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One-step purification and freeze stability of papain at acidic pH values

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Abstract: Papain is a proteolytic enzyme of great commercial value. It is a cysteine protease highly expressed in *Carica papaya* fruit latex, but also present in papaya leaves. Purification procedures mostly deal with the latex and include a combination of precipitation and/or chromatographic techniques. Due to its solubility, structure and activity characteristics, the pH and salt content play significant roles in handling papain extracts. Here we report a simple, rapid and easily scalable procedure for papain purification from papaya leaves, which contain different contaminants as compared to papaya latex. Sodium chloride precipitation of contaminants at pH 5 followed by ammonium sulphate precipitation resulted in the removal of other leaf proteins and protein fragments from papain solution and about a 3-fold purification. The procedure also benefits from the suppression of autoproteolysis and preservation of the native structure, as confirmed by FTIR analysis, and the high recovery of activity of over 80%.

Keywords: papain; papaya leaves; precipitation techniques; structural stability; activity recovery

INTRODUCTION

Papain is a cysteine protease isolated from papaya (*Carica papaya*) latex, a protective milky extrudate that flows through unripe fruit and young leaves. This enzyme shows high activity in the hydrolysis of proteins, oligopeptides, amino acid esters and amide bonds in general [1]. Papain belongs to the papain superfamily of cysteine proteases (C1 family of cysteine proteases). In addition to papain, this family includes other plant proteases such as chymopapain, caricain, bromelain, actinidin, ficin, as well as animal cysteine proteases – cathepsins. Members of the papain family are evolutionarily related by the similarity of their tertiary structures, the order of the catalytic residues in their sequence as well as the motifs around the catalytic center. Most proteases in this family are relatively small proteins with molecular weights between 20 and 35 kDa [2,3]. These enzymes are synthesized in the form of zymogens and are characterized by broad *in vitro* substrate specificity, sensitivity to cysteine protease inhibitors, and the requirement of reducing agents in order to be fully active *in vitro* [4].

Proteases are the most widely used enzymes in the food, pharmaceutical and detergent industries, and account for about 60% of total world enzyme production [5]. According to global market analysis, the protease market is expected to reach over $ 3 billion by the end of 2024, with papain as one of the most commonly used enzymes. Due to its broad substrate specificity, papain is widely used both in the laboratory and on an industrial scale, especially in meat softening [6], caries removal [7], preparation of clinically significant antibody parts [8], as a component in cosmetic products [9] and detergents [10].

Traditionally, papain is purified from fruit papaya latex by salt precipitation at slightly alkaline pH values (around 9) using ammonium sulphate and sodium chloride in two precipitation steps, respectively [11]. In the past two decades, aqueous two-phase extraction in papain purification was introduced, using mainly polyethylene glycol (PEG)-phosphate systems, with applicability in large-scale preparations [12]. In laboratory practice, various chromatographic techniques are in use, including covalent binding [13] and ion exchange [14].

Papaya leaf extract contains papain to a lesser extent than latex collected from unripe fruits, but as a papaya by-product, it has the potential to represent an alternative source of this commercially important enzyme [15].

The aim of this work was to purify papain from papaya leaf powder in a scalable, inexpensive and simple way while preserving the activity of the enzyme. This paper describes a method for the purification of papain in one step, with a high yield and without the use of expensive and/or toxic substances. The process presented here may be of fundamental importance for industrial, large-scale papain purification from papaya leaves because of the reduced time of purification, higher activity recovery and the higher purification fold in comparison to procedures that are currently in use.

