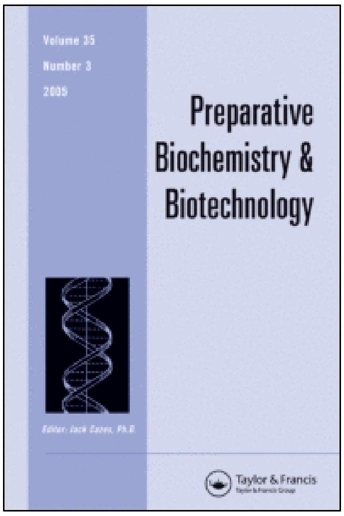

Preparative Biochemistry and Biotechnology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597291

SODIUM TETRATHIONATE EFFECT ON PAPAIN PURIFICATION FROM DIFFERENT *Carica papaya* LATEX CRUDE EXTRACTS

Carlos R. Llerena-Suster[a]; Nora S. Priolo[a]; Susana R. Morcelle[a]
[a] Laboratorio de Investigación de Proteínas Vegetales (LIPROVE), Depto. Cs. Biológicas, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, La Plata, Argentina

Online publication date: 26 March 2011

To cite this Article Llerena-Suster, Carlos R. , Priolo, Nora S. and Morcelle, Susana R.(2011) 'SODIUM TETRATHIONATE EFFECT ON PAPAIN PURIFICATION FROM DIFFERENT *Carica papaya* LATEX CRUDE EXTRACTS', Preparative Biochemistry and Biotechnology, 41: 2, 107 — 121

To link to this Article: DOI: 10.1080/10826068.2011.544230

URL: http://dx.doi.org/10.1080/10826068.2011.544230

Preparative Biochemistry & Biotechnology, 41:107–121, 2011

ISSN: 1082-6068 print/1532-2297 online
DOI: 10.1080/10826068.2011.544230

SODIUM TETRATHIONATE EFFECT ON PAPAIN PURIFICATION FROM DIFFERENT *Carica papaya* LATEX CRUDE EXTRACTS

Carlos R. Llerena-Suster, Nora S. Priolo, and Susana R. Morcelle
Laboratorio de Investigación de Proteínas Vegetales (LIPROVE), Depto. Cs. Biológicas, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, La Plata, Argentina

□ *Papain from latex of* Carica papaya *was purified up to matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry homogeneity by salt precipitation from two different crude extract sources: a refined preparation obtained in our laboratory and a commercial one. Sodium tetrathionate was tested in the purification process to preserve the enzymatic activity of the peptidase. Purification was checked by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) and cation exchange chromatography, using commercial pure papain as standard for a rapid comparison. The best purification yields (3.4%) were obtained in presence of 30 m*M *sodium tetrathionate for the crude extract prepared in our laboratory. The described purification method proved to be robust and reliable to obtain pure papain on a preparative scale.*

Keywords papain, plant cysteine peptidases, protein purification, sodium tetrathionate

INTRODUCTION

Papain is the most well-known plant peptidase due to its powerful proteolytic activity. This ability is the base of its multiple uses. It is obtained from the latex of unripe papaya fruit (*Carica papaya, Caricaceae*), a species originally from the tropical and subtropical areas of the Americas and extended throughout the tropical regions of the world for its commercial importance. Traditional application of papain mainly involves meat tenderizing, thus helping the digestion of proteins. Industrial uses of papain range from leather tanning to food manufacture as a softener in bakery, as a clarifying agent for beer and wine ferment, and in other food industries processes in which breakdown of proteins is needed. Papain is also an ingredient of laundry powder soaps.[1,2] Medicinal uses of papain include digestion promotion in digestive capsules, antiparasite action, debridement

Address correspondence to Susana R. Morcelle, LIPROVE, Fac. Cs. Exactas, UNLP, CC 711, La Plata (1900), Argentina. E-mail: morcelle@biol.unlp.edu.ar

of necrotic tissue from ulcers and burn wounds, teeth whitening, etc.[3] However, topical drugs containing papain (such as ophthalmic balanced salt solutions and ointments to treat acute and chronic lesions) were recently subjected to prohibition by the Food and Drug Administration (FDA) due to reports indicating allergic reactions, hypotension, tachycardia, permanent vision loss, etc.[4] Other uses of papain are in the dissociation of cells in the first step of cell culture preparations and the scission of the Fc portion of immunoglobulins from the Fab (antigen-binding) portion.[5]

Crude extract from papaya latex contains four endopeptidases, namely, papain, chymopapain, caricain, and glycyl endopeptidase. The isolated papain itself (EC 3.4.22.2) is the most acidic proteolytic fraction of the papaya latex extract.[6] The term "papain" itself is rather misleading, because it refers both to the crude protelytic extract obtained from a basic step of refinement, containing the four endopeptidases already mentioned, and to the pure proteolytic enzyme.

