Purification of Papain from Fresh Latex of *Carica papaya*

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ABSTRACT

In the present study we wish to report a method of crystallizing papain from fresh papaya latex which gave higher yields than previously reported. This method does not involve the use of sulphydryl reagents. The papain thus obtained is practically pure and shows a single band when submitted to electrophoresis on polyacrylamide gel, and is identical to the papain obtained by other methods. In routine enzymatic assays, specific activity was measured using Z-gly-pNP and BAEE as substrates. Papain crystallized by this method, without the use of high concentrations of salts or thiol-containing substances such as cysteine and dithiothreitol, is obtained in the form of a complex with natural inhibitors existent in latex which can be removed by dialysis.

Key Word: *papain; Carica papaya; cysteine proteinase; proteolytic enzymes; purification of papain*

INTRODUCTION

The papain present in the latex of *Carica papaya* has been extensively studied (Brocklehurst et al., 1981, Brocklehurst et al., 1987, apud Thomas 1994, Mellor et al., 1993) and is an enzyme of industrial use and of high research interest. Among the major applications of papain are its use in the food industry, (Neidlema, 1991) beer clarification, (Caygill, 1979) meat tenderizing, preparation of protein hydrolysates and others (Dupaigne, 1973). Balls et al., 1937, developed a process for the purification and isolation of papain in the native crystalline state from fresh latex. This method was later modified (Kimmel & Smith, 1954) using commercially available dry latex and has been the classical method for papain preparation for many years, with some later modifications (Arnon, 1970, Baines & Brocklehurst, 1979). According to Brocklehurst et al., 1981, Baines & Brocklehurst, 1979, 1982, Brocklehurst et al., 1981, aqueous extracts of Carica papaya latex contain some cysteine proteinases that can be separated by ion-exchange chromatography, and fully active forms can also be obtained by covalent chromatography using thiol-disulfide exchange (Brocklehurst et al., 1985, apud Mellor et al., 1993). The kinetics and ionization of the catalytic site of papain obtained by different methods ($Cys\text{-}S^-/His\text{-}Im^+H$ formation) are a matter of controversy. Studies carried out by one of the major groups specializing in research on the cysteine-proteinase family from the latex of Carica papaya have demonstrated the importance of the use of reversible inhibitors during their isolation and manipulation (Brocklehurst et al., 1981). The authors also emphasized that the multiple ionizations occurring in chymopapain and papain may be a general phenomenon for the cysteine-proteinase family (Thomas et al., 1994). In the present study we report on the development of a method for the purification and crystallization of native papain from fresh latex (Monti, 1983), which differs from the classical methods described in the literature. The method showed that papain precipitates spontaneously at low temperatures, presenting a high level of purity and excellent catalytic activity in comparison to classical methods of Kimmel & Smith, 1954.

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MATERIALS AND METHODS

Latex extraction. Fresh latex was obtained from developing green fruits directly picked from trees in the Araraquara region, State of São Paulo, Brazil. Three or four vertical incisions were made in the fruits with a sharp stainless steel instrument to a depth of 2 to 3 mm. The latex that surfaces after incision lasts only 1 to 2 minutes and then rapidly coagulates and can be collected into a glass container. After extraction, the latex was immediately used for the purification of papain in its native state or stored at -8 ^{o}C protected under a nitrogen atmosphere.