MATERIALS AND METHODS

Separation of proteins at different pH values of the solution

Five g of papaya leaf powder (Ettera, Serbia) was suspended in 20 mL of 10 mM Tris buffer pH 8. The pH was adjusted to 5.8 (using 1 M HCl) and mixed for 30 min. The precipitate was then separated by centrifugation at 9000 x *g* for 5 min, and the solution was filtered through dry, well-packed cotton wool. The suspension was again centrifuged for 5 min at 9000 x *g*, and the supernatant was separated by decantation. A 1-mL aliquot was taken as a control sample (C). Solid NaCl was added to the remainder of the solution to a saturation of 40% and after the complete solubilization of the salt (2.66 g of NaCl in 19 mL of solution), 7 1-mL aliquots were taken. To check papain solubility in the presence of salt at different pH values, 0.1 mL of buffers varying in their pH were added sequentially to each aliquot, and the samples were shaken gently. Buffers (1 M) used for pH adjustment were as follows: acetate buffers pH 4 and pH 5, citrate buffer pH 6, phosphate buffers pH 7 and 8, and Tris-HCl buffers pH 9 and 10. Buffer salts corresponding to 1 mole (sodium acetate or sodium citrate or sodium phosphate or Tris-HCl) were dissolved in 800 mL of deionized water and the pH was adjusted using 4 M HCl. Water was added to give a total volume of 1 L. The precipitates were then separated by centrifugation for 10 min at 9000 x *g* and dissolved in 1.1 mL of 10 mM acetate buffer pH 5. Both precipitates and remaining solutions were then dialyzed twice for 1 h against 1 L of 10 mM acetate buffer pH 5 to remove the salt.

Bradford assay for determining protein concentration

Protein concentration was determined by the Bradford assay [16] with minor modifications. Briefly, 5 µL of sample and 200 µL of Bradford reagent were mixed in the well of a microtiter plate. All measurements were performed in triplicate. Absorbance was recorded at 620 nm after 5 min. The calibration curve was created using bovine serum albumin (BSA) as standard at the concentration range 0.1-1 mg/mL.

Determination of proteolytic activity

Proteolytic activity towards synthetic substrate *N*α-Benzoyl-DL-arginine 4-nitroanilide hydrochloride (BAPNA) was determined as described previously [17]. Briefly, 50 µL of the sample, 50 µL of 1 M Tris-HCl buffer pH 7 and 100 µL of 1 mM BAPNA solution in 100 mM Tris buffer pH 9 with 5% DMSO were added to a 1.5-mL tube. In the blank sample, activity was first inhibited by 50 µL of 50% acetic acid. Together with the blank, all samples were heated at 50°C for 1 h to allow the reaction to proceed. Thereafter, 50 µL of 50% acetic acid was added to all samples except the blank to stop the reaction. After cooling, the solutions were centrifuged for 5 min at 9000 x *g*, and 200 µL of clear solution was applied to the well of a microtiter plate. Absorbance was recorded at 405 nm. All samples were tested in triplicate. One unit of activity is defined as the micromole of released colored product per 1 min of reaction.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was run according to Laemmli [18] using 12% polyacrylamide gels. Samples were prepared under reducing conditions. Briefly, 40 µL of each sample was mixed with 10 µL of a 5× concentrated reducing sample buffer, and all samples were heated for 15 min at 95°C. After that, 15 µL of each sample was added to wells of the stacking gel. After electrophoresis, gels were stained with Coomassie Brilliant blue R 250 (CBB R-250).

Papain purification

Twenty g of papaya leaf powder were resuspended in 80 mL of 33.3 mM Tris-HCl buffer pH 8. After shaking, the pH of the mixture was found to be about 6.3 and was adjusted to 5.8 using 1 M HCl. The mixture was stirred for 30 min and then centrifuged for 3 min at 9000 x *g*. The supernatant was filtered through dry, well-packed cotton wool and centrifuged again for 10 min at 9000 x *g* to completely remove the precipitate. To 48 mL of the resulting solution, 6.72 g of NaCl was added (corresponding to 40% of NaCl saturation), the pH was adjusted to 5 using 1 M HCl, and the mixture was centrifuged at 9000 x *g* for 10 min. Ammonium sulphate (14.2 g) was then added to the solution (corresponding to 40% of $(NH_4)_2SO_4$ saturation), the mixture was incubated 30 min at 4°C and centrifuged at 6000 x *g* for 10 min. The supernatant was discarded, and the precipitate was dissolved in 24 mL of 100 mM acetate buffer pH 5 or 24 mL of 100 mM Tris-HCl buffer pH 8. The resulting solution was dialyzed against the same buffer in which the precipitate was dissolved to remove residual salt.