Many approaches were performed to purify papain from the other components present in the crude protelytic mixture refined from latex. These methods are: salt precipitation using $(NH_4)_2SO_4$[7] and NaCl,[8] precipitation followed by affinity chromatography,[9] hydrophobic and ionic exchange chromatography,[6] precipitation at low temperature,[10] aqueous two-phase extraction using PEG and $(NH_4)_2SO_4$,[11] and by adsorption onto membranes.[12]

Bearing in mind that all these peptidases have a cysteine residue in the catalytic site, it is important to keep it in its active form, i.e., to avoid the irreversible oxidation of the –SH responsible for catalysis. Reducing agents such as SO_3^{2-}, 1,4-dithio-D,L-threitol, and cysteine itself, among others, were used during all steps of purification for this purpose. Reversible thiol blocking agents can be used as well: Methylmethanethiol sulfonate, 2,2′-dipyridyl disulfide, or tetrathionate ions convert them into mixed disulfide bonds, which can be regenerated as free –SH by addition of the reducing agents already mentioned.[9] The use of these kinds of reversible inhibitors can also minimize the risk of peptidases autodigestion. Sodium tetrathionate displayed the best protective effect for all these purposes, since 96% of initial activity can be recovered using this chemical to stabilize papain in crude preparations.[13]

In this opportunity, purification of papain is achieved on the bases of the salt precipitation method described by Baines and Brocklehurst[8] with some modifications, which include the use of sodium tetrathionate to maximize the recovered activity of the enzyme. The same purification method was made without tetrathionate for comparison. Two different sources of papain were employed: commercial papain crude powder, and hydrosoluble crude papain obtained in our laboratory. The purification steps were monitored by traditional methods, such as electrophoresis and ionic exchange chromatography determinations, as well as by matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS), to evaluate the purity achieved.

EXPERIMENTAL

Acronyms

The following acronyms are used in this article: BSA, bovine serum albumin; CPE, commercial crude papain extract; CPs, cysteine peptidases; CV, column volume; E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino) butane; EDTA, ethylendiamine tetraacetic acid; 4-HCCA, α-cyano-4-hydroxycinnamic acid; HPE, hydrosoluble crude papain extract obtained in our laboratory; IEF, isoelectric focusing; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; TT, sodium tetrathionate; and Tris, tris-(hydroximethyl)-aminomethane.

Chemicals

Casein (Hammarsten type) from bovine milk, Tris, agarose, cysteine, and BSA were obtained from Sigma. Tricine, sodium iodoacetate, Coomassie brilliant blue R-250 and G-250, acrylamide, bisacrylamide, and low-molecular-weight markers were obtained from Bio-Rad. Commercial crude papain powder (3.11 units/mg solid, 1 unit hydrolyzes 1.0 μmol of N^{α}-benzoil-L-arginine ethyl ester chloride [BAEE] per minute at pH 6.2 at 25°C) was from Fluka. Pure papain suspension (≥30 U/mg protein) was obtained from Roche. E-64 was from Bachem. All other chemicals were obtained from commercial sources and were of the highest purity available.

Purification of Papain

Papain was purified following the method described by Baines and Brocklehurst in the absence and presence of different concentrations of TT (1 m*M* and 30 m*M*). Two different sources of papain were used: CPE and HPE.

HPE Preparation

Latex from unripe *Carica papaya* fruits was collected in Jujuy province, Argentina, dried under controlled conditions of time and temperature and sent to our laboratory to obtain a refined hydrosoluble papain extract (HPE) with a final activity of 50,000 USP units/mg as described by López et al.[14]

Papain Salt Precipitation

One gram of CPE or HPE was dissolved in 10 mL of solution A (see Table 1) and mixed under magnetic stirring for 30 min at room temperature. In the case of CPE, an opalescent suspension was obtained. The pH levels of CPE and HPE mixtures were adjusted to 9.0 with NaOH and the resulting precipitates were eliminated by centrifugation (9600 × g, 30 min, 4°C). $(NH_4)_2SO_4$ up to 45% saturation was added to both supernatants, and after 20 min at 4°C in an orbital shaker (180 rpm), the supernatants were separated by centrifugation and discarded, whereas both precipitates were resuspended in 10 mL of solution A. $(NH_4)_2SO_4$ up to 40% saturation was added. The whole process was repeated as for the first precipitation step with $(NH_4)_2SO_4$. The resulting precipitates were resuspended in 10 mL of buffer B (Table 1) and NaCl was added up to 10% (w/v), and the process was repeated as described for the other precipitation steps. The precipitates were resuspended in 4 mL of buffer C (Table 1). The solutions were left at room temperature for 30 min and then stored at 4°C for 18 h. The insoluble material was separated by centrifugation, redissolved in solution D (Table 1), and the supernatant was discarded.

Protein Quantification

Protein content of crude extracts and the final purification products was measured according to Bradford's method,[15] using a curve of BSA as standard.

TABLE 1 Papain Purification Conditions Assayed for HPE and CPE

Assay	Sample	Solution A	Buffer B	Buffer C	Solution D
1a	HPE	20 m*M* Cys 1 m*M* EDTA	0.1 *M* phosphate, pH 7.5 20 m*M* Cys 5 m*M* EDTA	0.1 *M* phosphate, pH 6.5 20 m*M* Cys 5 m*M* EDTA	Deionized water
1b	CPE	20 m*M* Cys 1 m*M* EDTA	0.1 *M* phosphate, pH 7.5 20 m*M* Cys 5 m*M* EDTA	0.1 *M* phosphate, pH 6.5 20 m*M* Cys 5 m*M* EDTA	Deionized water
2a	HPE	1 m*M* TT 1 m*M* EDTA	0.1 *M* phosphate, pH 7.5 1 m*M* TT 5 m*M* EDTA	0.1 *M* phosphate, pH 6.5 1 m*M* TT 5 m*M* EDTA	Deionized water
2b	CPE	1 m*M* TT 1 m*M* EDTA	0.1 *M* phosphate, pH 7.5 1 m*M* TT 5 m*M* EDTA	0.1 *M* phosphate, pH 6.5 1 m*M* TT 5 m*M* EDTA	Deionized water
3a	HPE	30 m*M* TT 1 m*M* EDTA	0.1 *M* phosphate, pH 7.5 30 m*M* TT 5 m*M* EDTA	0.1 *M* phosphate, pH 6.5 30 m*M* TT 5 m*M* EDTA	Solution A (assay 3)
3b	CPE	30 m*M* TT 1 m*M* EDTA	0.1 *M* phosphate, pH 7.5 30 m*M* TT 5 m*M* EDTA	0.1 *M* phosphate, pH 6.5 30 m*M* TT 5 m*M* EDTA	Solution A (assay 3)