Isolation and crystallization of papain. The following procedure was used to obtain papain in crystalline form: 1) for extract preparation, ethylenediamine-tetraacetic acid (EDTA), pH 7.0, was added to the fresh latex to a final concentration of 1 mM and the preparation was kept under nitrogen for 1 hr at room temperature with constant shaking. Possible volatile dragged by nitrogen were qualitatively collected through bubbling distilled water and measured by absorbance in 230 nm. The suspension was then centrifuged at 12,000 x g for 30 min at room temperature in a Sorvall RC-2B centrifuge with an SS-34 rotor. The supernatant (fraction 1) was opalescent and yellow-greenish in color and the precipitate, which consisted of insoluble material, was discarded. 2) The pH of fraction 1 was increased from 5.4 to 9.0 by slow addition of 0.1 M sodium hydroxide with constant agitation, and then centrifuged at 12,000 x g for 10 min at room temperature. The clear supernatant (fraction 2) was placed in the first vessel connected with another through silicon hose. In the second flask were added 30 ml of distilled water. By bubbling with nitrogen in the first flask and then passing through distilled water, an absorbance measure was read on a spectrophotometer (Ultrospec 1000 Pharmacia). The fraction 2 was placed in an ice bath and kept in an ice box. 3) After 72 hr in an ice bath (0 ^{o}C), papain precipitated spontaneously (1st crystallization) and was collected by centrifugation at 12,000 x g for 20 min at 0 ^{o}C (fraction 3). 4) The fraction 3 was washed three times with a minimal necessary amount of 1 mM EDTA, pH 7.0, at 4^{o}C. Then collected by centrifugation at 12,100 x g for 20 min at 0^{o}C (fraction 4). 5) The fraction 4 was dissolved in a minimal necessary amount of 1 mM EDTA, pH 7.0, at 37 ^{o}C for 30 min at the proportion of 25 mg protein ml^{-1} EDTA and then placed in an ice bath for spontaneous precipitation (1st recrystallization). The precipitate was dissolved in the minimal necessary amount of 1 mM EDTA, pH 7.0, and stored at 4 ^{o}C (fraction 5). The entire procedure was carried out under bubbling with nitrogen for protection against atmospheric oxygen. Protein was measured by the method of Itzhaki & Gill (1964) and by absorbance at 280 nm.

Page. Non-denaturing electrophoresis was carried out by the method of Reisfield et al., 1962 for basic proteins, using 12% polyacrylamide gel, 34 mM ß-alanine buffer, pH 4.3, and a constant 4 mA current per tube. SDS-PAGE was carried out by the method of Laemmli, 1970 using 12% acrylamide. The samples were prepared in Tris-glicerol-β-mercaptoetanol and placed in a boiling water during 60 s. Gels were stained with Coomassie-Blue R-250 and Brilliant Blue G-colloidal concentrated by the method of Neuhoff, 1988.

Gel filtration of Sephadex G-75. To determine the molecular weight of papain obtained by the method described in this paper, a column (1.1 x 100 cm) of Sephadex G-75 was used, previously equilibrated with a 0.1 M sodium phosphate buffer, 1 mM EDTA, pH 7.0. The following patterns were used: anidrase carbonic (29 kDa), tripsinogen (24 kDa), cytochrome C (12.4 kDa), lysozyme (14.3 kDa) and classical papain obtained by Kimmel and Smith's method (1954). The exclusion volume (v_0) was determined with blue dextran.

Chromatography of fresh latex. Aliquots of proteins dissolved in 0.4 M sodium acetate buffer, pH 5.0, were applied to a CM-cellulose column (1.5 x 20 cm) equilibrated with the same buffer. The material was eluted using a discontinuous gradient (0.4 to 1.0 M) of sodium acetate buffer, pH 5.0. The peaks obtained were delimited, pooled and dialyzed against 1 mM EDTA at 4 ^{o}C with three successive changes of 10 hr each. After dialysis, they were lyophilized and then submitted to electrophoresis on acid gels.

Measurement of sulfhydryl groups. Sulfhydryl groups were determined by the method of Ellman. The reaction of papain with 5,5' - ditiobis - (2-nitrobenzoic) (DTNB) was buffered with 0.1 M sodium phosphate, pH 8.0, and monitored spectrophotometrically at 412 nm, 25 ^{o}C. The molar extinction coefficient of 13,600 was used for the DTNB reaction. When activated with DTE, papain samples were separated from the activator by gel filtration on Sephadex G-25 and were eluted with 0.1 M sodium phosphate buffer, pH 8.0, and 1 mM EDTA under a nitrogen atmosphere.

