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Bioseparation of papain from *Carica papaya* latex by precipitation of papain–poly (vinyl sulfonate) complexes

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ABSTRACT

The formation of insoluble complexes between enzymes and polyelectrolytes is a suitable technique for isolating these biomolecules from natural sources, because it is a simple and rapid technique that allows the concentration of the protein. This technique can be used in most purification protocols at the beginning of the downstream process.

The aim of this investigation is to isolate papain from *Carica papaya* latex by precipitation of insoluble complexes between this enzyme and poly (vinyl sulfonate).

The papain–poly (vinyl sulfonate) complex was insoluble at pH lower than 6, with a PVS/PAP stoichiometric ratio of 1:279. Ionic strength affected the complex formation. The presence of the polymer increased the enzymatic activity and protected the enzyme from autodegradation. The optimal conditions for the formation of insoluble papain–polyelectrolyte complex formation were applied to *C. papaya* latex and a high recovery was obtained (around 86%) and a purification factor around 2. This method can be applied as an isolation method of papain from *C. papaya* latex or as a first step in a larger purification strategy.

Introduction

The latex of *Carica papaya* is a rich source of cysteine endopeptidases, including papain (PAP),[1] glycyl endopeptidase, chymopapain and caricain, which constitute more than 80% of the whole enzyme fraction [1]. Papain (EC 3.4.22.2) is a minor constituent (5–8%) among the papaya endopeptidases [2,3]. It is a monomeric protein of 23.4 kDa; with a pI around 6.7 and a temperature of maximum activity of 37 °C. This enzyme is widely used as meat tenderizer, and also has several other applications, e.g. defibrinating wounds, treatment of edemas, shrink proofing of wool, etc.

Purification of papain from papaya latex has been traditionally achieved by precipitation methods [4–6]; however, the purified enzyme still remains contaminated with other proteases. An alternative purification strategy involves the use of various chromatographic techniques including ion exchange, covalent, or affinity chromatography [7], but in these cases the initial processing of the latex is essential before samples can be applied on chromatography [8,9].

Precipitation as a product concentration step offers several advantages since it is easy to scale up, uses simple equipment and can be based on a large variety of alternative precipitants [10]. When a protein–polyelectrolyte complex is specifically formed with one of the proteins in the crude extract followed by a phase separation, the process can be used as a convenient strategy for the isolation and purification of the target protein [11].

A wide variety of synthetic and natural polyelectrolytes can interact with globular proteins to form stable protein–polyelectrolyte complexes that result in the formation of soluble or insoluble complexes. The insoluble complex can be easily separated by simple decantation [12,13].

The interaction between proteins and polyelectrolytes has been extensively studied, in particular for the analysis of living processes, immobilization or stabilization of enzymes, modification of substrate affinity, changing properties of food products, and for the development of many pharmaceutical applications [14–16].

Here, we used spectroscopic techniques to obtain information about the molecular mechanism of interaction between papain and a negatively charged polyelectrolyte poly (vinyl sulfonic acid). The aim of this work is to apply this information to the formation of protein–polyelectrolyte complexes as a tool for papain separation from natural sources.

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[1] *Abbreviations used:* PAP, Papain; BAPNA, d-N-benzoyl DL-Arginine-p-nitroaniline; PF, purification factor; PVS, poly (vinyl sulfonic acid, sodium salt).

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Materials and methods

Chemicals

Papain (PAP) from *C. papaya*, d-*N*-benzoyl-DL-Arginine-*p*-nitroaniline (BAPNA), cystein and HCl were purchased from Sigma Chem. Co. (USA) and the polymer poly (vinyl sulfonic acid, sodium salt) (PVS) 25% w/w, d = 1.267, sol. in water, molecular average mass 170 kDa, was purchased from Aldrich and used without further purification. Sodium phosphate and Tris–HCl buffer solutions of different pH were prepared at a concentration of 50 mM. The pH was adjusted with NaOH or HCl.

