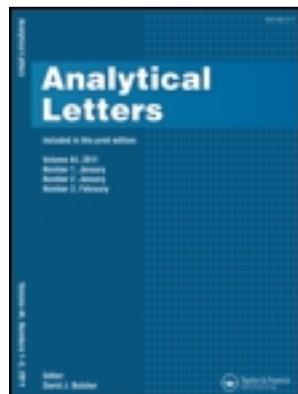


This article was downloaded by: [Susana R. Morcelle]

On: 13 August 2011, At: 11:31

Publisher: Taylor & Francis

Informa Ltd Registered in England and Wales Registered Number: 1072954 Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK



## Analytical Letters

Publication details, including instructions for authors and subscription information:

<http://www.tandfonline.com/loi/lanl20>

### Papain Purification Insights: Monitoring by Electrophoretic Approaches and MALDI-TOF Peptide Mass Fingerprint Analyses

Carlos R. Llerena-Suster<sup>a</sup>, W. David Obregón<sup>a</sup>, Sebastián A. Trejo<sup>b</sup> & Susana R. Morcelle<sup>a</sup>

<sup>a</sup> Laboratorio de Investigación de Proteínas Vegetales (LIPROVE), Departamento de Ciencias Biológicas, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, La Plata, Argentina

<sup>b</sup> Institut de Biotecnologia i Biomedicina "Vincent Villar i Palasi", Universitat Autònoma de Barcelona, Barcelona, Spain

Available online: 12 Aug 2011

**To cite this article:** Carlos R. Llerena-Suster, W. David Obregón, Sebastián A. Trejo & Susana R. Morcelle (2011): Papain Purification Insights: Monitoring by Electrophoretic Approaches and MALDI-TOF Peptide Mass Fingerprint Analyses, *Analytical Letters*, 44:12, 2124-2137

**To link to this article:** <http://dx.doi.org/10.1080/00032719.2010.546022>

PLEASE SCROLL DOWN FOR ARTICLE

Full terms and conditions of use: <http://www.tandfonline.com/page/terms-and-conditions>

This article may be used for research, teaching and private study purposes. Any substantial or systematic reproduction, re-distribution, re-selling, loan, sub-licensing, systematic supply or distribution in any form to anyone is expressly forbidden.

The publisher does not give any warranty express or implied or make any representation that the contents will be complete or accurate or up to date. The accuracy of any instructions, formulae and drug doses should be independently verified with primary sources. The publisher shall not be liable for any loss, actions, claims, proceedings, demand or costs or damages whatsoever or howsoever caused arising directly or indirectly in connection with or arising out of the use of this material.

## Bioanalytical

### PAPAIN PURIFICATION INSIGHTS: MONITORING BY ELECTROPHORETIC APPROACHES AND MALDI-TOF PEPTIDE MASS FINGERPRINT ANALYSES

Carlos R. Llerena-Suster,<sup>1</sup> W. David Obregón,<sup>1</sup>  
Sebastián A. Trejo,<sup>2</sup> and Susana R. Morcelle<sup>1</sup>

<sup>1</sup>Laboratorio de Investigación de Proteínas Vegetales (LIPROVE),  
Departamento de Ciencias Biológicas, Facultad de Ciencias Exactas,  
Universidad Nacional de La Plata, La Plata, Argentina

<sup>2</sup>Institut de Biotecnologia i Biomedicina “Vincent Villar i Palasi”,  
Universitat Autònoma de Barcelona, Barcelona, Spain

*Papain was purified from dried Carica papaya latex by fractioned salt precipitation in presence of sodium tetrathionate to preserve enzymatic activity. Purification was followed by different electrophoretic methods. Identification of the purified product was afforded by submitting the peptides obtained by tryptic digestion of papain to matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF/TOF MS) analysis. Comparison of the peptide masses analyzed by peptide mass fingerprinting (PMF) MALDI-TOF and those obtained by theoretical tryptic digestion, revealed the presence of some peptides belonging the other three endopeptidases contained in papaya latex (very similar to papain in molecular weight and pI) in the purified fraction of papain. The PMF by MALDI-TOF could be applied as a method to follow papain purification.*

**Keywords:** Caricain; Chymopapain; Glycyl endopeptidase; MALDI-TOF peptide mass fingerprint; Papain

## INTRODUCTION

Papain (EC 3.4.22.2) has been the most studied plant peptidase for many reasons and from many aspects. It is the prototype of the cysteine peptidases family that has its name (clan CA, family C1, according to the Merops classification system of proteases), as it is the model for the 3D structure and catalytic mechanism studies (Barrett 2001; Barrett, Rawlings, and Woessner 2004). Due to its powerful proteolytic activity,

Received 29 December 2009; accepted 21 August 2010.

The present study was supported by grants from ANPCyT (PICT N° 38088 and PICT N° 02224) and Universidad Nacional de La Plata (Project X-445). C. R. Llerena-Suster was awarded a CONICET fellowship.

Address correspondence to Susana R. Morcelle, Laboratorio de Investigación de Proteínas Vegetales (LIPROVE), Departamento de Ciencias Biológicas, Facultad de Ciencias Exactas, Universidad Nacional de La Plata. C.C. 711, B1900AWV, La Plata, Argentina. E-mail: morcelle@biol.unlp.edu.ar

papain is one of the most used enzymes in many industries, such as pharmaceutical, brewery, meat, dairy, textile, tanning, cosmetic, detergents, food, and leather. These facts turn papain into one of the most important peptidases in the worldwide market of enzymes. Raw papain (i.e., dry latex) is used for most of these applications. A further step of refinement removes the insoluble fraction of raw papain, mainly consisting in gums and other polysaccharides, to obtain a crude extract enriched in proteolytic enzymes, which consists of four endopeptidases: papain, chymopapain, caricain, and glycy endopeptidase. All endopeptidases are similar in their biochemical features, which makes them very difficult to purify. Each has a molecular weight of approximately 23 kDa, 212–218 amino acids, and very similar pI (from 8.75 to 11.7). The isolated papain itself is the most acidic proteolytic fraction of the papaya latex extract (Azarkan et al. 2003; El Mossaoui et al. 2001; Monge et al. 2008).