Enzymatic assays. Papain activity was determined with N-carbobenzoxiglycil p-nitrophenyl ester (Z-Gly-pNP) by the method of (Kirsch & Igelström, 1966). The reaction was monitored at 400 nm and 25 ^{o}C in 0.1 M sodium phosphate buffer and 1 mM EDTA, pH 7.0, plus 6.7 % acetonitrile and ionic strength was adjusted to 0.3 M with KCl. Corrections for spontaneous hydrolysis were made. Specific activity is defined as µmol of p-nitrophenol produced per min and per mg protein under the above conditions. The molar extinction coefficient of p-nitrophenol was calculated as 9,368 M^{-1} cm^{-1}. Papain activity was also determined using α-N-benzoyl-L-arginin ethyl ester (BAEE) as substrate (Davis & Smith, 1955, Jacobsen et al., 1957). The rate of hydrolysis was measured by titration from the amount of base (1 M Tris) added to the unbuffered solution of enzyme and substrate in order to maintain a given pH value during the reaction. The reactions were carried out in a thermostatic cuvette at 25 ^{o}C. A Beckman model SS-2 potentiometer sensitive to variations of 0.01 units in pH was used and Tris was added with a Nimetrics microsyringe capable of measuring amounts of up to 0.1 µl. The solution was thoroughly mixed by constant shaking and protected against atmospheric molecular oxygen by constant bubbling with nitrogen. The initial rate of reaction was calculated directly from the linear portion of the curves for substrate hydrolysis as a function of time and the catalytic constant for BAEE was determined by the ratio between maximum velocity and active enzyme concentration.

RESULTS AND DISCUSSION

Extraction of fresh latex. Maximum latex flow was obtained between 5:00 and 10:00 a.m. After latex extraction, fruits weighing 1 to 2 kg ripen and fruits weighing 200 to 300 g stop developing, many of them deteriorating. Medium-sized soil supply fruits weighing 0.5 to 1.0 kg are those containing the largest amounts of latex. A new latex extraction can be made 5 to 6 days later, but smaller amounts are obtained. We noted that for papaya trees growing on damp, the latex obtained was highly diluted with low papain concentrations. Using a total of 176 fruits we obtained 1524 g of latex, with a mean value of 9 g latex per fruit.

Papain crystallization, electrophoresis and gel filtration. When fresh latex is bubbled with nitrogen for 1 hr at room temperature under constant shaking, a volatile substance is released which absorbs light at 250 nm. When present in the latex extract (fraction 2) this substance prevents the spontaneous precipitation of part of the papain, that wasn't identifield (Monti, 1983). A total of 1396 mg papain was obtained from 927 g fresh latex, with a value of 1.51 mg papain per g latex. When fraction 2 was treated by bubbling with nitrogen, 1238 mg papain was obtained from 510 g fresh latex, for a total of 2.43 mg papain per g latex, with a consequent improvement in spontaneous papain crystallization. Electrophoresis of fresh latex on acid gel (fraction 2) revealed the presence of seven protein components migrating toward the cathode (Fig. 1, column 1, 4 and 6). Two proteins bands were detected in sample of fraction 4 (column 2 and 3) and electrophoresis of fraction 5 (column 5) showed a single protein band. This protein component had a relative mobility of 0.5 $\pm$ 0.03, corresponding to band 3 of the column 1, 4 and 6 and was identified as papain since papain prepared in our laboratory by the classical method (Kimmel & Smith, 1954) has the same relative mobility. Gel electrophoresis denaturing of papain obtained by the method described here showed only one band (results not showed). The pure papain exhibited aparent molecular masses of 21 kDa and the classical papain 21,3 kDa, when G-75 Sephadex was used (Fig.2). Throught of methodology that we used in this work, was concluded that the papain prepared here have the same properties of

the papain obtained from classical method.