Fresh latex was collected from locally grown *C. papaya*. Initially, four to six longitudinal incisions were made on the unripe fruit using a stainless steel knife. The exuded latex was allowed to run down the fruit and drip into collecting devices attached around the trunk. After collection, the latex was transferred to a glass bottle and stored at −20 °C. Total protein and nucleic acid concentrations were quantified by Warburg method [17].

Phase diagrams of the PAP–PVS complex

To study the effect of the pH on the formation of the insoluble PAP–PVS complex at 25 °C, three solutions containing PAP and PVS were prepared at different molar ratios (1:46; 1:183 and 1:279) with 50 mM sodium phosphate buffer, pH 5.00, and titrated with alkali and acid in order to cover the whole pH range. The absorbance was measured at 420 nm (turbidity) every 0.50 units of pH using a Jasco 520 spectrophotometer with a thermostatized cell of 1 cm of path length. Finally, turbidity was plotted vs. pH. These phase diagrams show the pH range where the PAP–PVS complex is soluble or insoluble.

Turbidimetric titration curves at different ionic strengths

The formation of the insoluble PAP–PVS complex was followed by means of turbidimetric titration at different conditions of ionic strengths. PAP solutions (27 μM) were prepared with sodium phosphate buffer solutions 50 mM pH 6.00 with different concentrations of NaCl (0–0.25 M). They were titrated at 25 °C with a PVS solution (0.25 w/w%, aliquot volume 10 μL). The turbidity increase was used to follow the formation of the insoluble PAP–PVS complex. The absorbance was measured as described above and plotted vs. the PAP/PVS molar ratio.

To avoid changes in pH during titration, both the PAP and the PVS stock solutions were adjusted to the same pH value.

Kinetics of the formation of the PAP–PVS complex

The time needed to form the complex was evaluated measuring the time required to obtain the maximum turbidity. This experiment was performed mixing a PVS solution and a PAP solution in a PVS/PAP molar ratio of 1:46 [20]. The experiment was performed in 50 mM sodium phosphate buffer, pH 6.00, and absorbance was measured at 420 nm.

Assays of enzymatic activity

PAP activity was determined with the substrate d-*N*-benzoyl DL-Arginine-*p*-nitroaniline (BAPNA) using a modified Gildberg and Overbo method. BAPNA was used at a final concentration of 0.85 mM in Tris–HCl buffer 100 mM pH 8.20 cystein 4.2 mM. The reaction was followed by measuring the absorbance of the released reaction product, *p*-nitroanilide, at 400 nm (molar absorptivity of 18,100 $M^{-1}cm^{-1}$) for 5 min. The enzymatic activity was calculated from the slope of the absorbance vs. time curve [21].

In order to evaluate the stability of the enzyme in the presence of a polymer excess, PAP was incubated with PVS and the activity was measured for 24 h. The selected PVS/PAP molar ratio was 1:46 and a PAP solution was incubated in the same condition without PVS as an activity control of the enzyme.

Precipitation of PAP with PVS

A solution of PVS was added to a PAP solution at a final molar ratio of 1:46 in sodium phosphate buffer 50 mM pH 6.00 (volume = 4.00 mL). The insoluble complex formed was incubated for 5 min at 25 °C and centrifuged at 1000x*g* for 10 min to separate the supernatant and the precipitate fraction. Then, the precipitate was redissolved by addition of 200 μL of Tris–HCl buffer 100 mM pH 8.20 NaCl 0.5 M and 3.80 mL of Tris–HCl buffer 100 mM pH 8.20. The enzymatic activity in the supernatant and in the redissolved precipitate was measured. Both values were compared with the activity of a PAP control (without PVS) at the same conditions.

Then, this protocol was applied to the *C. papaya* latex in order to isolate PAP from a natural source. The enzymatic activity in the supernatant and in the redissolved precipitate was measured. Total protein concentration was estimated by the Warburg and Christian method [17].

