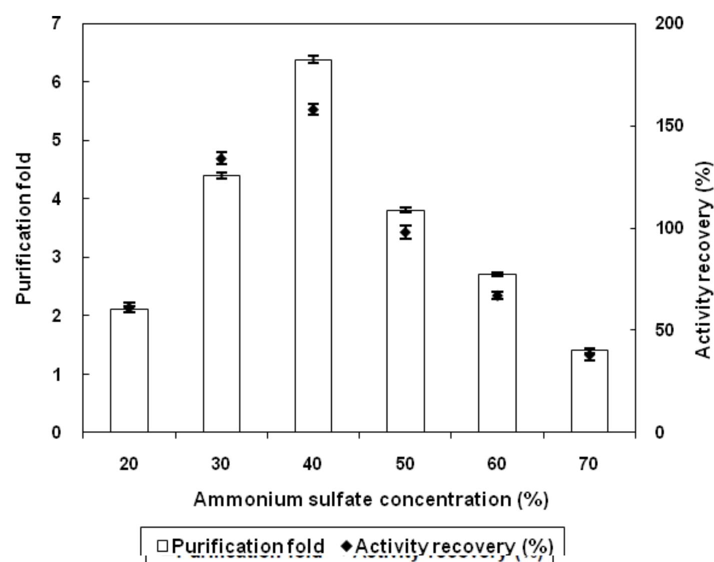

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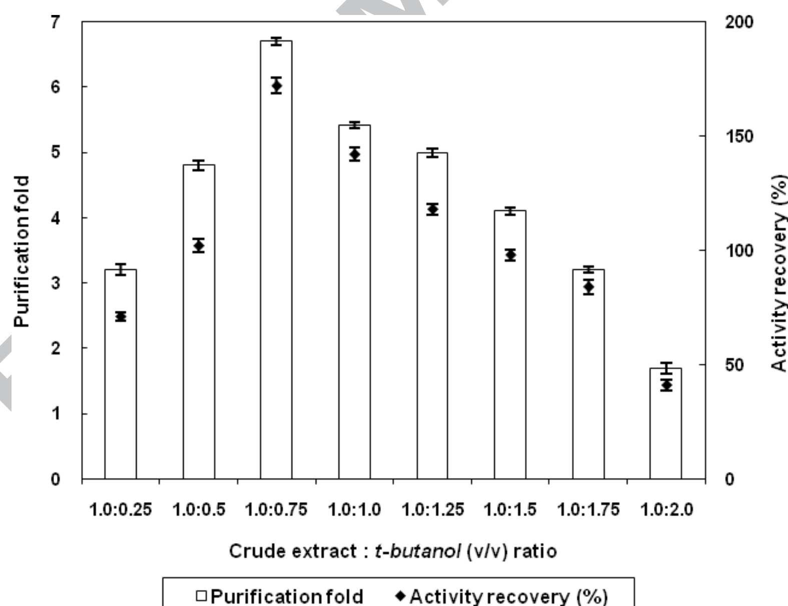

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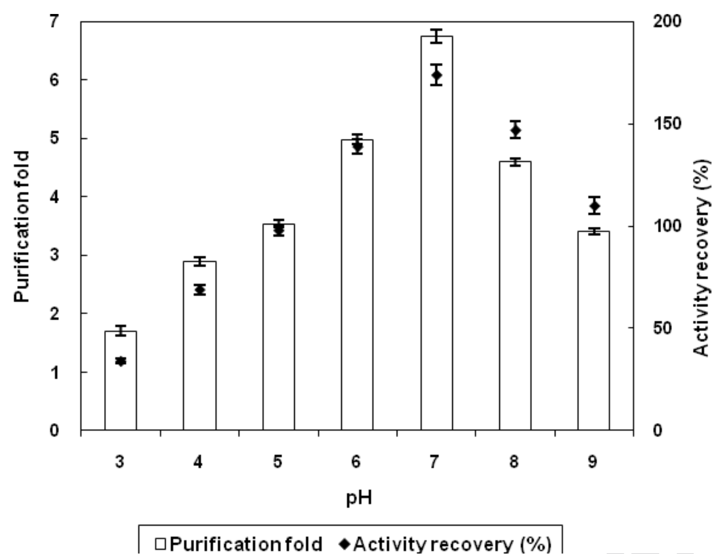

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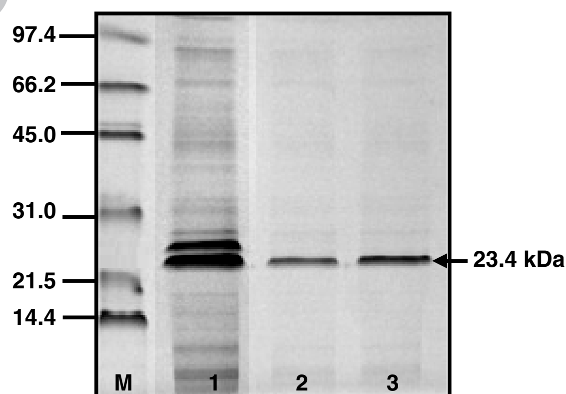

