

Evaluation of drying techniques measuring proteolytic activity of Papain obtained from unripe fruit and skin juice

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The papain is one of the most used enzymes in the industry. A comparative study between four drying process types: Tray drier, oven, vacuum oven and a lyophilizator at different temperatures was made with the aim of evaluating the impact of the treatment on the enzyme proteolytic activity of the crude papain. No significant statistical difference was found for the different drying process at the selected conditions. The activity measurements were realized by casein hydrolysis and molecular weight was determined by SDS-PAGE electrophoresis finding an average weight of 22086 Da. The crude enzyme extract was purified by means of a salting out and an ionic exchange chromatography. The purification procedure enhanced up to 20-folds the specific enzyme activity (7.5×10^{-4} and 1.5×10^{-2} U mg⁻¹ of protein for crude and purified enzyme, respectively). In Colombia there is not an industrial papain production; due to the large papaya crops that exist, the evaluation of these drying and purification procedures as potential alternatives for the purified and crude papain production is important for future industrial investment.

1. Introduction

The papaya is the fruit of the papaya tree (*Carica papaya*) native of Central America. The fruit ripens from 4 to 6 months depending on the climate where it is grown (Salunkhe and Kadam, 1995). The cultivation of this fruit has two main purposes: the sale of the fruit for human consumption and the extraction of enzymes that constitute 40% of the latex in 1 mM concentrations (Azarkan *et al.*, 2004). The papain is a natural proteolytic enzyme that is extracted from the latex in the leaf, the stem and the papaya's unripe fruits (Baeza *et al.*, 1989). Papain is used in a many industrial fields (like pharmaceutical, brewery, meat, dairy, textile, photographic, optical, tanning, cosmetic, detergents, food and leather industry), because a synthetic enzyme is not capable of simulating the properties of the natural enzyme, which increased its demand. The process to obtain raw papain consists of two main stages: latex extraction and drying. A third stage, purification, may be used if a purified papain is wanted. This work compares the crude enzymatic activity obtained from locally *Carica papaya* using the unripe fruit and the skin juice under different drying processes and evaluates the enzyme activity for the proposed purification procedure.

2. Materials And Methods

2.1 Materials

The papaya fruit, *C. papaya* grown locally (Cundinamarca, Colombia) was used as starting latex material. Polyacrylamide, bis-acrylamide, ammonium persulfate and casein were purchased from Sigma–Aldrich (USA). Molecular weight marker and Q-Shepadex™ were purchased to Bio-Rad (USA). Others reagents were analytical grade.

2.2 Latex extraction

The extracted latex was obtained by several longitudinal incisions with a rustless-steel blade on the unripe fruits using Nitsawang's protocol *et al.* (2006). This latex was allowed to run down the fruit and drip in plastic containers. Before being stored at -20°C NaOH 0.3 M was added to avoid oxidation (Ortiz *et al.*, 1980). The other used latex was obtained from the unripe fruits skin, which were peeled and crushed in a food processor obtaining a humid paste. NaOH 0.3 M was added to this paste before being stored at -20°C.

2.3 Drying process

To evaluate the different drying process, 10 g samples of each latex source were arranged in aluminum trays of 15cm². A tray drier with an air flow of 10 Km h⁻¹, a conventional oven (Memmert), a vacuum oven (Cole-Parmer 5053-20) and a lyophilizator (Freezone 6 plus Labconco) were used to dry the obtained latex and to establish the temperature effect on the crude enzyme activity. Table 1 presents the operational conditions for the different dryers. To each condition three different assays were made.

Table 1. Operational conditions for the different dryers used in the latex drying.

| Number | Origen | Drier type | Temp (°C) | Pressure (mbar) | Time (h) |
|--------|--------|---------------|-----------|-----------------|----------|
| 1 | Latex | Oven | 40 | 746.6 | 8 |
| 2 | Skin | Oven | 50 | 746.6 | 8 |
| 3 | Latex | Oven | 40 | 746.6 | 8 |
| 4 | Skin | Oven | 50 | 746.6 | 8 |
| 5 | Latex | Tray Drier | 40 | 746.6 | 2 |
| 6 | Skin | Tray Drier | 50 | 746.6 | 2 |
| 7 | Latex | Tray Drier | 40 | 746.6 | 2 |
| 8 | Skin | Tray Drier | 50 | 746.6 | 2 |
| 9 | Latex | Vacuum Oven | 40 | 137.06 | 18 |
| 10 | Skin | Vacuum Oven | 50 | 137.06 | 18 |
| 11 | Latex | Vacuum Oven | 40 | 137.06 | 18 |
| 12 | Skin | Vacuum Oven | 50 | 137.06 | 18 |
| 13 | Latex | Lyophilizator | -30 | 0.1 | 24 |
| 14 | Skin | Lyophilizator | -40 | 0.1 | 24 |
| 15 | Latex | Lyophilizator | -30 | 0.1 | 24 |
| 16 | Skin | Lyophilizator | -40 | 0.1 | 24 |

2.4 Enzymatic activity determination

Protease enzymatic activity was determined by Sigma's SSCASE01.001 protocol (1999). This uses casein as protease substrate. Dried samples of 0.05 g were dissolved in 5 mL sodium acetate buffer 10 mM (pH 7.5) and 5 mL calcium acetate buffer 10 mM (pH 7.5). For each sample 455 μL Casein 65%^(w/v) were preheated in a thermal bath at $37\pm 1^\circ\text{C}$ for 10 minutes and then 20 μL of these were added. After 10 min of reaction, the reactions were stopped by the addition of 455 μL trichloroacetic acid 110 mM. and were kept in the thermal bath for another 30 min. Each reaction has its negative control, which did not have enzyme during preincubation, but it was added after the trichloroacetic acid addition.