The purification factor (PF) was calculated by the ratio between the PAP specific activity in the supernatant (or in the redissolved precipitate) and the specific activity in the latex. PAP recovery percentage (*R*%) was calculated as the ratio between the activity in the redissolved precipitate and the activity in the fresh latex [11].

Results and discussion

Phase diagrams of the PAP–PVS complex

Fig. 1 shows the plots of absorbance at 420 nm vs. pH obtained for three solutions containing PAP and PVS (PVS/PAP molar ratios = 1:46; 1:183 and 1:279). It can be seen that the formation of the PAP–PVS complex was highly influenced by the pH medium since proteins and polyelectrolytes mainly interact by electrostatic interaction. At pH lower than 6.00, PAP and PVS molecules posses opposite charges and so, the insoluble complex between the two is formed. pH values above 6.00 induced a large decrease in turbidity

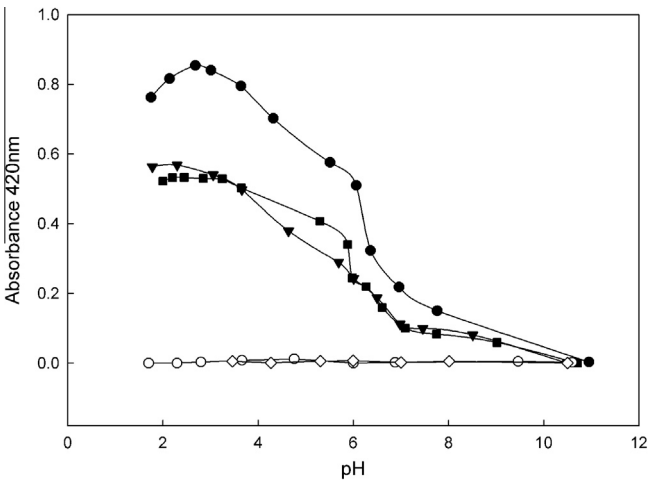

Fig. 1. Phase diagrams: absorbance at 420 nm vs. pH at three different PVS/PAP molar ratios = 1:46 (■); 1:183 (▼) and 1:279 (●). Controls: PAP 27 μM (◇); PVS 0.018 w/w% (○). Temperature 25 °C.

values, which suggested a minor amount of insoluble complex formed [22]. The selected pH to perform the subsequent assays was 6.00 in order to obtain the insoluble PAP–PVS complex and avoid acid denaturation of the enzyme.

Turbidimetric titration curves at different ionic strengths

Fig. 2 shows the turbidimetric titration curves of PAP at pH 6.00. In the absence of NaCl the turbidity of the system increased as the concentration of PVS increased, indicating that PAP and PVS interact to form the insoluble complex. Also, it can be seen that turbidity disappeared when the ionic strength increased, thus indicating that the formation of the insoluble complex was inhibited. This is consistent with the presence of coulombic forces [19].

This finding may be interesting because it is the basis of the PAP isolation method which allows precipitation using charged polymers, followed by the dissolution of the precipitate by addition of a NaCl solution at low concentration.

Kinetics of the formation of the PAP–PVS complex

Fig. 3 shows that only three minutes were needed to achieve the maximum turbidity and so, the maximum quantity of the insoluble PAP–PVS complex. After three minutes, turbidity remains constant indicating that no more insoluble complex is formed.

The kinetics of this process is considered fast and is related to the time required to form the PAP–PVS complex [20].

This is a major finding since the application of a fast methodology to purify PAP is very important to avoid its denaturation and lose of catalytic activity.

PAP activity through time, in the absence and presence of PVS

Fig. 4 shows the PAP activity in the presence of PVS at a polymer/protein ratio of 1:46. The presence of the PVS increases the PAP activity when the enzyme is in solution. On the other hand, the PAP activity in the redissolved precipitate was the same as the PAP control. Both, the insoluble PAP–PVS complex and the precipitate fraction, present more activity after 24 h of incubation than the PAP control, indicating that PVS stabilizes the structure of the enzyme through time, preventing the process of autodegradation of PAP.