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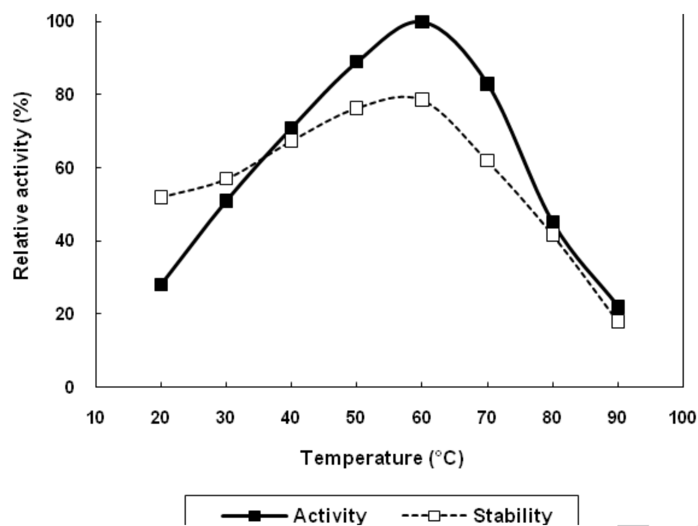

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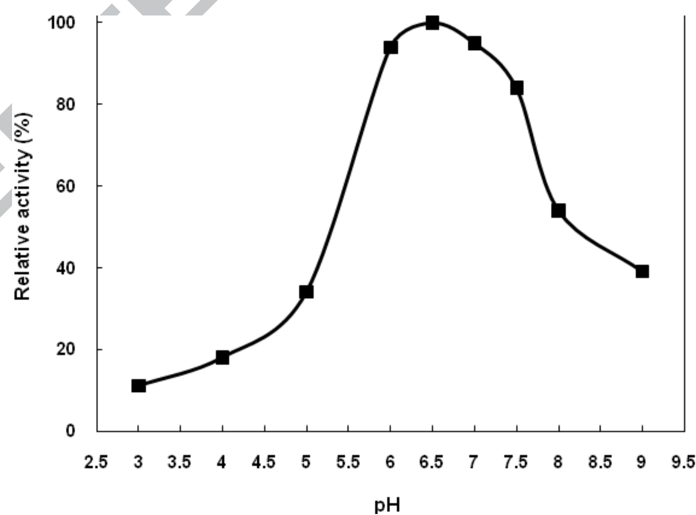

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Table 1. Three-phase partitioning purification and recovery profile of Ficin from fresh Mediterranean *Ficus carica* latex.[1]

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
Crude extract	10.65	7.89	1.35	1	100
Interfacial phase of TPP	17.78	2.18	8.15	6.04	167
TPP aqueous phase	0.002	0.06	0.03	0.02	0.02

[1] The ammonium sulfate (40%, w/v) was added to the crude extract of *Ficus carica* latex (2ml containing 21.34U) and then pH was adjusted to pH 7.0. This was followed by addition of *t-butanol* in a ration of 1.0:0.75 (v/v) (crude extract:*t-butanol*). 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.

Table 2. Comparison of different processes from literature for ficin purification

Source	Purification method steps	MW (kDa)	Optimum pH	Optimum temperature (°C)	Thermal stability	References
Ficus carica	SP-Sepharose chromatography[a]	23.1	7	-	70[b]	[16]
F. carica cv. Horaishi	CM-cellulose, CM-Sephadex and crystallization	26	8	60	67[c]	[39]
Ficus glabrata	Ammonium sulfate precipitation, CM-cellulose chromatography and gel filtration	25 – 26	5 – 8	45 – 65	-	[35, 36, 41]
Ficus racemosa	Ammonium sulfate precipitation, Size exclusion chromatography and anion exchange chromatography	44.5	4.5 – 6.5	60	70[d]	[42]
Ficus carica	Three phase partitioning system	23.4	6.5	60	40 – 70	Present study

[a] Commercial lyophilized ficin extract was used.
[b] Ficin loses 37% of its activity after 10 min of incubation.
[c] Inactivation temperature at pH 7.0 for 30 min.
[d] The enzyme exhibit 50% of its original activity after 1h.

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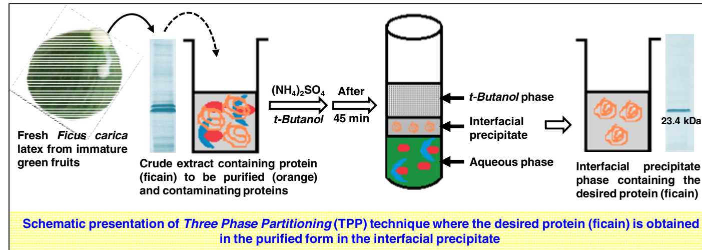
Graphical abstract SEPPUR_11797



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*Highlights (for review)

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Highlights

- The paper provides a study of downstream operations for *Ficus carica* ficin recovery.
- This is the first report on ficin separation using TPP.
- TPP was optimized to concentrate and purify ficin effectively.
- Ficin recovery activity using TPP was better than conventional purification techniques.

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