The two form phases were separated by centrifugation at 9000 rpm and 4°C during 20 min (Fresco 17 Thermo) in order to discard the solid formed. The supernatant was taken for protease assays. Aliquots of 625 μL supernatant were added to 1570 μL sodium carbonate 500 mM and 250 μL of Folin – Ciocalteus reagent. The protease activity was detected spectrophotometrically since the released tyrosine developed a blue coloration. Each sample was read in a spectrophotometer (Thermospectronic Genesys 5) at 660nm and compared with a calibration curve. One protease unit was defined as the amount of casein hydrolyzed to produce color equivalent to 1.0 μmole (181 μg) of tyrosine per minute at pH 7.5 and 37°C (color by Folin and Ciocalteu's reagent) and was calculated by Eq. 1. (Sigma, 1999).

$$\frac{\text{Units}}{\text{mL Enzyme}} = \frac{(\text{mol Tyrosine})(V_T)}{(V_E)(t)(V_C)} \quad (1)$$

where V_T is the total assay volume in mL, V_E is the volume of the enzyme used mL, t is the reaction time in min, and V_C is the volume used in the colorimetric reaction in mL.

The protein presented in each sample was determined by Biuret's method, where 5 μL of sample was added to 5 μL of water and 20 μL of Biuret reagent stirring gently for 30 min. The absorbance was determined (Nanodrop, spectrophotometer ND-1000) at 540nm and compared with a pattern curve constructed with albumin. Biuret's test was only applied to latex sample and not to skin samples due to color interference. The specific activity was calculated as the ratio between the activity calculated by Eq. 1 and the mg of protein determined per mL of enzyme extract.

2.5 Papain purification and molecular weight determination

Non-denaturing electrophoresis was carried out by the method of Reisfield *et al.*, 1962 for basic proteins, using 12% polyacrylamide gel, 34 mM β -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 Trisglycerol-b-mercaptoethanol and placed in 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).

To determine the molecular weight of papain obtained by the method described in this paper. To 1 mL sample was added 65%^(w/v) ammonium sulphate solution until complete precipitation and centrifuged at 13000 rpm and 4°C during 20 min. The precipitated obtained was reconstituted with sodium acetate 10mM (pH 5.0), after that was dialyzed in a sodium acetate buffer 10mM (pH 5.0) with a volumetric ratio (1:100), then the sample was lyophilized and reconstituted in 1 mL of sodium acetate buffer 10mM (pH 5.0) to be pass through a column (11x100 mm) of Q-SephadexTM fast flow previously equilibrated with a 0.1 M sodium acetate buffer (pH 5.0). The elution was made with NaCl solutions from 0.1 a 0.5 M, for the obtained fractions protease activity was evaluated. The fraction that presented activity was dialyzed in a sodium acetate buffer 10mM (pH 5.0) with a volumetric ratio (1:100) and its final activity was determinated.

3. Results And Analysis

Table 2 presents for the different drying conditions and latex source the activity obtained in units mg⁻¹ of sample.

Table 1. Enzymatic activity for the different drying processes and latex source.

| | | Activity (units mg ⁻¹ solid sample) | | | | | |
|---------------|-----------|--|--------|--------|------------------------|--------|--------|
| | | Latex | | | Skin x 10 ² | | |
| Drying | Temp (°C) | Test 1 | Test 2 | Test 3 | Test 1 | Test 2 | Test 3 |
| Oven | 40 | 0.067 | 0.109 | 0.175 | 1.024 | 0.137 | 2.139 |
| Oven | 50 | 0.143 | 0.146 | 0.175 | 7.351 | 0.296 | 1.001 |
| Tray Drier | 40 | 0.143 | 0.144 | 0.197 | 0.228 | 0.501 | 0.273 |
| Tray Drier | 50 | 0.037 | 0.041 | 0.037 | 1.866 | 1.821 | 1.843 |
| Vacuum Oven | 40 | 0.158 | 0.102 | 0.173 | 1.547 | 0.273 | 0.660 |
| Vacuum Oven | 50 | 0.161 | 0.152 | 0.165 | 1.707 | 1.752 | 1.684 |
| Lyophilizator | -30 | 0.104 | 0.143 | 0.154 | 1.866 | 1.297 | 1.365 |
| Lyophilizator | -40 | 0.140 | 0.144 | 0.172 | 0.614 | 0.660 | 1.070 |

For the different drying process is observed that the enzymatic activity obtained for latex is major than for the skin, however by a statistical analysis. A factorial experiment design was selected for the analysis of drying temperature effect and latex source over the protease activity after latex drying in the oven, tray drier and vacuum oven, while lyophilization was analyzed separately because the temperature differences (Montgomery, 2005).