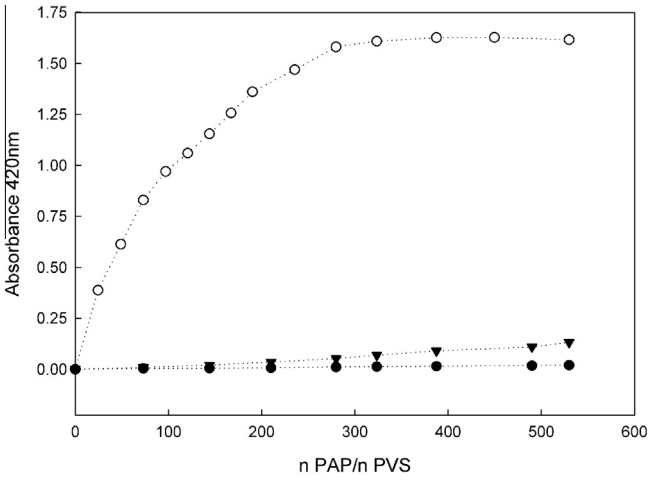

Fig. 2. Effect of the ionic strength on the turbidimetric titration curves: medium phosphate buffer 50 μM pH 6.00 (○) 0 M NaCl; (▼) 0.125 M NaCl; (●) 0.250 M NaCl. Temperature 25 °C.

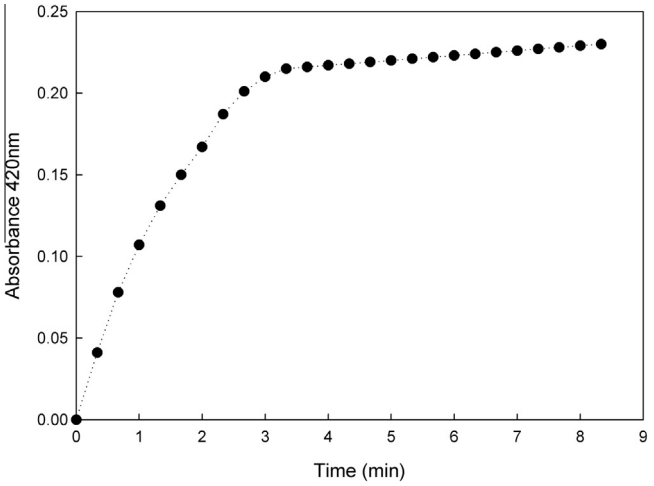

Fig. 3. Kinetics of formation of the insoluble complex. PVS/PAP molar ratio = 1:46. Medium phosphate buffer 50 mM pH 6.00. Temperature 25 °C.

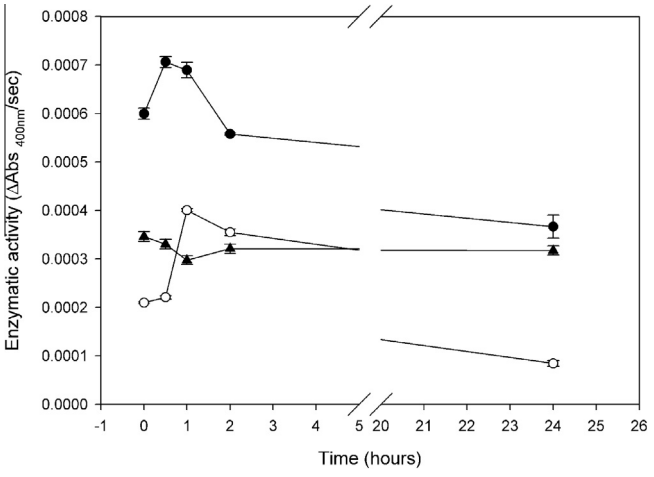

Fig. 4. PAP activity in the absence (○), the presence of PVS (●) and after precipitation (▲) through time. PVS/PAP molar ratio = 1:46. Medium phosphate buffer 50 mM pH 6.00. Temperature 25 °C.