The similar properties of these peptidases turn their identification into a very difficult task after their purification in a fast and efficient manner. This could impact on the application of purified enzymes for medical use in humans, in which the degree of purification demanded is quite different to the one required for manufacturing purposes.

Electrophoretic methods are considered as a good analytical technique to resolve and separate the individual components of protein mixtures in general. However, papaya latex peptidases are very similar in the characteristics that consist on the basis of electrophoretic separations. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), for example, is able to characterize proteins in terms of their molecular size, whereas isoelectric focusing (IEF) is able to separate proteins according to their charge properties (Dunn 1989). In this sense, protein identification using mass spectrometry would be the most appropriate choice, and is typically accomplished by either PMF or amino acid sequencing of the peptide using tandem mass spectrometry (MS/MS). Both techniques consist on high-throughput approaches, a very important advantage when compared with other methods, such as western blotting or kinetic determinations in the case of enzymes (Damodaran et al. 2007; Obregón et al. 2009).

In this opportunity, purification of papain is achieved on the basis of the salt precipitation method described by Baines and Brocklehurst (1979) with some modifications, which include the addition of sodium tetrathionate to maximize the recovered activity of the enzyme. The same purification method was made without tetrathionate for comparison. The purification steps were monitored by traditional methods, such as electrophoresis, and by modern proteomic tools (e.g., peptide mass fingerprint, PMF) to confirm the identity of the purified protein.

## MATERIALS AND METHODS

### Chemicals

Casein (Hammarsten type) from bovine milk, Tris, agarose, cysteine, iodoacetamide, and BSA were obtained from Sigma. Tricine, sodium iodoacetate, Coomassie Brilliant Blue R-250 and G-250, acrylamide, bisacrylamide, low molecular weight, ampholytes of broad and alkaline pH range, and pI markers were obtained from Bio-Rad. Pure papain suspension ( $\geq 30$  units/mg protein) was obtained from

Roche. The E-64 was from Bachem. The DTT was purchased from Invitrogen. All other chemicals were obtained from commercial sources and were of the highest purity available.

### Purification of Papain

Papain was purified by a two-step  $(\text{NH}_4)_2\text{SO}_4$  precipitation (45% saturation and 40% saturation, respectively) and a third NaCl (10% w/v) precipitation stage (Baines and Brocklehurst 1979) in presence of 30 mM TT from dried latex collected from unripe *Carica papaya* fruits grown in Jujuy province, Argentina. Protein content was measured by Bradford's method (Bradford 1976) using a calibration curve of BSA as standard. Proteolytic activity was performed using casein as substrate (Priolo et al. 1991). A quick pre-incubation step of purified papain in presence of cysteine (20 mM, 5 min at room temperature) was performed before caseinolytic assay to revert the inhibitory effect of TT.

To the aim of comparison, the purification was repeated in absence of TT according to the original method.

### Characterization of Purified Papain

**SDS-PAGE.** Samples, inhibited with 30 mM sodium iodoacetate, were submitted to denaturing SDS polyacrylamide (12%) gel electrophoresis using tricine buffer (Schägger and von Jagow 1987). Gels were stained using the colloidal Coomassie method after the electrophoretic run (Neuhoff et al. 1988).

**IEF.** The IEFs were developed on immobilized pH gradient gels of polyacrylamide (5%) in the different pH range: from 3 to 10 (broad pH range) (Morcelle et al. 2004) and 8.0 to 10 (alkaline pH range) (BioLyte 3–10 and 8.0–10 carrier ampholytes, Bio-Rad) in a Mini IEF Cell (Model 111, Bio-Rad). For the IEF developed in alkaline pH range, a piece of filter paper was soaked in fresh 6.0 M NaOH and placed in the IEF cell. The resulting gels were then fixed and stained with Coomassie Brilliant Blue R-250.

**Analysis of tryptic peptides from papain by PMF MALDI-TOF.** Lyophilized purified papain was submitted to tryptic digestion in two different conditions. In one case, papain (previously inhibited with E-64) was incubated with trypsin in 50 mM  $\text{NH}_4\text{COOCH}_3$  buffer pH 8.0 at 37°C overnight, and the resulting peptides were submitted to an analysis by MALDI-TOF MS using 4-HCCA as a matrix in an Applied Biosystems 4800 Analyzer. In the second case, lyophilized pure papain (0.5 mg protein) was resuspended in 100  $\mu\text{L}$  of Milli-Q<sup>R</sup> water. An amount of 50  $\mu\text{L}$  of 1.0 M GndCl in 50 mM  $\text{NH}_4\text{HCO}_3$  buffer pH 8.0 were added to 4  $\mu\text{L}$  of papain suspension and heating at 100°C (5 min). Then, reduction with 10 mM DTT and carbamidomethylation with 20 mM iodoacetamide were performed (20 min). The sample was diluted with 150  $\mu\text{L}$  of 50 mM  $\text{NH}_4\text{HCO}_3$  buffer pH 8.0 and submitted to tryptic digestion for 2 h at 37°C, and the resulting peptides were submitted to an analysis by MALDI-TOF MS using 4-HCCA as a matrix in an ultrafleXtrem mass spectrometer (Bruker).