Freeze-thaw cycles

Purified papain solutions at pH 5 and pH 8 were subjected to freeze-thaw cycles to examine the freeze-induced damage to protein and potential optimal cold storage conditions. The prepared samples were frozen at a rate of 1°C/min, and thawed at a rate of >10°C/min as recommended [19]. The freezing/thawing procedure was repeated up to 3 times. After each thawing and before the next freezing, the activity of the papain samples was measured using BAPNA as described above. All experiments were performed in triplicate.

Fourier transform infrared spectroscopy with attenuated total reflectance (ATR-FTIR)

Infrared spectra of freeze-thawed papain samples in attenuated total reflectance (ATR) mode were recorded at 1 cm^{-1} resolution using the Nicolet 6700 FTIR instrument (Thermo Scientific, USA). Sample aliquots of 6 µL were applied onto diamond crystal (Smart Orbit, Thermo Scientific, USA) and evaporated in a nitrogen stream to obtain a thin film. Each spectrum was recorded by a collection of 64 scans. Raw spectra were corrected using OMNIC software by applying ATR correction, automatic baseline correction and automatic smoothing, all according to the manufacturer's instructions. The analysis of spectra was based on the Amide I region, which was decomposed to constituents assigned to secondary structure elements according to the previously published article [13]. Positions of peaks contributing to certain secondary structures were recognized by the secondary derivative spectra in OMNIC software, followed by automatic Amide I region decomposition. Secondary structure content was calculated by integrating the areas under the resolved peaks and normalizing each of them to the total area under the Amide I region.

RESULTS

The effect of pH on papain solubility and activity in the presence of NaCl

Fig. 1A shows that the maximum solubility of papaya leaf proteins is at about pH 7. Moving away from this value, the solubility decreases with almost identical

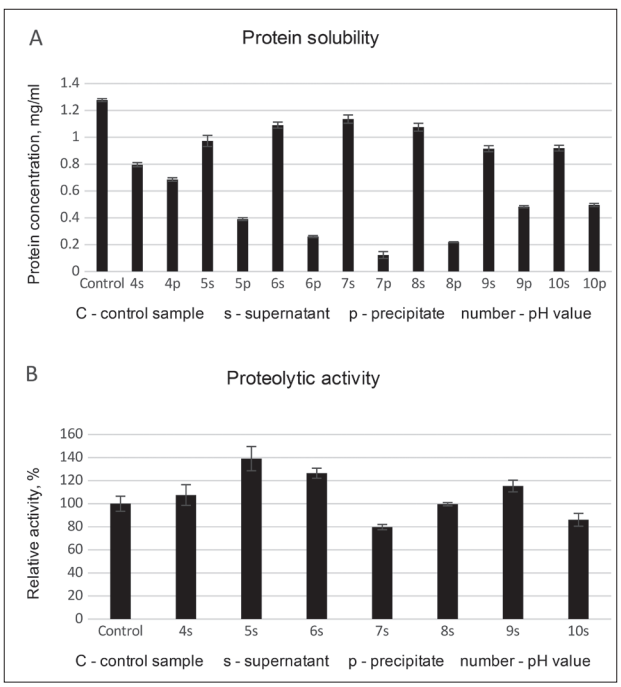

Fig. 1. Protein solubility (**A**) and proteolytic activity (**B**) at different pH values and in the presence of NaCl (40% of saturation). **C** – control, initial sample; numbers 4-10 indicate the pH values of the solutions at which the precipitate was formed; s – supernatant; p – precipitate.