Determination of Proteolytic Activity

Measure of enzymatic activity of crude extracts and the purified enzymes was performed using casein as substrate according to Priolo et al.[16] For the caseinolytic activity determination, the reaction mixture consisted on 0.1 mL of sample and 1.1 mL of 1% casein containing 5 m*M* cysteine, in 0.1 *M* Tris-HCl buffer (pH 8.0). The reaction was carried out at 37°C and stopped after 10 min by addition of 1.8 mL of 5% TCA. The absorbance of the supernatant was measured at 280 nm after centrifugation at 3900 × g for 10 min in an Agilent 8453E ultraviolet (UV)–visible spectroscopy system. Caseinolytic activity was expressed in terms of the caseinolytic unit (U_{cas}), defined as the amount of protease that produces an increment of 1 absorbance unit per minute in the assay conditions.

For the samples containing TT, a preincubation step (5 min at room temperature) in the presence of 20 m*M* Cys in distilled water of a convenient diluted sample (1:100 in the case of the crude extracts and 1:25 for the purified papain) was performed before caseinolytic activity determination.

SDS PAGE-Determinations

Samples of HPE, CPE, the purification products, and commercial pure papain were precipitated with cold acetone, redissolved in sample buffer containing SDS and β-mercaptoethanol, inhibited with 30 m*M* sodium iodoacetate, and submitted to denaturing SDS-PAGE using tricine buffer according to Shägger and von Jagow.[17] The running conditions were 40 V for the stacking gel and 150 V for the resolution gel. After the electrophoretic run, the resulting gels were stained using the Coomassie colloidal method.[18] Molecular weight of the protein bands was estimated by using the Scion Image software.[19]

Cation-Exchange Chromatography

Samples of purified papain, as well as the different crude papain extracts, were submitted to a chromatrographic analysis in an Äkta Purifier 10 (GE Healthcare) by cation exchange chromatography. Samples (100 μL) were loaded onto a Resource S column (1 mL, GE Healthcare). Chromatographic conditions were: mobile phase A, 0.1 *M* acetic acid-sodium acetate buffer (pH 5.5); mobile phase B, 0.1 *M* acetic acid–sodium acetate buffer (pH 5.5) with 0.9 *M* NaCl. Flow rate was 0.5 mL/min. Elution of proteins was achieved in steps: 2 CV (column volumes) with 0% B; 22% B (2 CV); 25% B (2 CV); 25% to 100% B linear gradient (1 CV); and a final wash step with 100% B (2 CV). Detection was made at 280 nm.

Mass Spectrometry Analysis of Purified Papain

MALDI–TOF MS was used for the determination of the molecular masses, as well as the degree of purity of purified papain in the presence of 30 m*M* TT. One milligram was precipitated twice with cold acetone and redissolved in deionized water to eliminate salts, inhibited with E-64 (30 m*M*), and liophylized. Mass spectrometry was acquired on an Applied Biosystems 4800 analyzer in linear positive ion mode using sinapinic acid as matrix for the sample. Proteins of known molecular mass were used as standards for mass calibration.

RESULTS AND DISCUSSION

Papain, the most acidic peptidase from *Carica papaya* latex, was purified according to the method described by Baines and Brocklehurst[8] with some modifications. This method was selected among others because of its applicability at industrial scale. The modifications assayed in this opportunity include the use of sodium tetrathionate (TT), which is known as a reversible inhibitor of cysteine peptidases (CPs). TT forms disulfide bonds with the free thiol groups of proteins; in this case, the disulfide bond is formed with the catalytic –SH of proteases active sites (Figure 1).

The use of TT has two main goals: to protect the catalytic cysteine from irreversible oxidation, and to avoid autodigestion of proteases. TT has the advantages of being not as toxic as other reversible inhibitors, like Hg^{2+} salts, relatively cheap, and easy to remove. On the other hand, it has been proved to be quite effective for the stabilization of papain preparations.[6,13] Two concentrations of TT were chosen in this option: 1 m*M* and 30 m*M*.

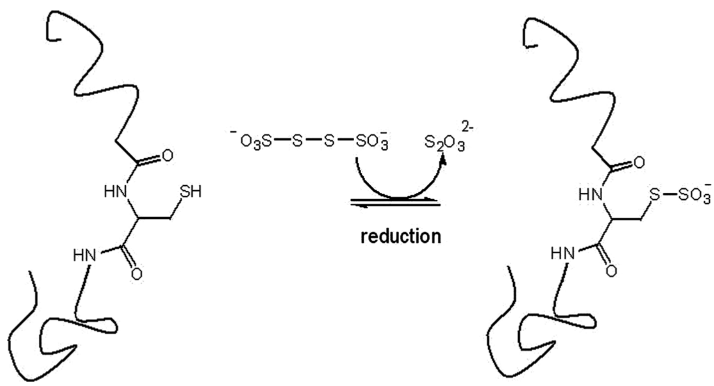

FIGURE 1 Mechanism of reversible inhibition of TT on CPs.