**Identification by MALDI-TOF/TOF MS/MS.** For the second case, identification of papain was achieved by subsequent analysis of three peptides by MALDI-TOF/TOF MS/MS using an ultrafleXtrem mass spectrometer (Bruker).

**PMF by MALDI-TOF MS from electrophoretic bands.** Purified papain was submitted to SDS-PAGE and visualized by staining according to the colloidal Coomassie method. The bands were cut, washed with 25 mM  $\text{NH}_4\text{HCO}_3$  and acetonitrile several times to remove the dye, and dried under vacuum. The gel fragments were rehydrated with 10 mM DTT for 30 min at 37°C, then centrifuged and washed with acetonitrile for 5 minutes. The fragments were incubated in a solution of 25 mM  $\text{NH}_4\text{HCO}_3$  containing 50 mM iodoacetamide for 20 min at room temperature in darkness, and washed with Milli-Q<sup>R</sup> water and acetonitrile alternatively. The tryptic digestion was achieved by using a 25 mM  $\text{NH}_4\text{HCO}_3$  buffer pH 8.5 containing trypsin (4 ng/ $\mu\text{l}$ ) for 2 h at 37°C. The resulting peptides were recovered by extraction with 50% (v/v) acetonitrile and then dried in a SpeedVac, redissolved in 0.1% (v/v) TFA. The sample was spotted on an MTP GroundSteel target, and analyzed by an UltraFlex MALDI-TOF mass spectrometer using a 4-HCCA as matrix.

Identification of the resulting peptides from the tryptic digestion was tried by searching with the Mascot tool (Perkins et al. 1999) (<http://www.matrixscience.com/>). Search parameters: (1) Full MS: (a) MS tolerance, 100.00 ppm; (b) Enzyme, trypsin; (c) Database, MSDB (version MSDB\_20060831.fasta). (2) MS/MS: (a) MS tolerance, 100.00 ppm; (b) MS/MS tolerance: 0.500000 Da; (c) Enzyme, trypsin; (d) Database, NCBIInr (version NCBIInr\_20100728.fasta). Probability Based Mowse Score: Protein score is  $-10 \cdot \log(P)$ , where P is the probability that the observed match is a random event.

## RESULTS AND DISCUSSION

### Papain Purification: Electrophoretic Analyses

Papain, the most acidic peptidase from *Carica papaya* latex was purified according to the classical salt precipitation method firstly described by Kimmel and Smith (1954), modified by Baines and Brocklehurst (1979) to render a papain without chymopapain as the main contaminant (chymopapain is the most abundant peptidase present in *Carica papaya* latex) and in presence of 30 mM TT to preserve the -SH of the active sites from oxidation, thus protecting the enzymatic activity of the final product (Azarkan et al. 2003; Espin and Islam 1998). The use of TT has the advantage of being not as toxic as other reversible inhibitors, like  $\text{Hg}^{2+}$  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 (Azarkan et al. 2003; Espin and Islam 1998; Takahashi et al. 1973). When papain was purified in presence of TT, a product with higher specific activity was achieved (Table 1). This could be due by the protection role exerted on the active site by TT.

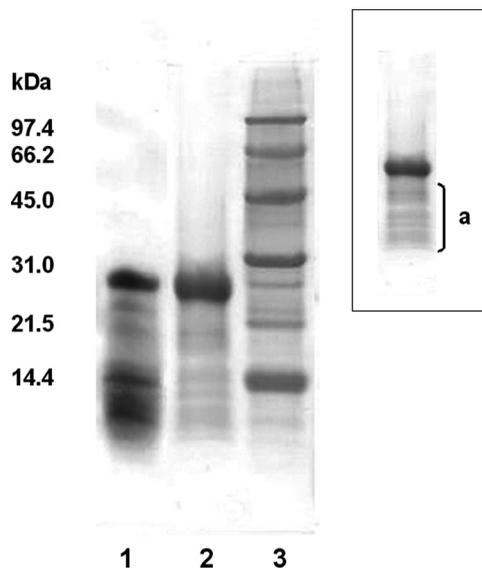
The SDS-PAGE determination for both crude papain extract and the purified enzyme (Fig. 1) shows that the main protein fraction is in the range of 23–24 kDa. As mentioned in the Introduction section, this range corresponds to the cysteine endopeptidases present in *Carica papaya* latex.

**Table 1.** Papain purification results

	Total protein (mg/g)		Specific activity (Ucas/mg protein)		Purification (times)		Yield (%)	
	30mM TT	no TT	30mM TT	no TT	30mM TT	no TT	30mM TT	no TT
Crude papain extract	202.4	321.5	5.3	4.1	–	–	100.0	100.0
Purified papain	6.8	16.0	6.7	4.5	1.3	1.1	3.4	5.0

The SDS-PAGE is considered a general method to assure protein purity, as well as for monitoring the progress of a purification procedure and for the identification of fractions containing the protein of interest (Dunn 1989). However, in this case, there is no certainty that the purified fraction corresponds only to papain, because it could be contaminated by the other three peptidases, which have similar molecular weights. Additionally, there is no certain evidence that the identity of the purified protein is really papain and not any of the other proteases that forms part of the crude extract.