Analysis of fresh latex. Chromatography on CM-cellulose using a discontinuous sodium chloride gradient (Fig. 3) yielded a reproducible pattern of protein elution (Basílio, 1987). The chromatography of fresh latex revealed the presence of 5 protein components. Since in an identical experiment (Schack, 1967) peak 2 was characterized as papain, the fraction corresponding to peak 2 in Fig. 3 was pooled, concentrated with ammonium sulfate (600 g /l), dialyzed and submitted to electrophoresis of acid gel, revealing a single protein band with the same mobility as band 3 on the total latex gel (Fig. 1, column 1). An additional electrophoretic run was then carried out on acid gel using a mixture of peak 2 with the protease extracted by the method described in the present paper (Fig. 4, gel 1) and a mixture of peak 2 with papain extracted by the classical method (Fig. 4, gel 2). Thus, we conclude that the protease obtained by the method described here is provenly the papain and the methodology here described adds to the ones already in use.

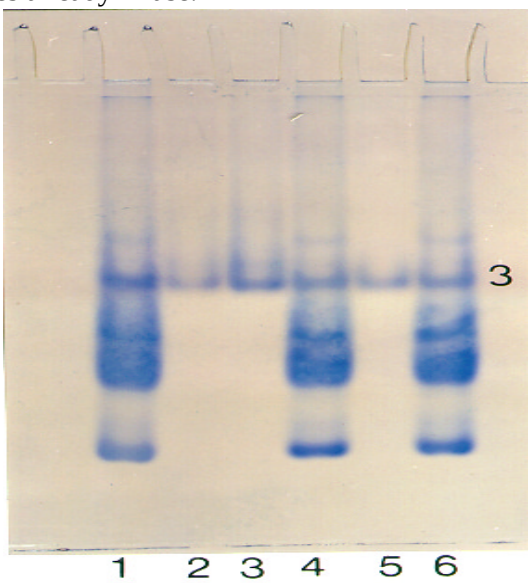

Figure 1 - Nondenaturing polyacrylamide gel electrophoresis using 12% acrylamide gel in the β-alanine acetic acid buffer at pH 4.3. Staining was by Brilliant Blue G-colloidal. In the columns 1, 4 and 6, were added aliquot of the fraction 2 (crude latex). In the column 2 and 3, aliquot of fraction 4. In the column 5, aliquot of fraction 5 (recrystalization). The purity of the band 3 was confirmed electrophoresis and immunologic assay (Basílio, 1993).

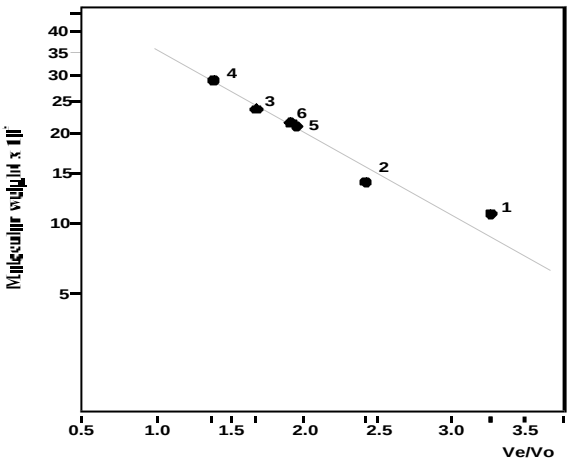

Figure 2 - Molecular weight estimation of papain by Sephadex G - 75 gel filtration colunm (1.1 x 100 cm), equilibrated with 0,1 M sodiun phosphate buffer , EDTA 1 mM, pH 7,0, flow rate 20 mL/hour at room temperature. 1) Cytochrome c; 2) Lysozym; 3) Tripsinogen; 4) Carbonic Anhydrase; 5) Papain from Kimmel & Smith, 1954; 6) Papain from our methodology