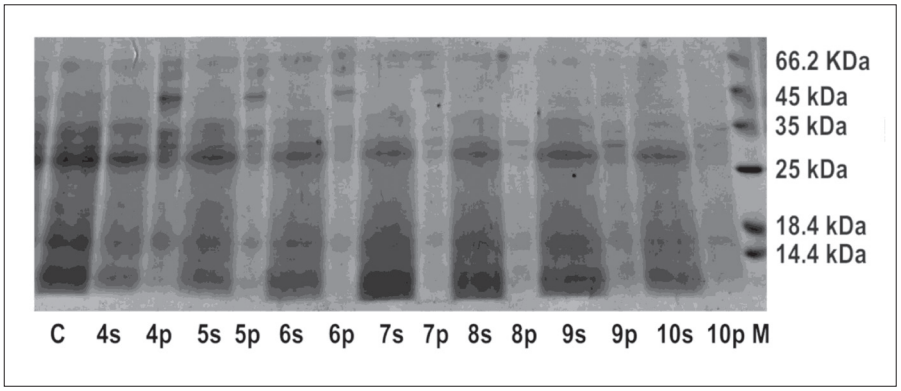

Fig. 2. SDS-PAGE of papaya leaf extract proteins soluble or precipitated at different pH values in the presence of NaCl (40% of saturation). C – control, initial sample; numbers 4-10 indicate the pH values of the solutions at which the precipitate was formed; s – supernatant; p – precipitate; M – molecular weight markers.

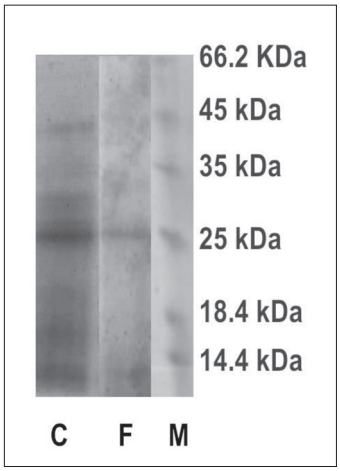

Fig. 3. SDS-PAGE of papaya leaf extract (C) and final purified papain (F) dissolved at pH 8. M – molecular weight markers.

Table 1. Purification of papain from papaya leaf extract.

	C, g/L	V, L	Activity(U)	Sp (U/g)	Activity recovery (%)	Fold purification
Papaya leaf extract	1.50±0.03	0.48	541±29	7.51±0.56	-	-
Purified papain	0.82±0.01	0.24	440±12	22.35±0.88	81.3	2.98

Sp – specific activity; C – protein concentration; V – sample volume.

solubility at pH 9 and 10. Conversely, the measured proteolytic activity was highest at pH 5 and 9, with a minimum at pH 7 (Fig. 1B). Since a very modest proteolytic activity was detected in precipitates, native and active papain remained soluble in the entire pH range.

Analysis of protein contaminant removal

The electrophoretic profiles of soluble and insoluble proteins at different pH values and in the presence of NaCl are shown in Fig. 2. The band attributed to papain (~25 kDa) was strongly present in all the supernatants, regardless of the pH value of the solution. At the same time, other proteins were more abundant in precipitates obtained at lower pH values (4 and 5). The most intense staining in the lower part of the gel (at about 10 kDa) can be observed in samples of pH 7 and 8 and to a lesser extent in samples of pH 6 and 9.

Ammonium sulphate precipitation of papain

Papain precipitation with ammonium sulphate led to the removal of autolytic bands of molecular weight lower than 10 kDa (Fig. 3). Using this simple, fast and inexpensive procedure, papain was purified 2.98-fold from papaya leaf extract with an activity recovery of 81.3% (Table 1).