Purification Yields

Purification results can be seen in Tables 2a–2c. EDTA and Cys were added as protective and reducing agents, respectively (according to the original purification method[7,8]). As a general rule, better purification yields were obtained for the HPE preparation. The presence of unknown additives in CPE could be the reason for the lower yields of purification obtained in this case. Partial solubilization of CPE in the first step of the process was observed. The presence of insoluble material in CPE could be the reason for the lower yields in papain purification: After the centrifugation steps, part of the proteolytic enzymes present in CPE could co-precipitate, thus provoking lower yields. According to Tables 2a, 2b, and 2c, HPE rendered a better yield in pure papain; however, CPE gave a more active papain than the purified protease from HPE in absence of TT (Table 2a).

The influence of TT in papain purification depended on the concentration used. Using 1 m*M* TT, no significant improvement was observed not for HPE nor CPE (Table 2b). This could be due to two factors: (a) the absence of Cys in solution A, and (b) the concentration of TT, which in this case could have been not enough to protect all the active sites of the proteolytic enzymes. Both factors could have allowed the irreversible oxidation of the Cys present in the catalytic site of the endopeptidases, provoking even lower yields and lower activity recovered than in the first assay, i.e., the original method dscribed by Baines and Brocklehurst.[8] However, better specific activity and purification rates were obtained in the presence of 30 m*M* TT for both papain extracts (Table 2c). The use of TT was proved to be good for chromatographic CPs purification.[20] It was also demonstrated that TT resulted a good stabilizing agent for papain activity in papaya peels after drying overnight at 55°C when compared with other chemicals such as antioxidants (sodium ascorbate, sodium erythorbate, *t*-butyl hydroquinone, rutin, and α-tocopherol), polyphenol oxidase inhibitors (4-hexylresocinol), and other reducing sulfur-containing agents (sodium metabisulfite).[13] Although TT has been claimed as a potential antidote for cyanide poisoning,[21,22] it is also known that TT is not innocuous to be applied in human use.[21,23] However, it could be considered as less

TABLE 2a Papain Purification Results Without TT

	Total Protein (mg/g solid)		Specific Activity (Ucas/mg protein)		Purification (times)		Yield (%)	
	HPE	CPE	HPE	CPE	HPE	CPE	HPE	CPE
Initial crude material	321.5	299.1	4.1	3.5	—	—	100	100
Purified papain	16.0	5.6	4.5	8.2	1.1	2.0	5.0	1.9

TABLE 2b Papain Purification Results in the Presence of 1 m*M* TT

	Total Protein (mg/g)		Specific Activity (Ucas/mg protein)		Purification		Yield (%)	
	HPE	CPE	HPE	CPE	HPE	CPE	HPE	CPE
Initial crude material	254.6	323.5	4.0	3.2	—	—	100	100
Purified papain	1.9	0.4	3.3	1.9	0.8	0.6	0.7	0.1

harmful than other compounds (like those derived from Hg^{2+} salts) used to protect the –SH group of active cysteine and it is easy to remove by the action of reducing agents as Cys itself, all of which can be eliminated by a dialysis step. In this opportunity, the better concentration for papain purification was 30 m*M* TT for the hydrosoluble papain obtained in our laboratory. Interestingly, for commercial papain, the best results were obtained in absence of TT, probably due to the presence of other stabilizing agents that could interfere with TT and thus with papain purification, lowering the yields of the process (Table 2a). In this study, a quick preincubation with 20 m*M* Cys (5 min, room temperature) was performed before caseinolytic activity assay to revert TT inhibitory effect.

Considering that papain represents the 8% of the proteases present in papaya latex, the yields obtained in the different purifications (between 2% and 5%) are very attractive. It is important to point out that the apparent low purification rates are due to the fact that at least 50% of the proteins present in latex are peptidases in the case of *Carica papaya* latex,[24] and proteases count for more than the 80% of the whole enzyme fraction.[25] This was also proved for other proteases purified from plant lattices: Laticifers are organs where proteases represent the bulk of protein content of crude extract.[26–30] Papaya latex includes other enzymes and proteins such as glycosyl hydrolases (chitinases, lysozyme), a lipase (*Carica papaya* lipase, CPL), a glutaminyl cyclotransferase (PQC), and also protease inhibitors of the phytocystatine family and the Kunitz-type inhibitors,[25] all of which are present to a much lower extent. Some of these proteins are insoluble in aqueous media (for example, CPL), so they are eliminated in the very first steps of soluble crude papain preparation.[31]

TABLE 2c Papain Purification Results with 30 m*M* TT

	Total Protein (mg/g)		Specific Activity (Ucas/mg protein)		Purification		Yield (%)	
	HPE	CPE	HPE	CPE	HPE	CPE	HPE	CPE
Initial crude material	202.4	256.6	5.3	3.8	—	—	100	100
Purified papain	6.8	2.8	6.7	2.9	1.3	0.8	3.4	1.1

Electrophoretic Determinations

A first inspection of HPE and the purified papain SDS-PAGE (Figure 2) shows that the main protein fraction is comprised in the range of ∼23 kD (according to the analysis performed with Scion Image software[19]), which corresponds to the endopeptidases. Both crude proteolytic extracts, HPE and CPE, seemed to be good as sources for purification of papain. Comparison of the purified papain from both sources with the commercial standard in SDS-PAGE (Figure 3) showed a similar degree of purity.