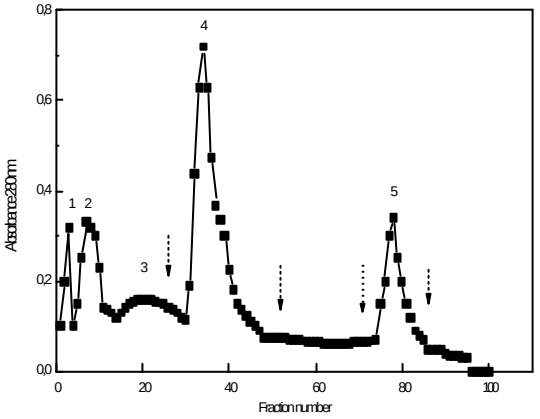

Figure 3 - CM-cellulose Chromatography of crude latex of the Carica papaya. The column (1,5 x 20 cm) after equilibrated with 0.4 M sodium acetate buffer, pH 5.0, was added 60mg of the protein, and eluted with 0.4, 0.6, 0.8 and 1.0 M with the same buffer at a flow rate of 30 mL/h. Fraction of 9 mL were collected.

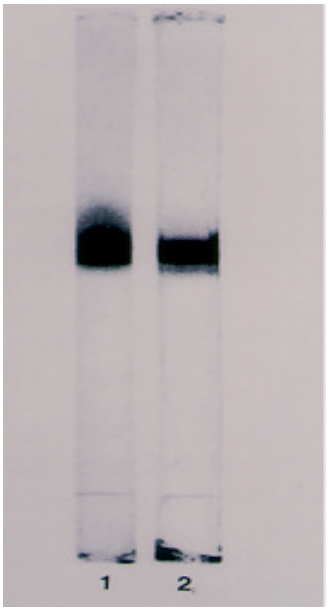

Figure 4 - Non-denaturing polyacrylamide gel electrophoresis using 12% acrylamide gel and ß-alanine buffer, pH 4.3. Staining was by Coomassie Blue R250. Gel 1 - Mixture of peak 2 with classic papain. Gel 2 - Mixture of peak 2 with the papain extracted by this method.

Number of sulfhydryl groups per mol papain. The number of free sulfhydryl groups in the papain prepared by the method described here was determined with 5,5'-dithiobis-(2-nitrobenzoic) acid (DTNB). Different numbers of sulfhydryl groups have been reported in the literature. Finkle & Smith, 1958, Glazer & Smith, 1965 determined to the papain by classical method 0.5 mol SH per mol papain. Surprisingly, the number of sulfhydryl groups in papain obtained by the present method was 1.5 mol SH per mol papain. However, freshly prepared papain filtered through Sephadex G-25 or activated with dithioerythritol (DTE) and then filtered presented a mean value of 0.76 mol SH per mol papain, result also obtained for Jorge, 1977, showing, this way, the liberation of some reducible substances. The enzyme dialysed twice against distilled water presented a slightly lower value due to the oxidation of sulfhydryl groups. The mean value detected for the dialysed enzyme was 0.62 SH per mol enzyme, being detected the liberation of the natural inhibitory of papain during the purification process.

Assay of enzyme activity. Daily kinetic studies of papain were performed using carbobenzoxyglycyl p-nitrophenyl ester (Z-Gly-pNP) as substrate because of the rapid and easy preparation, the possibility of working under conditions of saturation with the substrate and finally because of the small amounts of enzyme (approximately 20 µg) needed for an assay. We carried out a systematic study of the kinetics of papain because we had modified the method for purification and also because the prepared papain presented a hight value of SH per mol enzyme. Table 1 shows the results obtained with three different preparations. We determined the activity of freshly prepared papain without treatment, of freshly prepared papain filtered through Sephadex G-25, and of freshly prepared papain activated with DTE and then filtered through Sephadex G-25. Assuming that the number of SH groups of untreated papain (0.76) is equal to the number detected in the enzyme filtered through Sephadex G-25, it can be seen that the $Kcat.s^{-1}$ $(mol\ SH)^{-1}$ was not modified when compared to the value obtained for papain filtered through Sephadex G-25. However, treatment with DTE did not lead to a great increase in $Kcat.s^{-1}$ $(mol\ SH)^{-1}$. The most probable hypothesis to explain these results may be the presence of natural inhibitors forming a complex with the enzyme prepared by this method and released from the enzyme during DTE treatment and filtration. On the basis of these results, we carried out an experiment in which catalytic activity was measured at different enzyme concentrations (Table 2). A 55% increase in $Kcat.s^{-1}$ $(mol\ SH)^{-1}$ was observed when the enzyme concentration was decreased, demonstrating once again the presence of an inhibitory substance. Enzymatic assays were also carried out using the synthetic substrate α-N-benzoyl-L-arginine ethyl ester (BAEE) as described by Davis & Smith, 1955 and modified by Jacobsen et al., 1957. Papain prepared by the present method was used for these assays and the enzyme was always treated with DTE for reduction of sulfhydryl groups and filtered through Sephadex G-25. Table 3 summarises the results obtained for four different preparations. In brief, the enzyme papain prepared by the method described in this work is the same the enzyme obtained by the classic method, with the advantage of not receiving treatment for salts in high concentrations (or thiol-containing substances), providing like this, papain with minimum alterations in its kinetic properties. This preparation method allowed to detect the presence of inhibitor substances forming a