The ANOVA factorial design analyses were made by the used MINI-TAB[®] v.15. The ANOVA showed that the temperature factor was not significant for any design ($\alpha = 0,05$), which indicated that enzymatic activity was not affected by the selected temperatures in the drying processes but the latex source did. The enzyme activities obtained according to the latex source were likewise to results reported by Baeza *et al.* (1989). The obtained ANOVA for the specific enzymatic activity analyses using all the drying process presented no significant statistic difference among them (Table 3-4).

These results differed to report by Baeza *et al.*, (1989) who found that lyophilization is the best drying procedure, nevertheless the drying conditions were different.

Table 3. Specific enzymatic activity obtained for the latex from the unripe fruits. The number process is associated with the drying process described in Table 1.

| Number | Enzyme Activity (units ml ⁻¹ sample) x 10 ⁴ | | | Enzyme Activity (units mg ⁻¹ protein) x 10 ⁴ | | |
|--------|--|--------|--------|---|--------|--------|
| | Test 1 | Test 2 | Test 3 | Test 1 | Test 2 | Test 3 |
| 1 | 6.8 | 11.0 | 17.5 | 4.0 | 8.1 | 11.3 |
| 2 | 14.3 | 14.6 | 17.5 | 9.7 | 9.3 | 10.2 |
| 5 | 14.3 | 14.5 | 19.7 | 8.5 | 9.3 | 12.7 |
| 6 | 3.8 | 4.2 | 3.7 | 2.1 | 2.5 | 2.3 |
| 9 | 15.9 | 10.3 | 17.4 | 8.8 | 5.6 | 11.5 |
| 10 | 16.2 | 15.2 | 16.5 | 8.0 | 5.5 | 8.9 |
| 13 | 10.4 | 14.3 | 15.4 | 3.8 | 7.9 | 6.0 |
| 14 | 14.0 | 14.4 | 17.2 | 7.3 | 6.9 | 8.7 |

Table 2. Variance analysis to establish the effect of the drying process on the specific enzyme activity.

| Factor | GL | Seq SS | Adj SS | F | P-value |
|----------------|-----------|------------------|-----------|------|---------|
| Drying Process | 3 | 0.0000002 | 0.0000001 | 0.92 | 0.450 |
| Error | 20 | 0.0000017 | 0.0000001 | | |
| Total | 23 | 0.0000020 | | | |

An electrophoresis test was made with non-denatured samples, to observe if the drying process had denatured the papain enzyme. As a result the same colored pattern was obtained to each treatment indicating that there was no denaturizing effect due to the drying process. Therefore the tyrosine measured corresponded to the one produced through the hydrolysis reaction. Figure 1 presents SDS-PAGE for latex samples dried under different conditions. The average molecular weight obtained was 22086.7 Daltons. This molecular weight is similar to reported by Daliya and Ruey-Shin (2005).

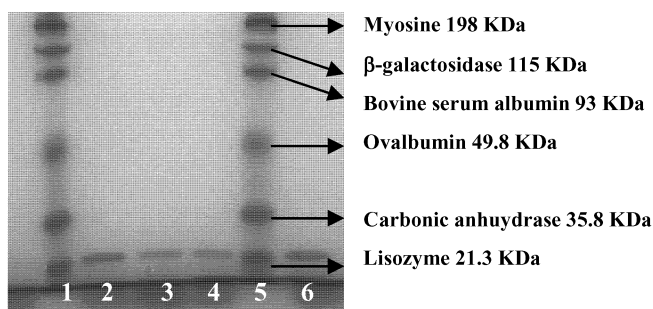


Figure 1. SDS-PAGE electrophoresis gel. Samples 1 and 5 are molecular weight markers and 2, 3, 4 and 6 belongs to latex dried in conventional oven, tray dryer, vacuum oven and lyophilizer, respectively.

The purification process displayed an increase in the specific enzyme activity of papain obtained from the unripe fruit. It was 20-fold higher than the obtained from the crude latex extract: 7.5×10^{-4} and 1.5×10^{-2} U mg⁻¹ of protein for crude and purified enzyme, respectively.

4. Conclusions

The locally papaya fruit is a potential alternative for the papain extraction, now that the latex from these unripe fruits presented a high activity compared with the fruit skin.

Under the temperature evaluated conditions does not exist a significant statistic difference for the specific enzymatic activity for the selected drying processes. The only main difference presented was obtained according to the latex source.

The purified papain presented an average molecular weight of 22086.79 Da and specific enzymatic activity of 1.5×10^{-2} U mg⁻¹ of protein 20-fold higher than the activity obtained in the crude extract 7.5×10^{-4} U mg⁻¹ of protein.

5. Recommendations

It would be interesting to compare the four processes of drying using a wider range of temperatures and ratifying the independence of the proteolytic activity obtained and the process used.

6. References

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