Freeze-thaw stability of purified papain at pH 5 and pH 8

After ammonium sulphate precipitation and solubilization of the purified enzyme, the final papain preparation was freeze-thawed at pH 5 and 8. Residual activity of purified papain after three freeze-thaw cycles is shown in Fig. 4. The activity of papain at pH 8 after 3 freeze-thaw cycles is mostly preserved compared to the starting sample. On the other hand, it can be observed that in acidic conditions (pH 5), papain loses activity significantly after each freeze-thaw cycle. After only three freeze-thaw cycles under these conditions, papain activity decreased to 19% of the starting value.

Structural perturbations induced by freezing of protein were analyzed using FTIR. Assignment of specific secondary structures to peaks within Amide I region of FTIR spectra is shown in Fig. 5, which shows that there are no significant changes in the FTIR spectra of papain samples in solution at pH 8. On the

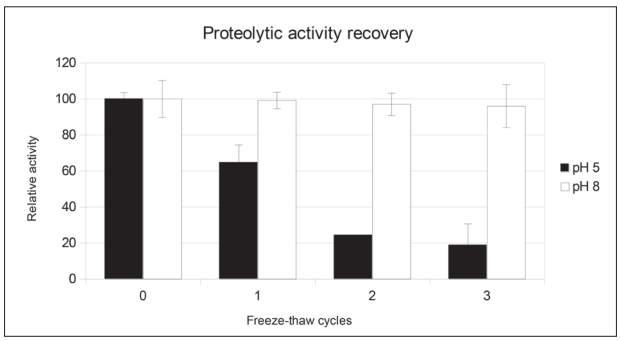

Fig. 4. Effect of freeze-thaw cycles on remaining papain activity at pH 8 and pH 5.

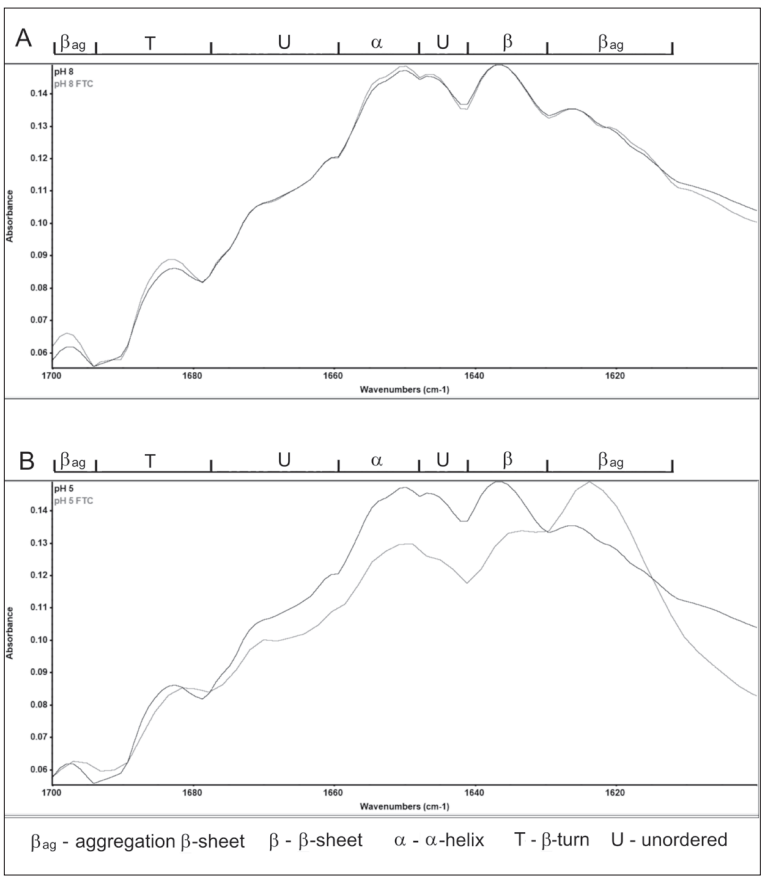

Fig. 5. Amide I region of papain samples FTIR spectra, before and after 3 freeze-thaw cycles at pH 8 (**A**) and pH 5 (**B**).