Cation Exchange Chromatography

Many kinds of chromatographic separation were used to purify papain from the other components of papaya latex, especially different types of affinity chromatography (having ligands as different protease inhibitors, such as peptidic molecules,[32,33] organomercurial[6] or cyano[34] compounds, or electrophilic moieties having pyridyl disulfide derivatives[35,36]).

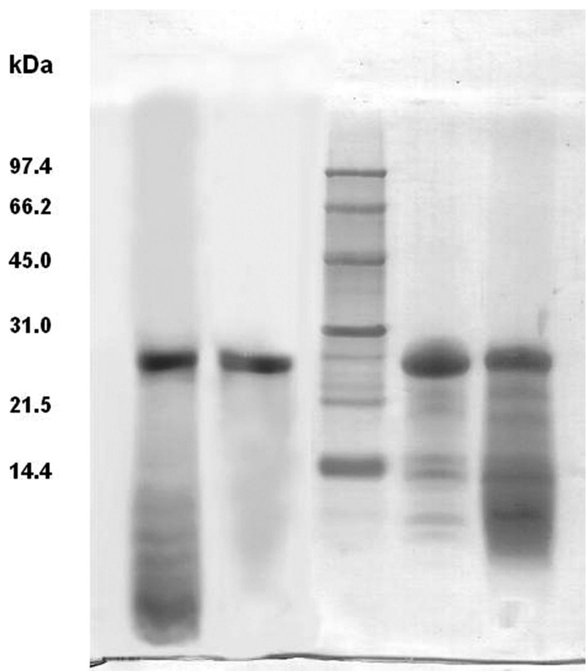

FIGURE 2 SDS-PAGE of purified papain. Lane 1: commercial pure papain. Lane 2: purified papain (assay 3a, see Table 1). Lane 3: Bio-Rad molecular mass markers: phosphorylase b, 97.4 kD; serum albumin, 66.2 kD; ovalbumin, 45.0 kD; carbonic anhydrase, 31.0 kD; trypsin inhibitor, 21.5 kD; and lysozyme, 14.4 kD. Lane 4: HPE with 30 m*M* TT. Lane 5: HPE without TT (assay 1a).

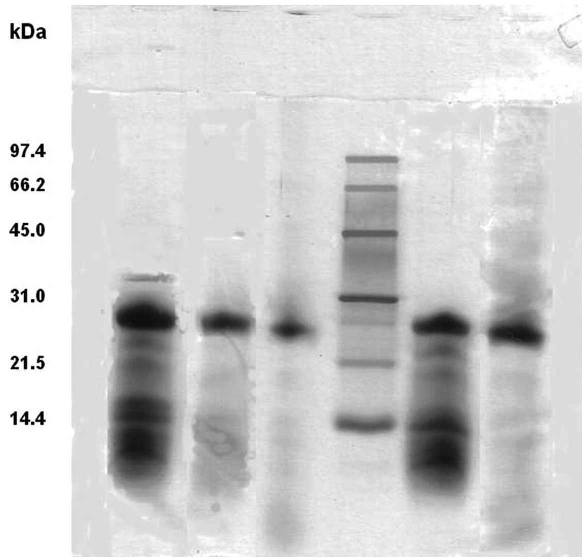

FIGURE 3 SDS-PAGE papain purification. Lane 1: CPE without TT. Lane 2: Commercial standard of pure papain. Lane 3: Purified papain from CPE (assay 1b). Lane 4: Low-molecular-weight standards (see Figure 2). Lane 5: HPE without TT. Lane 6: Purified papain from HPE (assay 1a).

Some of these methods are very interesting since they can separate fully active papain molecules from inactive ones.[9] Cation-exchange chromatography seems to be the most popular, due to its simplicity and effectiveness.[9] Nevertheless, this method allows the purification of small quantities of papain, which sometimes is not pure enough, as proved by SDS-PAGE.[6] On the other hand, all these methods are very unlikely to be applied in industry due to the difficulty in scaling up the process.

In this study, cation-exchange chromatography was used as a tool to ensure the purity of the purified papain. Chromatographic profiles of HPE with 30 m*M* TT, the purified papain according to assay 3, and commercial pure papain standard are shown in Figure 4. It is clearly seen that the peaks of the purified papain (Figure 4b) and the commercial pure standard (Figure 4c) have similar elution patterns.

Molecular Weight Determination

Mass spectrometry analysis by MALDI-TOF of papain purified from HPE revealed a homogeneous protein with molecular mass of 23,693.6875 Da (Figure 5). Although papain theoretical molecular mass is around 23,400 Da,[37] the shift to slight higher molecular masses could

