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Treball Final de Grau

Papain extraction from papaya and determination of the enzyme activity.

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June 2.021

UNIVERSITAT DE BARCELONA

Viu com si haguessis de morir demà, aprèn com si el món durés per sempre.

Mahatma Gandhi

Agrair al meu tutor de TFG, el Dr. Roger Bringué, per la seva ajuda, consell i paciència al llarg de la realització del treball. També agrair al Dr. Jaume Giménez, el meu tutor al llarg de la carrera, per tots els seus consells i ajuda durat les tutories, i a tota la resta de professors que s'han esforçat per motivar-me i perquè aprengués a les seves matèries.

Agrair a la meva família i amics per acompanyar-me i donar-me tot el seu suport i confiança durant els estudis i la resta de la meva vida.

CONTENTS

SUMMARY

Papain is an enzyme from papaya. It digests protein substrates from meat and vegetables better than pancreatic proteases. Therefore, it is used in food industry, specially in the preparation of meat for human consumption (as it has tenderizer properties) and beer production. In addition, it is used in different laboratories studies as it has some applications on cell dissociation. In medicine, it has anti-inflammatory and detox properties. For all these reasons, in the last years papain has had a large increase of its production.

This project will be a bibliographic research of all the proceedings useful to extract this enzyme from the latex of papaya on a laboratory. Moreover, it will include the proceedings needed to determine the enzyme activity of the extraction. The object of this project is to have a document with all these proceedings to help anyone who wants to carry out this experiment on a laboratory.

Keywords: Papain, enzyme, enzyme activity, purification of papain, cysteine proteinase.

RESUM

La papaïna és un enzim que s'extreu del làtex de la papaia. Digereix els substrats de proteïnes d'origen càrnic i vegetal millor que les proteases del pàncrees. Per aquest motiu, s'utilitza en la industria de l'alimentació, especialment, en la preparació de carns per al consum humà (per estovar-les) i en la producció de cervesa. A més, s'utilitza en diferents estudis de laboratori per les seves aplicacions en dissociació cel·lular. En medicina, s'utilitza per les seves propietats antiinflamatòries i detox. Per tots aquests motius, la producció d'aquest enzim s'ha incrementat en els últims anys.

Aquest projecte consistirà en una recerca bibliogràfica dels procediments existents per extreure aquest enzim del làtex de la papaia en un laboratori. També s'inclourà els procediments per determinar la seva activitat enzimàtica. L'objectiu d'aquest projecte consisteix en un recull bibliogràfic de tots aquests procediments per tal d'ajudar a qualsevol que vulgui dur a terme un experiment al laboratori.

Paraules clau: Papaïna, enzim, activitat enzimàtica, purificació de papaïna, cisteïna proteïnasa.

1. INTRODUCTION

Papaya is a fruit from South America, and nowadays its production has extended worldwide, especially in Africa and the Southeast of Asia, where climate conditions allow and favour its production. This fruit is used for eating and extracting enzymes, and papain enzyme is one of them.

Papain, also known as papaya proteinase I, is a cysteine protease enzyme extracted from the latex of raw papaya fruits. It was discovered by G.C. Roy, who published an article in 1.873 on the Calcutta Medical Journal called "The solvent action of Papaya Juice on Nitrogenous Articles of Food". The enzyme of papain was firstly partially purified by Wurtz and Bouchut in 1.879, but it was not until mid-twentieth century when it was totally purified. The structure of papain was the second structure of an enzyme being determined by x-ray crystallography, by Drenth in 1.968. The first company to sell it was the Green Cross Corporation, a Japanese company, in 1.969.

It can break peptide bonds better than human gastric juices, therefore it is widely used in nutrition, beer clarification and meat tenderizing. It has also anti-inflammatory and detox properties, which makes it perfect for athletes. The presence of the enzyme depends on the ripeness of the fruit. The more unripe the papaya is, the more active the enzyme is. A person can easily see how ripe the fruit is by its colour. The red-orange colour of the fruit shows how ripe it is, the redder it is, the riper it is. On the Figure 1 from García et al. (2.019) we can see this difference of colour, where the orange ones are the ripest.