complex with the papain. They were isolated and separated by chromatography in SP Sephadex C-25 three inhibitor substances, of small molecular masses, and the results will be presented later on. A possible hypothesis is that the complex formation with one of these inhibitors can induce alterations in the results of the catalytic constant and number of sulfhydryl groups per mol papain.

Table 1 - Esterase activity of papain at different treatments

Preparation	Treatment	$Kcat.s^{-1}$	$Kcat.s^{-1}$
		mol protein	mol SH
1	None	6.00	7.90
	G-25	5.74	7.55
	DTE and G-25	7.85	10.30
2	None	3.90	5.20
	G-25	4.00	5.33
	DTE and G-25	7.30	9.73
3	None	5.20	6.50
	G-25	5.00	6.25
	DTE and G-25	7.71	9.64

The following conditions were used: kcat was determined using the substrate Z-Gly-pNP 2 x 10^{-4} M, enzyme concentration of 2 x 10^{-8} M in 0.1 M sodium phosphate buffer and 1 mM EDTA, pH 7.0; ionic strength 0.3, and acetonitrile 6.7%; temperature, 25°C.

Table 2 - Esterase activity measured at different papain concentrations

Enzyme concentration in the reaction	$Kcat.s^{-1}$ mol protein	$Kcat.s^{-1}$ mol SH
2.30 x 10^{-8}M	3.10	4.08
1.74 x 10^{-8}M	3.40	4.48
1.17 x 10^{-8}M	4.00	5.28
5.92 x 10^{-9}M	4.80	6.32

The following conditions were used: kcat was determined using 2 x 10^{-4} M Z-Gly-pNP as substrate, in 0.1 M sodium phosphate buffer and 1 mM EDTA, pH 7.0; ionic strength 0.3, and acetonitrile 6.7%; temperature, 25°C

Table 3 - Determination of Km, Vmax and Kcat using BAEE as substrate

Preparation	Km (μM)	V_{max}(μmols.L^{-1}.s^{-1})	$Kcat.s^{-1}$ mol SH
1	17.9	22.20	28.5
2	20.6	20.40	28.0
3	20.0	22.70	28.1
4	20.8	23.80	29.0

The following conditions were used: BAEE in 0.3 M KCl, pH maintained at 6.0 with 1.0 M Tris at 25°C. Enzymatic concentration from 1 to 2 x 10^{-6} M, activated with DTE and filtered through Sephadex G-25.

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RESUMO

No presente trabalho apresenta - se um método de cristalização da papaína oriunda do látex fresco de mamão, o qual apresenta uma alta produtividade em relação aos métodos previamente descritos. A metodologia aqui descrita não envolve o uso de reagentes sulfidrílicos, a papaína foi obtida de forma praticamente pura, apresentando uma simples banda quando submetida a eletroforese, e com propriedades idênticas àquelas obtidas por outros métodos. A atividade específica foi determinada utilizando Z-gly-pNP e BAEE como substrato. A papaína obtida por essa metodologia, sem uso de substâncias tais como cisteína e ditiotreitol, apresenta - se na forma de um complexo com inibidores naturais, os quais podem ser removidos por diálise.

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