The IEF is not generally used for monitoring the progress of purification procedures. It is commonly used as a method for the characterization of the final purified protein product, since it provides complementary information to SDS-PAGE. When used in conjunction with a parallel analysis of a sample by SDS-PAGE, these two techniques form a powerful method for assessing protein purity with IEF detecting charge heterogeneity and SDS-PAGE detecting size heterogeneity. Since papain

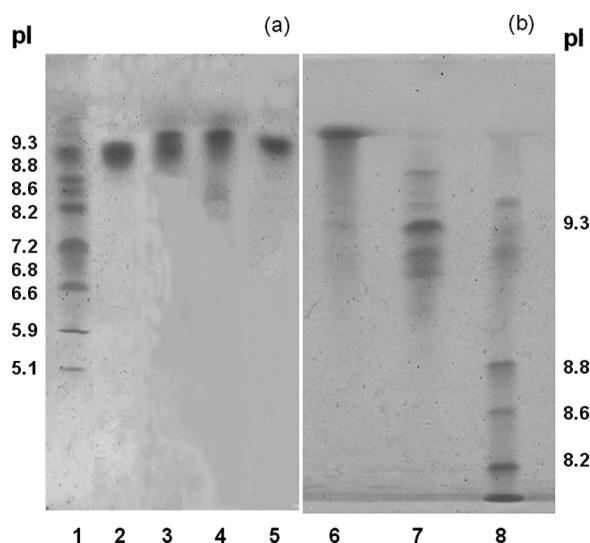


**Figure 1.** SDS-PAGE of purified papain. Lane 1: papain crude extract. Lane 2: purified papain. Lane 3: Bio-Rad molecular weight markers: phosphorylase b, 97.4 kDa; serum albumin, 66.2 kDa; ovalbumin, 45.0 kDa; carbonic anhydrase, 31.0 kDa; trypsin inhibitor, 21.5 kDa; and lysozyme, 14.4 kDa. Inset: detail of Lane 2 (purified papain) showing the bands of low molecular weight (a) that were submitted to PMF MALDI-TOF analysis.

is the most acidic peptidase of the four (i.e., the one with the lowest pI), IEF could be considered a more accurate method to identify the final product. According to bibliographic data, papain pI is in the range of 8.7–8.9 (Barrett et al. 2004). The IEF of broad and alkaline pH range were performed to determine the pI of the purified protein (Fig. 2a and 2b, respectively).

As can be seen in Fig. 2a, purified papain moves to the cathode region up to the zone corresponding to pI 9.3. This is also observed for the commercial pure papain. For the whole crude extract, the main protein fraction moves to a more alkaline region (i.e., pI > 9.3). This behavior is coincident with the expected for the rest of the proteolytic enzymes from *Carica papaya* latex, which have higher pI values (10.3 to 10.7 for chymopapain, 11.7 for caricain and above 10.0 for glycyl endopeptidase) (Domsalla and Melzig 2008). It can be seen that, after the second precipitation with  $(\text{NH}_4)_2\text{SO}_4$ , there is an important enrichment in papain, which was still contaminated with another important more alkaline fraction. This more alkaline fraction is supposed to be chymopapain, which seems to disappear after the precipitation with NaCl (Baines and Brocklehurst 1979).

Due to the high pI determined for the purified product, a second IEF in alkaline conditions was performed (Fig. 2b). The majority of proteins that compose the crude papain extract have pI values higher than 9.3. Purified papain revealed several bands with a pI below 9.3. However, this result does not necessarily mean that the purification is incomplete. The IEF technique has high resolution and frequently resolves proteins into more bands than expected. This could be due to artifacts



**Figure 2.** (a) IEF of papain purification in presence of 30 mM TT (broad range). Lane 1: Sigma IEF-Mix pI markers (amyloglucosidase from *Aspergillus niger*, 3.6; trypsin inhibitor from soybean, 4.6;  $\beta$ -lactoglobulin A from bovine milk, 5.1; carbonic anhydrase II from bovine erythrocytes, 5.9; carbonic anhydrase I from human erythrocytes, 6.6; myoglobin from horse heart, 6.8, 7.2; lectin from *Lens culinaris* 8.2, 8.6, 8.8; trypsinogen from bovine pancreas, 9.3). Lane 2: purified papain. Lane 3: resuspended precipitate obtained after precipitation with 45%  $(\text{NH}_4)_2\text{SO}_4$ . Lane 4: crude papain extract. Lane 5: commercial pure papain. (b) IEF of papain purification (alkaline range). Lane 6: crude papain extract. Lane 7: purified papain. Lane 8: Sigma IEF-Mix pI markers (the same as in Lane 1).

and protein-ampholytes interactions, protein microheterogeneity such as the presence of different conformers of the protein, slight damage during the purification procedure that can provoke the loss or gain of charged groups and differences in oxidation states, the presence of disulfide bonds, the folding state, and the environment, among others, which have a key influence in the determination of this parameter (Gianazza and Righetti 1980). In other cases, as for CPs obtained from bromelain or other plants belonging to *Bromeliaceae*, differences in the charged state of the purified peptidase result in more than one band in IEF; this could be due to the change of one amino acid in its sequence (Napper et al. 1994; Bruno et al. 2008). It is also important to point out that the IEF performed in alkaline conditions amplifies a narrow pH range (comprised between 8.0 and 10.5), and thus highlighting even more all the phenomena already mentioned. All these conformational alterations detected by IEF could have important effects on protein functionality and interactions, indicating that the purified product could be partially inactive and thus not completely effective for medical use in humans.