Table 2. Fitted values for the secondary structure content of papain before and after 3 freeze-thaw cycles (FTC) at pH 8 and pH 5 determined by ATR FTIR spectroscopy.

Samples	α-helix	β-sheet	β-turns	Disordered	Error
Papain pH 8	25.6	25.1	7.7	41.6	1.2
Papain FTC pH 8	24.6	27.0	8.3	40.1	1.8
Papain pH 5	24.7	26.4	8.6	40.3	1.4
Papain FTC pH 5	19.9	53.5	7.9	18.7	1.7
Papain crystal structure	26	25	7	42	-

other hand, the papain sample freeze-thawed at pH 5 (Fig. 5B) showed changes in the Amide I region of FTIR spectra. The most prominent change is a more pronounced band at 1624 cm^{-1}, which is attributed to an intermolecular aggregation-specific β-sheet. The most significant changes in calculated secondary structures content are reduction (about 55%) in an disordered polypeptide content, and an ordered α-helix content (about 20% reduction) on account of the doubled β-sheet content (Table 2).

DISCUSSION

Papaya fruit latex is a common papain source [11,12]. However, it is reported that this enzyme is highly present in leaf extract as well [15]. Besides papain, there are many different proteins present in the papaya leaf extract (such as Rubisco, various oxidoreductases, chloroplast associated proteins [20]), varying in size and distribution of charged and hydrophobic regions on the protein surface.

To develop a simple one-step precipitation procedure for papain purification, we took into account that both the pH value of the solution and its ionic strength influence specific protein solubility. The pH value of a solution affects the charge of ionizable side chains of amino acids, and consequently, the solubility of a particular protein. Changing the pH value also affects the solubility of different proteins in the solution depending on their structure [21]. Thus, varying the pH value should affect the solubility of different proteins in a different manner, depending on their amino acid content and overall structure [21]. Strong electrolytes, salts that dissociate to charged cations and anions when dissolved in water in lower concentrations, help in dissolving proteins in general by interacting with ionized groups on their surface, while at higher concentrations, they promote protein aggregation by expelling uncharged, hydrophobic parts of proteins from the solution [22]. It follows

that it is theoretically possible to adjust the salt concentration and the charge of the protein by adjusting the pH value of the solution so that papain is sufficiently charged and remains dissolved while other proteins that are less charged at a given pH are precipitated. The most common examples of strong electrolytic salts used in protein biochemistry are sodium chloride, ammonium sulphate and potassium chloride.

The traditional papain purification procedure from papaya latex employs two-step precipitation of papain using mainly ammonium sulphate in the first step [11]. Since we wanted to explore the effect of pH on salt precipitation of proteins present in papaya leaf extract, we used neutral salt sodium chloride. The experiment was performed by changing the pH value of the protein solution containing salt (40% saturation of NaCl) and analyzing the samples of supernatant and precipitate by SDS-PAGE electrophoresis and by testing protein concentration and proteolytic activity.

A slight decrease in the proteolytic activity of papain at the lowest and the highest pH (4 and 10, respectively) could be attributed to structural perturbations within the native structure and aggregation of the destabilized enzyme. The observed transition usually occurs at lower pH values of around 2-3, but structural destabilization also contributes, to a smaller extent, to papain activity loss at pH 4 [17,23,24]. A decrease in proteolytic activity at pH 7, however, could be explained by autoproteolysis since papain shows optimal activity in the pH range of 6-8 with a maximum at pH around 7 [25].