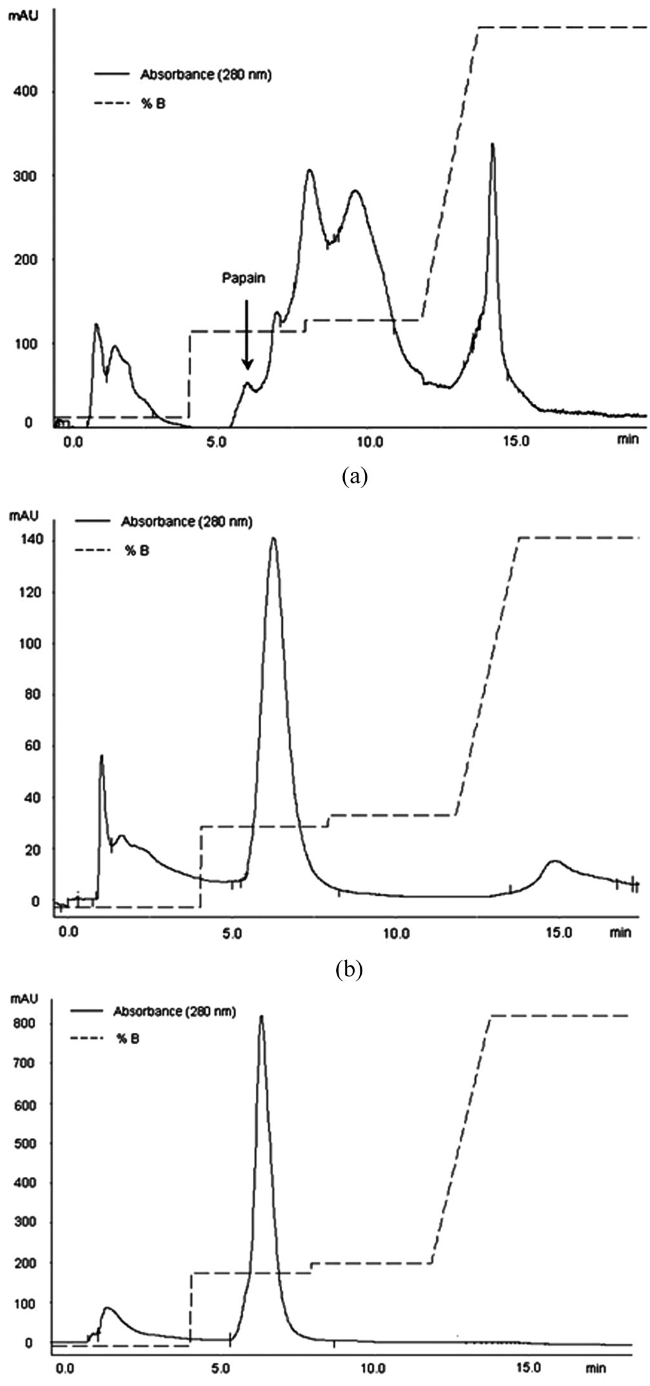

FIGURE 4 Cation exchange chromatography of (a) HPE with 30 m*M* TT; (b) purified papain according to assay 3 (Table 1); and (c) commercial pure papain.

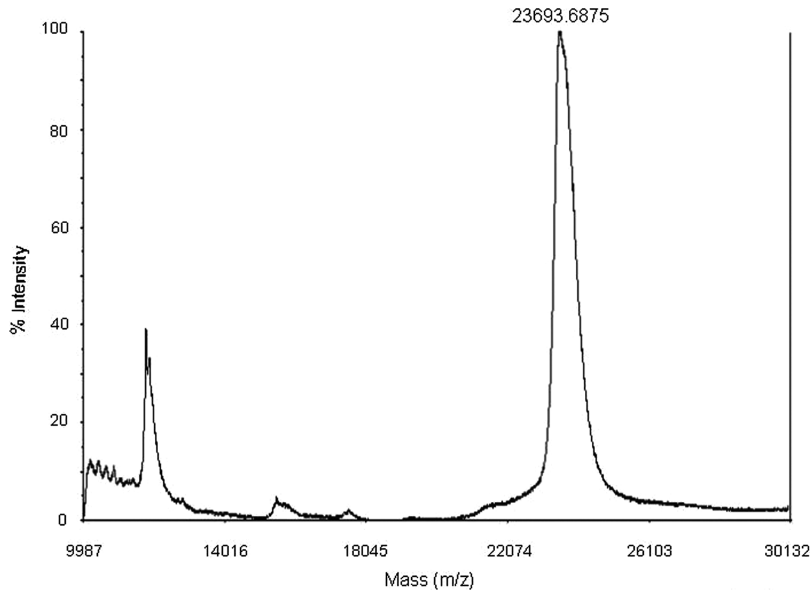

FIGURE 5 MALDI-TOF mass spectrometry of purified papain.

be due to the formation of the complex between the irreversible inhibitor E-64 (molecular weight: 357.4) and the enzyme.

CONCLUSIONS

The purification of papain was achieved by a very simple method from two different sources: a water-soluble latex extract obtained in our laboratory and a commercial papain extract. Two fractionation steps of precipitation with $(NH_4)_2SO_4$ (0.45 and 0.4 saturation, respectively), followed by a final step of precipitation with NaCl, were performed in the presence of different concentrations of sodium tetrathionate to preserve the enzymatic activity of the purified papain. It is important to point out that chymopapain is the most abundant endopeptidase in papaya latex. This was the papain purification method we chose due to its simplicity and because other methods, like different types of chromatography, are very unlikely to be applied in industry due to the difficulty in scaling up the process. Homogeneity of the purified product was verified by SDS-PAGE and MALDI-TOF mass spectrometry.