### **Papain Purification: Identification by Different MALDI-TOF Mass Spectrometry Approaches**

The ambiguous results obtained by traditional methods such as SDS-PAGE and IEF, led us to try identification of the isolated protein by means of modern proteomic tools, such as analysis by PMF. This technique consists in breaking down the unknown protein into peptides by a protease (trypsin is one of the most used) which does not create too many peptides. The absolute masses of these peptides are then determined by mass spectrometry (e.g., MALDI-TOF). Finally, the complete sequence of the studied protein should be present in the available databases. The great advantage of this method for protein identification is that only the masses of the peptides have to be known; other specific determinations, as the whole amino acid sequence of the protein, are not necessary (Obregón et al. 2009). As can be concluded, the results obtained by this technique for identification are more accurate than other biochemical parameters that were used in the past, such as molecular weight determinations or the calculation of kinetic constants using certain synthetic substrates in the case of enzymes (Baines and Brocklehurst 1979). On the other hand, tandem mass spectrometry (MS/MS) of peptides is currently the primary method to identify proteins in complex samples (Falkner et al. 2007).

### **Identification of Papain by MALDI-TOF/TOF MS/MS of the Selected Peptides Obtained After Tryptic Digestion**

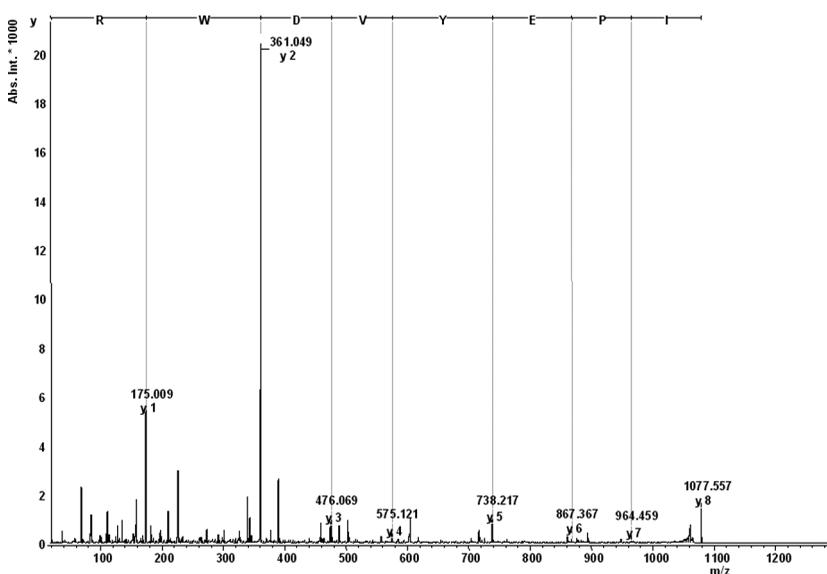
Purified papain was submitted to tryptic digestion without and with the previous reduction and alkylation steps (see Materials and Methods Section), and the peptides were analyzed by MALDI-TOF MS. Those peptides showing the highest intensity signals (1077.615, 1226.671, and 1596.772 Da) were submitted to fragmentation by MALDI-TOF/TOF MS/MS (Figs. 3, 4, and 5). The use of this methodology established the amino acidic sequence of these peptides and, thus, confirmed the identity of the purified protein by comparison with the information from the available databases with the Mascot search tool. The combination of both

approaches to peptide sequence interpretation (database search and de novo sequencing) is used, since the de novo sequencing methodology produces less accurate results (Frank et al. 2007). According to the Mascot search from PMF MALDI-TOF/TOF MS/MS results, the identity of the purified sample was established with papain.

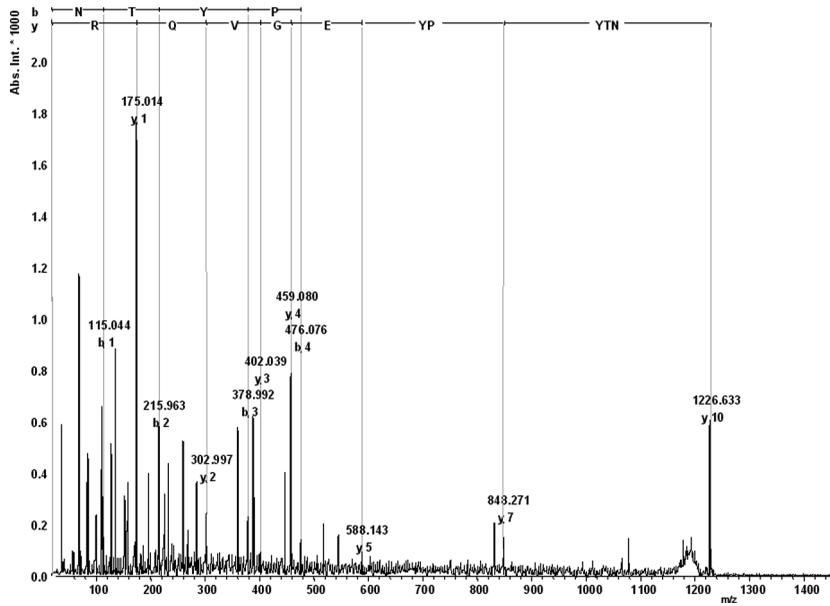
### Identification of Other Peptides Obtained After Tryptic Digestion

Peptides produced after tryptic digestion of purified papain, with and without the previous reduction and alkylation steps, were analyzed by MALDI-TOF MS. In the first case, twelve peptides were positively identified with papain, covering 88% of its sequence, whereas in the second case only two peptides were identified, being the sequence coverage of 8% (see previous section).