Precipitation of PAP with PVS by formation of insoluble complexes

Table 1 shows the recovery of PAP in the fractions collected after precipitation with PVS: supernatant and precipitate. The aim of this experiment is to determine if the formation of the insoluble PAP–PVS complex is a useful technique to precipitate PAP. It can be seen that the recovery of the PAP activity was very high, yielding 91% in the redissolved precipitate. This indicates that PVS is suitable for precipitating PAP and that this methodology can be applied to the latex.

Precipitation of PAP with PVS from latex

The methodology used in the previous item was applied to the fresh latex of *C. papaya*. PAP activity and total protein concentrations were quantified in three fractions: redissolved precipitate,

Table 1
PAP enzymatic activity and recovery of PAP after precipitation.

	PAP enzymatic activity (U/mL)	Recovery (%)
PAP control	24.46	100
Redissolved precipitate	22.28	91.12
Supernatant	3.76	15.00

Table 2
PAP enzymatic activity and PAP concentration in different fractions: control, redissolved precipitate and supernatant.

Fractions	Enzymatic activity		Proteins (μg/ml)	PF	Recovery (%)
	U/mL	%			
PAP control	8.348	100	363 ± 3	–	–
Redissolved precipitate	7.258	86.98	152 ± 3	2.08	86.98
Supernatant	1.194	14.32	282 ± 3	–	–

supernatant and latex control. These allowed to determined de purification factor and the recovery for the purification of PAP. All this information is summarized in Table 2. Because these media cannot be reproduced to make a calibration curve, it was not possible to carry out activity balances. However, the determination of the PAP activity in both fractions demonstrates a high tendency of the enzyme to precipitate.

It can be seen that the recovery obtained after the precipitation of PAP with PVS from latex was high (around 87%). However, the purification factor obtained was approximately 2, due to the presence of contaminants that may have the same charge as the PAP.

These results prove that the methodology is very effective in concentrating the enzyme or as first extractive step in purification protocols that may include, for example, chromatography.

Conclusions

Although precipitation of proteins with polyelectrolytes has been studied for many years, few theoretical studies have been directed toward understanding the mechanism of precipitation [17].

Our experimental findings agree with other authors who have shown that the formation of the complex is strongly dependent on the pH and the presence of salts [18].

This study showed that PAP could interact with PVS and form insoluble complexes depending on the solution pH, ionic strength, etc. Insoluble complexes were formed at pH values where the PAP and the PVS molecules have opposite electrical charges (pH lower than 6) because of a strong electrostatic attraction between the PAP and the PVS. Moreover, assays of turbidity showed that a great number of PAP molecules are bound to a single PVS molecule (279 PAP molecules per PVS molecule). These results are consistent with the model proposed by Kokufuta et al. [19], where one polymer molecule has the capability to interact with many protein molecules.

The presence of low ionic strength (0.25 M) can easily redissolve the complex, confirming that electrostatic forces are involved in the interaction between protein and polymer.

PAP activity in the presence of PVS is higher than in its absence. The activity of the complex measured after incubating the enzyme for 24 h was also higher than the activity in the absence of PVS. Besides, the enzymatic activity in the redissolve precipitate was similar to the PAP control but more stable along time. This shows that PVS not only protects the enzyme from autodegradation but also make its structure more stable through time. These effects were observed in other protein–polyelectrolytes complexes [23,24].

When the protocol of precipitation was applied to a solution of PAP, a recovery of 91.12% was obtained. On the other hand, when the same protocol was applied to a solution of the latex from *C. papaya*, the recovery obtained was 86.98% and the purification factor was 2.08.

This method is suitable for applying as a first step for PAP isolation from its natural source because is simple, fast, uses low quantities of PVS, concentrates the enzyme in the sample and moreover, it stabilizes the structure of PAP. Even more, if the enzyme is expected to be used in industrial processes, there is no need of further purification since the contaminants are proteases that could aid with the PAP activity. The removal of the polymer is also optional, being unnecessary if the PAP will be used in, for example, leather softening.

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