In order to avoid the active-site oxidation of purified papain and thus preserve its proteolytic activity, sodium tetrathionate (TT) was proved as a protective agent due to its ability to form a reversible disulfide bond with the thiol group of the active Cys. In this method, the best concentration for papain purification was 30 m*M* TT for the hydrosoluble papain obtained in our laboratory, furnishing yields of around 3% (6.8 mg/g of solid with the maximum activity). Interestingly, for commercial papain, the best results were obtained in the absence of TT (5.6 mg/g of solid with the maximum activity, 2% yield), probably due to the presence of other stabilizing agents that could interfere with TT and with papain purification, lowering the yield of the process. Better purification yields could be achieved by collecting the latex from the plant organs (fruits, stems, etc.) in the presence of TT to avoid oxidation from the very early steps. This procedure would stabilize the enzymes during the drying and transport stages to the places were refinement and purification processes are achieved.

ACKNOWLEDGMENTS

This work was supported by grants from ANPCyT (PICT number 38088) and Universidad Nacional de La Plata (project X-445). C.R.L.S. was awarded a CONICET fellowship. S.R.M. is a CONICET Assistant Researcher. MALDI-TOF mass spectrometry determinations were done at the Institut Pasteur de Montevideo (Uruguay). The authors thank Dr. Laura López and Julio R. Mercerat for HPE production and supply.

REFERENCES

1. Monge, A.; Barreiro, E.J.; Huenchuñir, P.; Pinzón, R.; Mora, G.; Núñez, A.; Chiriboga, X.; Cáceres, A.; Rivera, G.; Bocanegra-García, V.; Gupta, M.; Ferro, E.A.; Peralta, I.; Lock, O.; Flores, D.; Salazar, L.; Guzmán, O.D.; Cerezetto, H.; González, M. Functional Foods: Reflections on an Expanding Market. *Chem. Int.* **2008**, *30*(5), 9–13.
2. Uhlig, H. *Industrial Enzymes and Their Applications.* John Wiley & Sons, New York, 1998.
3. Telgenhoff, D.; Lam, K.; Ramsay, S.; Vasquez, V.; Villareal, K.; Slusarewicz, P.; Attar, P.; Shroot, B. Influence of Papain Urea Copper Chlorophyllin on Wound Matrix Remodeling. *Wound Rep. Reg.* **2007**, *15*(5), 727–735.
4. *FDA News*, September 23, 2008. FDA Warns Companies to Stop Marketing Unapproved Ophthalmic Balanced Salt Solution Drug Products and Topical Drug Products Containing Papain. http://www.fda.gov/bbs/topics/NEWS/2008/NEW01890.html, accessed December 2008.
5. Luo, Q.; Mao, X.; Kong, L.; Huang, X.; Zou, H. High-Performance Affinity Chromatography for Characterization of Human Immunoglobulin G Digestion With Papain. *J. Chrom. B* **2002**, *776*, 139–147.
6. Azarkan, M.; El Moussaoui, A.; van Wuytswinkel, D.; Dehon, G.; Looze, Y. (2003) Fractionation and Purification of the Enzymes Stored in the Latex of *Carica papaya*. *J. Chrom. B* **2003**, *790*, 229–238.
7. Kimmel, J.R.; Smith, E.L. Crystalline Papain. I. Preparation, Specificity, and Activation. *J. Biol. Chem.* **1954**, *207*, 515–531.
8. Baines, B.S.; Brocklehurst, K. A Necessary Modification to the Preparation of Papain from Any High-Quality Latex of *Carica papaya* and Evidence for the Structural Integrity of the Enzyme Produced by Traditional Methods. *Biochem. J.* **1979**, *177*, 541–548.