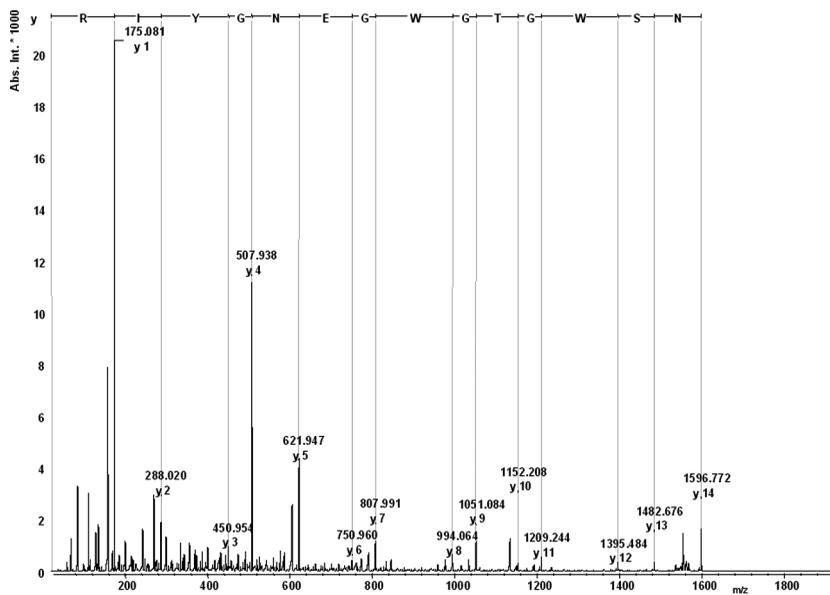
After the comparison made between the theoretical tryptic digestion of papain and the PMF MALDI-TOF MS using the Biotoools 3.1 software (Bruker Daltonics), the presence of some peptides that appeared in the spectra which did not belong to papain were analyzed. Tryptic theoretical digestions of chymopapain, caricain and glycyI endopeptidase were made in silico by means of the mentioned software. Some of these resulting peptides matched with some of those obtained from the analyzed samples by spectrometry, indicating that the purified papain was contaminated with the other CPs, or peptides resulting from the breakdown of those CPs (Table 2). The presence of two specific peptides from chymopapain (1064.5 and 1174.5 Da), and one peptide (1592.8 Da) from glycyI endopeptidase were detected. The other three peptides shown in Table 2 could not be found.



**Figure 3.** MALDI-TOF/TOF MS/MS of 1077.6 Da peptide obtained from tryptic digest of purified papain. The amino acid sequence was IPEYVDWR, corresponding to the N-terminal region of the protein up to the 8th amino acidic residue.



**Figure 4.** MALDI-TOF/TOF MS/MS of 1226.7 Da peptide obtained from tryptic digest of purified papain. The amino acid sequence was NTYPYEGVQR, covering from the 84th to 93rd amino acidic residue of the protein.



**Figure 5.** MALDI-TOF/TOF MS/MS of 1596.7 Da peptide obtained from tryptic digest of purified papain. The amino acid sequence was NSWGTGWGENGYIR, covering from the 175th to 188th amino acidic residue of the protein.

**Table 2.** Identification of non-papain peptides obtained by PMF MALDI-TOF

m/z (observed)	m/z (calculated) <sup>1</sup>	Sequence [start-end] <sup>2</sup>	Enzyme
1001.5768	1001.505	LPESVDWR [1–8]	Glycyl endopeptidase
1064.5930	1064.516	YPQSIDWR [1–8]	Chymopapain
1174.6187	1174.528	NSWGPNWGEK [179–188]	Chymopapain
1266.6812	1266.590	ASGNSPGVCGVYR [196–208]	Glycyl endopeptidase
1592.8383	1592.724	NSWGPWGGENGYIR [179–192]	Glycyl endopeptidase
2932.5156	2932.419	GAVTPVRHQSGSCWAFSAVATVEGINK [11–39]	Caricain

<sup>1</sup>Theoretical PMF calculated using the Biotoools 3.1 software.

<sup>2</sup>Papain numeration.

In order to determine if those non-papain peptides came from tryptic breakdown of the other entire CPs (i.e., chymopapain, caricain, and glycyl endopeptidase) or were already present in the purified sample (with and without TT), PMF MALDI-TOF MS determination was performed from the SDS-PAGE band of 23 kDa. Reduction with DTT and carbamidomethylation were carried out before tryptic digestion.

Results of both tryptic digestions are shown in Table 3. In this case, 12 and 11 peptides obtained from the digestion of the corresponding bands (purified with and without TT, respectively) matched with papain theoretical tryptic digestion, covering 84% of its sequence. According to the results obtained by the Mascot search tool, the sample purified without TT showed a protein score of 186, whereas for the one purified in presence of TT the protein score obtained was 134; protein scores higher than 78 are significant. The absence of peptides belonging to the other CPs in the electrophoretic band would indicate that papain is highly pure in those bands, and no intact chymopapain, not caricain nor glycyl endopeptidase were present in the purified sample. This finding would indicate that the non-papain peptides found could be already present or produced by tryptic digestion of larger peptides of the other CPs. These larger peptides could be those spurious bands of molecular weight lower than 23 kDa that appear weakly stained in the SDS-PAGE of the purified papain (see Fig. 1, lane 2). To confirm this idea, the electrophoresis gel containing those bands was cut (Fig. 1, inset), submitted to tryptic digestion after reduction and carbamidomethylation, and a subsequent PMF MALDI-TOF MS analysis. The masses of the obtained peptides were subjected to comparison with the theoretical tryptic digest calculated in silico.

The results of this analysis are shown in Table 4. Some of the identified peptides from the low molecular weight bands matched to those found in the PMF MALDI-TOF MS of the whole purified papain fraction. The coincident peptides correspond to those resulting from digestion of the other *Carica papaya* latex CPs (peptides marked in gray in Table 4). Nevertheless, other peptides resulting from digestion of pure papain were also found, indicating that SDS-PAGE would be a sort of further purification step, isolating papain from other degradation peptides.