Electrophoresis in denaturing conditions was performed to check if autoproteolysis occurred at near-neutral pH values as well as to analyze the efficiency of papain separation from other proteins present in the starting sample. The control sample, the crude papaya leaf extract, shows heterogeneity in its protein content with the most prominent bands at molecular weights of 16 kDa and 45 kDa (RUBISCO small and large subunits, attributed to photosynthesis), 26 kDa – papain-like proteases, and 33 kDa – chitinase [20]. Protein contaminants were more efficiently separated from papain by precipitation at lower pH values (4 and 5). Autoproteolysis occurred to a different extent in the supernatant samples according to the variable appearance of smeared bands containing small peptides (with a molecular weight less than 10 kDa), with maximal staining at around neutral pH values. These results confirmed our assumption that autoproteolysis could be responsible for the observed proteolytic activity decrease detected in the supernatant at pH 7.

The observed high solubility of papain, precipitation of unwanted proteins, lowest activity loss due to both autolysis and structural destabilization at pH 5 with 40% of NaCl saturation pointed to the most optimal condition for the removal of contaminants from papain solution. To remove autolytic products and concentrate the purified protein, papain was precipitated from the supernatant using a 40% saturation of ammonium sulphate, resulting in a 3-fold purification and activity recovery higher than 80%. Even though not fully comparable because of differences in the protein composition of the starting material, traditional purification of papain from papaya latex using two-step salt precipitation is far less successful, judging by lower purification fold (about 1.2) and the very low activity recoveries (5-12%) [12,26], probably due to autoproteolysis of papain. On the other hand, the more challenging aqueous two-phase extraction shows similar purification folds (about 3) and similar activity recoveries (60-88%) [12,27-29]. The purification of papain using the procedure described herein can be accomplished in several hours in a simple and straightforward manner (at a two-fold reduced timescale in comparison to other procedures).

As it was reported that pH as low as 2-4 could affect papain 3D structure both at low and elevated temperatures and in the presence of chaotropic agents [13,17,30], we sought to evaluate whether the acidic pH used for papain purification could affect its freeze-thaw stability, and consequently influence its storage. A massive loss of papain activity, which is observed at pH 5 (81% after 3 freeze-thaw cycles), occurs as a consequence of freezing-induced denaturation. Protein denaturation at low temperatures is caused by disruption of the protein hydrophobic core due to stresses associated with ice formation in the aqueous protein solution leading to the aggregation of misfolded polypeptide [13,19].

Structural perturbations induced by freezing the protein were analyzed using FTIR. The Amide I region in the FTIR spectra of proteins (1700-1600 cm^{-1}) arises

from C=O stretching vibrations of peptide bonds. The position of specific bands within the Amide I region depends on the conformation of the peptide bonds, and thus on the protein secondary structure. The absence of structural changes of papain in solution at pH 8 indicates structural preservation in line with activity preservation. The formation of an aggregation-specific β-sheet of the sample at pH 5 indicates structural perturbation and aggregation of proteins [13,31]. A similar effect was observed in the case of trypsin stored in acidic conditions, which was attributed to cold denaturation of the protein [19]. The determined content of secondary structures further shows that three repeated freeze-thaw cycles at pH 5 induced structural changes in papain. The described changes in secondary structures correspond to cold denatured proteins [32].

The presented results regarding the purification level and activation recovery of papain point to a high efficiency of acidic precipitation in contaminant removal. Using an acidic buffer of pH 5 positively affects papain activity and prevents autoproteolytic activity but, based on our results, should be avoided when freeze-thawing a papain sample. Slightly basic conditions of pH 8 provide better preservation of papain structure and activity when consecutively frozen/thawed.

CONCLUSIONS

Purification of papain from papaya leaf powder at pH 5 was shown to be more successful than at near-neutral pH values due to slower autoproteolysis and precipitation of protein contaminants. Subsequent papain precipitation with ammonium sulphate led to the removal of autolytic peptides from papain preparation. Despite destabilization upon freezing, solubilization of finally purified papain in a slightly alkaline buffer (pH 8) to avoid the freezing-induced denaturation of papain purified at pH 5 is successful in preventing freezing-induced damage to the protein.

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