9. Burke, D.E.; Lewis, S.D.; Shafer, J.A. A Two-Step Procedure for Purification of Papain from Extract of Papaya Latex. *Arch. Biochem. Biophys.* **1974**, *164*, 30–36.
10. Monti, R.; Basilio, C.A.; Trevisan, H.C.; Contiero, J. (2000). Purification of Papain from Fresh Latex of *Carica papaya. Brazilian Arch. Biol. Technol.* **2000**, *43*(5), 501–507.
11. Nitsawang, S.; Hatti-Kaul, R.; Kanasawud, P. Purification of Papain from *Carica papaya* Latex: Aqueous Two-Phase Extraction Versus Two-Step Salt Precipitation. *Enzyme Microb. Technol.* **2006**, *39*, 1103–1107.
12. Nie, H.-L.; Chen, T.-X.; Zhu, L.-M. Adsorption of Papain on Dye Affinity Membranes: Isotherm, Kinetic, and Thermodynamic Analysis. *Sep. Purif. Technol.* **2007**, *57*, 121–125.
13. Espin, N.; Islam, M.N. Stabilization of Papain from Papaya Peels. *Food Sci. Technol. Int.* **1998**, *4*, 179–187.
14. López, L.M.I.; Mercerat, J.R.; Briones-Martínez, R. Refined and Stabilized Papain for Industrial Use. *Proceedings of the International Congress of Biotechnology and Agriculture BIOVEG 2005*, Ciego de Ávila, Cuba, February 7–11, 2005, CD ROM.
15. Bradford, M.M. A Rapid and Sensitive Method for the Quantitation of Micrograms Quantities of Protein Utilizing the Principle of Protein–Dye Binding. *Anal. Biochem.* **1976**, *72*, 248–254.
16. Priolo, N.; López, L.M.I.; Arribére, M.C.; Natalucci, C.L.; Caffini, N.O. New Purified Plant Proteinases for the Food Industry. *Acta Aliment.* **1991**, *20*(3–4), 189–196.
17. Schägger, H.; von Jagow, G. Tricine-Sodium Dodecyl Sulfate Polyacrilamide Gel Electrophoresis for the Separation of Proteins in the Range from 1 to 100 kDa. *Anal. Biochem.* **1987**, *166*(2), 368–379.
18. Neuhoff, V.; Arold, N.; Taube, D.; Ehrhardt, W. An Improved Procedure for Staining of Proteins Following Separation in Polyacrylamide Gels is Described Which Utilizes the Colloidal Properties of Coomassie Brilliant Blue G-250 and R-250. *Electrophoresis* **1988**, *9*(6), 255–262.
19. Scion Image Software. http: //www.scionimage.com, accessed October 8, 2008.
20. Takashasi, N.; Yasuda, Y.; Kashiko, G.; Tokiko, M.; Murachi, T. Multiple Molecular Forms of Stem Bromelin. *J. Biochem.* **1973**, *74*(*2*), 355–357.
21. Baskin, S.I.; Kirby, S.D. The Effect of Sodium Tetrathionate on Cyanide Conversion to Thiocyanate by Enzymatic and Non-enzymatic Mechanisms. *J. Appl. Toxicol.* **1990**, *10*(5), 379–382.
22. Hatch, R.C.; Laflamme, D.P.; Jain, A.V. Effects of Various Known and Potential Cyanide Antagonists and a Glutathione Depletor on Acute Toxicity of Cyanide in Mice. *Vet. Hum. Toxicol.* **1990**, *32*(1), 9–16.
23. Philips, F.; Gilman, A.; Koelle, E.S.; Allen, R.P. The Effect of Tetrathionate *In Vivo* and *In Vitro* on the Activity on Succinoxidase. *J. Biol. Chem.* **1947**, *167*, 209–217.
24. Boller, T. *Plant Proteolytic Enzymes*, Vol. 1; CRC Press, Boca Ratón, FL, 1986.
25. El Mossaui, A.; Nijs, M.; Paul, C.; Witjens, R.; Vincentelli, J.; Azarkan, M.; Looze, Y. Revisiting the Enzymes Stored in the Laticifers of *Carica papaya* in the Context of Their Possible Participation in the Plant Defence Mechanism. *Cell. Mol. Life Sci.* **2001**, *58*(4), 556–570.
26. Priolo, N.; Morcelle del Valle, S.; Arribére, M.C.; López, L.M.I.; Caffini, N. Isolation and Characterization of a Cysteine Protease from the Latex of *Araujia hortorum* Fruits. *J. Protein Chem.* **2000**, *19*(1), 39–49.
27. Obregón, W.D.; Arribére, M.C.; Morcelle del Valle, S.; Liggieri, C.; Caffini, N.O.; Priolo, N.S. Two New Cysteine Endopeptidases Obtained from the Latex of *Araujia hortorum* Fruits. *J. Protein Chem.* **2001**, *20*(4), 17–25.
28. Trejo, S.A.; López, L.M.; Cimino, C.V.; Caffini, N.O.; Natalucci, C.L. Purification and Characterization of a New Plant Endopeptidase Isolated from Latex of *Asclepias fruticosa* L. (*Asclepiadaceae*). *J. Protein Chem.* **2001**, *20*(6), 469–477.
29. Vairo Cavalli, S.E.; Arribére, M.C.; Cortadi, A.; Caffini, N.O.; Priolo, N.S. Morrenain b I, A Papain-like Endopeptidase from the Latex of *Morrenia brachystephana* Griseb. (*Asclepiadaceae*). *J. Protein Chem.* **2003**, *22*(1), 15–22.
30. Morcelle, S.R.; Trejo, S.A.; Canals, F.; Avilés, F.X.; Priolo, N.S. Funastrain c II: A Cysteine Endopeptidase Purified from the Latex of *Funastrum clausum. Protein J.* **2004**, *23*(3), 205–215.
31. Domínguez de María, P.; Sinisterra, J.V.; Tsai, S.-W.; Alcántara, A.R. *Carica papaya* Lipase (CPL): An Emerging and Versatile Biocatalyst. *Biotechnol. Adv.* **2006**, *24*(5), 493–499.
32. Blumberg, S.; Schechter, I.; Berger, A. The Purification of Papain by Affinity Chromatography. *Eur. J. Biochem.* **1970**, *15*, 97–102.

33. Albeck, A.; Kliper, S. Mechanism of Cysteine Protease Inactivation by Peptidyl Epoxides. *Biochem. J.* **1997**, *322*(3), 879–884.
34. Buttle, D.J.; Kembhavi, A.A.; Sharp, S.L.; Shute, R.E.; Rich, D.H.; Barrett, A.J. Affinity Purification of the Novel Cysteine Proteinase Papaya Proteinase IV, and Papain from Papaya Latex. *Biochem. J.* **1989**, *261*(2), 469–476.
35. Brocklehurst, K.; Carlsson, J.; Kierstan, M.P.J.; Crook, E.M. Preparation of Fully Active Papain from Dried Papaya Latex. *Biochem. J.* **1973**, *133*(3), 573–584.
36. Thomas, M.P.; Verma, C.; Boyd, S.M.; Brocklehurst, K. The Structural Origins of the Unusual Specificities Observed in the Isolation of Chymopapain M and Actinidin by Covalent Chromatography and the Lack of Inhibition of Chymopapain M by Cystatin. *Biochem. J.* **1995**, *306*(3), 39–46.
37. Mitchel, R.E.J.; Chaiken, I.M.; Smith, E.L. The Complete Amino Acid Sequence of Papain. Additions and Corrections. *J. Biol. Chem.* **1970**, *245*(14), 3485–3492.