**Table 3.** Purified papain peptides obtained after tryptic digestion from electrophoretic bands

Observed m/z <sup>a</sup>	Observed m/z (TT) <sup>b</sup>	Calculated m/z <sup>c</sup>	Sequence [start-end] <sup>d</sup>
1077.545	1077.609	1077.536	IPEYVDWR [1–8]
-----	1106.609	1106.595	SREKGPYAAK [97–106]
1226.604	1226.615	1226.580	NTYPYEGVQR [84–93]
1333.732	1333.712	1333.690	IPEYVDWRQK [1–10]
1596.797	1596.717	1596.719	NSWGTGWGENGYIR [175–188]
1900.112	1900.014	1900.033	VDHAVAAGVYGPNIILIK [157–174]
2055.983	-----	2055.892	TGNLNEYSEQELLDCDR [42–58]*
2212.074	2212.008	2211.994	TGNLNEYSEQELLDCDRR [42–59]*
2271.103	2271.049	2271.039	GTGNSYGVCGLYTSSFYVPVK [192–212]*
2427.202	2427.177	2427.140	RGTGNSYGVCGLYTSSFYVPVK [191–212]*
-----	2668.413	2668.319	IKRGTGNSYGVCGLYTSSFYVPVK [189–212]*
2986.600	2986.822	2986.561	GGIFVGPCKGKVDHAVAAGVYGPNIILIK [146–174]*
3780.901	-----	3780.960	QVQPYNEGALLYSIANQPVSVVLEAAGKDFQLYR [112–145]
3967.854	-----	3967.861	SYGCNGGYPWSALQLVAQYGIHYRNTYPYEGVQR [60–93]*

<sup>a</sup>Data obtained from the purified papain without TT.

<sup>b</sup>Data obtained from the purified papain in presence of TT.

<sup>c</sup>Theoretical PMF MALDI-TOF calculated using the Biotools 3.1. Software (Bruker Daltonics).

<sup>d</sup>Papain numeration.

\*Carbamidomethylated peptides.

All the results obtained after analyzing in detail the purified product show that papain was the main component. However, the presence of peptides belonging to the other cysteine proteases was detected by electrophoresis and PMF MALDI-TOF MS methods (see Tables 2 and 4). The co-purification of these peptides could be due to two factors: (a) the presence of these peptides in the starting raw material (resulting from the proteases self-digestion), and/or (b) the formation of these peptides during the purification process in spite of the presence of a reversible protease inhibitor (TT in this case). The sensitivity of this method turns peptide mass fingerprint by MALDI-TOF MS combined with in MALDI-TOF/TOF peptide sequencing into a powerful tool, not only to evaluate the purity of a protein, but also the identification of the accompanying impurities. Then, the information obtained by this modern proteomic technique complements and goes beyond all data that could be extracted from traditional methods such as SDS-PAGE and IEF, and other identification methods used in the past, such as kinetic determinations, which are less reliable and time-consuming (Buttle et al. 1989).

## CONCLUSIONS

The purification of papain by salt precipitation in presence of sodium tetrathionate from raw papain extract was followed by traditional methods, such as SDS-PAGE and IEF at broad and alkaline range. Due to the marked similitude

**Table 4.** Comparison between peptides obtained by tryptic digestion of the low molecular weight electrophoretic bands resulting from purified papain SDS-PAGE and theoretical digests of crude papain CPs

m/z (calculated)	m/z (observed)	Sequence [start-end] <sup>a</sup>	Enzyme
1001.576	1001.505	LPESVDWR [1–8]	Glycyl endopeptidase
1064.516	1064.542	YPQSIDWR [1–8]	Chymopapain
1077.536	1077.552	IPEYVDWR [1–8]	Papain
1135.517	1135.564	NSWGTAWGEK [179–188]	Caricain
1174.619	1174.528	NSWGPNWGEK [179–188]	Chymopapain
1226.580	1226.630	NTYPYEGVQR [84–93]	Papain
1266.681	1266.590	ASGNSPGVCGVYR [196–208]	Glycyl endopeptidase
1323.611	1323.671	ASGNSPGVCGVYR [196–208]*	Glycyl endopeptidase
1475.742	1475.767	RAPGNSPGVCGLYK [195–208]*	Caricain
1592.724	1592.812	NSWGPWGGENGYIR [179–192]	Glycyl endopeptidase
1596.719	1596.809	NSWGTGWGENGYIR [175–188]	Papain
2035.036	2035.109	GYQSTSLQYVAQNGIHLR [66–83]	Glycyl endopeptidase
2055.892	2055.965	TGNLNEYSEQELLDCCR [42–58]*	Papain
2384.137	2384.008	NQGSCGSCWAFSAVVVTIEGIK [18–39]*	Papain
2775.438	2775.373	VQSNNEGSLNIAIAHQPVSVVVESAGR [113–139]	Glycyl endopeptidase
2932.516	2932.419	GAVTPVRHQGSCGSCWAFSAVATVEGINK [11–39]	Caricain

<sup>a</sup>Papain numeration.

\*Carbamidomethylated peptides.

Rows in gray indicate the tryptic peptides coincident with those obtained from the PMF MALDI-TOF of the whole purified papain.

in molecular weight and pI of the proteases contained in papaya latex, identification of the purified papain was tried by modern proteomic techniques (PMF by MALDI-TOF MS and identification by MALDI-TOF/TOF MS/MS spectrometry). These last methods revealed that the final product was mainly papain; however, it contained peptides resulting from the breakdown of the other three endopeptidases (chymopapain, caricain, and especially glycyl endopeptidase). Although the resolution of the traditional methods could be considered good enough for purity determination, and available in all laboratories where purification of proteins routinely takes place, PMF by MALDI-TOF MS (and identification by MALDI-TOF/TOF MS/MS) brings the possibility of knowing the identity of impurities contained in a fast and simple assay.

## REFERENCES

- Azarkan, M., A. El Moussaoui, D. van Wuytswinkel, G. Dehon, and Y. Looze. 2003. Fractionation and purification of the enzymes stored in the latex of *Carica papaya*. *J. Chrom. B* 790: 229–238.
- Baines, B. S., and K. Brocklehurst. 1979. 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.* 177: 541–548.

- Barrett, A. 2001. *Proteolytic Enzymes: Nomenclature and Classification, Proteolytic Enzymes, A Practical Approach*, eds. R. Beynon and J. S. Bond. Oxford: Oxford University Press.
- Barrett, A., N. D. Rawlings, and J. F. Woessner. 2004. *Handbook of Proteolytic Enzymes*. London, UK: Academic Press.
- Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of micrograms quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* 72: 248–254.
- Bruno, M. A., S. A. Trejo, N. O. Caffini, and L. M. López. 2008. Purification and characterization of hieronymain III. Comparison with other proteases previously isolated from *Bromelia hieronymi* Mez. *Protein J.* 27: 426–433.
- Buttle, D. J., A. A. Kembhavi, S. L. Sharp, R. T. Shute, D. H. Rich, and A. J. Barrett. 1989. Affinity purification of the novel cysteine proteinase papaya proteinase IV, and papain from papaya latex. *Biochem. J.* 261: 469–476.
- Damodaran, S., T. D. Wood, P. Nagarajan, and R. A. Rabin. 2007. Evaluating peptide mass fingerprinting-based protein identification. *Geno. Prot. Bioinfo.* 5: 152–157.
- Domsalla, A., and M. F. Melzig. 2008. Occurrence and properties of proteases in plant latices. *Planta Med.* 74: 699–711.
- Dunn, M. J. 1989. Initial planning. Electrophoretic analysis methods. In *Protein Purification Methods: A Practical Approach*, eds. R. Beynon and J. S. Bond. New York: IRL Press, Oxford University Press.
- El Mossaoui, A., M. Nijs, C. Paul, R. Witjens, J. Vincentelli, M. Azarkan, and Y. Looze. 2001. 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.* 58: 556–570.
- Espin, N., and M. N. Islam. 1998. Stabilization of papain from papaya peels. *Food Sci. Technol. Int.* 4: 179–187.
- Falkner, J. A., M. Kachman, D. M. Veine, A. Walker, J. R. Strahler, and P. C. Andrews. 2007. Validated MALDI-TOF/TOF mass spectra for protein standards. *J. Am. Soc. Mass Spectrom.* 18: 850–855.
- Frank, A. M., M. M. Savitski, M. N. Nielsen, R. A. Zubarev, and P. A. Pevzner. 2007. De novo peptide sequencing and identification with precision mass spectrometry. *J. Proteome Res.* 6: 114–123.
- Gianazza, E., and P. G. Righetti. 1980. Facts and artifacts in isoelectric focusing. In *Electrophoresis '79*, ed. B. J. Radola. Berlin: Walter de Gruyter.
- Kimmel, J. R., and E. L. Smith. 1954. Crystalline papain. I. Preparation, specificity, and activation. *J. Biol. Chem.* 207: 515–531.
- Matsumoto, K., D. Yamamoto, H. Ohishi, K. Tomoo, T. Ishida, M. Inoue, T. Sadatome, K. Kitamura, and H. Mizuno. 1989. Mode of binding of E-64-c, a potent thiol protease inhibitor, to papain as determined by X-ray crystal analysis of the complex. *FEBS Lett.* 245: 177–180.
- Monge, A., E. J. Barreiro, P. Huenchunir, R. Pinzón, G. Mora, A. Núñez, X. Chiriboga, et al. 2008. Functional foods: Reflections on an expanding market. *Chemistry Int.* 30: 9–13.
- Morcelle, S. R., S. A. Trejo, F. Canals, F. X. Avilés, and N. S. Priolo. 2004. Funastrain c II, a cysteine endopeptidase purified from the latex of *Funastrum clausum*. *Protein J.* 23: 205–215.
- Napper, A. D., S. P. Bennett, M. Borowski, M. B. Holdrige, M. J. C. Leonard, E. E. Rogers, Y. Duan, R. A. Laursen, B. Reinhold, and S. L. Shames. 1994. Purification and characterization of multiple forms of the pineapple-stem-derived cysteine proteinases ananain and comosain. *Biochem. J.* 301: 727–735.
- Neuhoff, V., N. Arold, D. Taube, and W. Ehrhardt. 1988. 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* 9: 255–262.

- Obregón, W. D., C. S. Liggieri, S. R. Morcelle, S. A. Trejo, F. X. Avilés, and N. S. Priolo. 2009. Biochemical and PMF MALDI-TOF analyses of two novel papain-like proteinases. *Protein Pept. Lett.* 16: 1323–1333.
- Perkins, D. N., D. J. C. Pappin, D. M. Creasy, and J. S. Cottrell. 1999. Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* 20: 3551–3567.
- Priolo, N., L. M. I. López, M. C. Arribére, C. L. Natalucci, and N. O. Caffini. 1991. New purified plant proteinases for the food industry. *Acta Aliment.* 20: 189–196.
- Schägger, H., and G. von Jagow. 1987. Tricine-sodium dodecyl sulfate polyacrilamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* 166: 368–379.
- Takahashi, N., Y. Yasuda, G. Kashiko, M. Tokiko, and T. Murachi. 1973. Multiple molecular forms of stem bromelin. *J. Biochem.* 74: 355–357.