Figure 1. Extracted from García et al. (2.019)

1.1. APPLICATIONS

1.1.1. Food industry

Papain is one of the most used catalyst on the food industry worldwide. Papain can modify myofibrillar and connective tissue proteins to improve meat tenderization. Papain also acts as a clarifying agent in food industry processes for beer production. During the maturation process of beer papain is used to avoid the coagulation and precipitation of some proteins.

1.1.2. Pharmaceutical industry

Papain is being used for contact lens cleansing solutions. The use of the enzyme in cleansing solutions lengthens the use of contact lenses in a day. Even though there have been reported allergic reactions to these solutions due to papain, it is still being used. Papain is also used as an anti-inflammatory and digestive natural medicine, usually combined with other medicines or drugs. It is recommended for athletes after a long session of sport for muscles recovery.

Some studies like I. Petushkova et al. (2.020) show how some viruses, like SARS-CoV or Hepatitis E create a papain-like protease to help virus cells to process viral polyproteins and enable viral spread. It has been recommended by experts to use inhibitors against this papain-like protease to treat them. Even though it is a papain-like protease, there are no studies which show a link between the consumption of papain and the spread of these viruses.

1.1.3. Cosmetics industry

Papain is very used in cosmetics industry. It is sold for peelings as it has exfoliating properties to remove dead surface skin. There are also papain-based shampoos for pets to clean the fur and brush it easier. It is also used as a whitener in toothpastes combined with bromelain, a similar enzyme that comes from pineapple.

It is a compound for skin drugs because of its properties in skin regeneration after a surgery or after being wounded to avoid scars.

1.1.4. Other industries

Papain is also being used in textile, leather, paper and detergents industry due to its ability to dissolve fibres. In the textile industry, the use of biocatalysts is being developed because of its harmless effluents and good effectiveness. For example, regarding to wool fabrics, papain is considered one of the most proteolytic effective for wool fibre morphology. In the detergent industry, it is usually used a chemical modified papain enzyme to increase its range of pH, its stability and its retention power when added in detergents. On the leather industry, papain is used to dissolve animal fats on leather.

1.2. GLOBAL MARKET

The global enzymes market, according to Allied Market Research, was valued over 8.000 million €. This market is expected to grow to more than 14.000 million € by the end of 2.027. Even though there has been an impact to the enzymes market because of the COVID-19 pandemic, this impact is not related to a decrease of the global enzymes market, but to a big change inside the market. With the reduction of the mobility due to the pandemic, the consumption of enzymes used for the productions of biofuels, one of the biggest enzyme consumers, has decreased. On the other hand, the enzymes market related to food and pharma has increased.

The increase of health awareness in society has a positive impact on the use of natural extracts. The demand on papain has increased in the last years due to the shift on industry into biocatalysts and the great demand on natural extracts and herbal products. The papain market is segmented into 5 regions:

- North America
- Europe
- Asia Pacific
- Latin America
- The Middle East and Africa

COVID-19 restriction measures have had a negative impact on the production of papain. This last year, the production of papain has decreased, and it is not expected to recover again until the next two years. In the future, the demand of papain is expected to increase.

North America is now the biggest consumer of papain, followed by Europe, and these two positions on the global market will last for the following years. In the region of Asia Pacific, there is going to be the highest increase on the demand of papain, due to the high increase of meat and beer industry on the region. However, it is not expected to reach the levels of North America and Europe due to the high spending power of people on food, beverage and medicine products in these two regions. In Latin America and Africa and Middle East, there is going to be a steady increase, but they are not expected to reach the levels of demand of other world regions. On the Figure 2, it is shown the food industry enzymes market in 2.021 and what is forecasted for 2.026 in the different regions of the world.

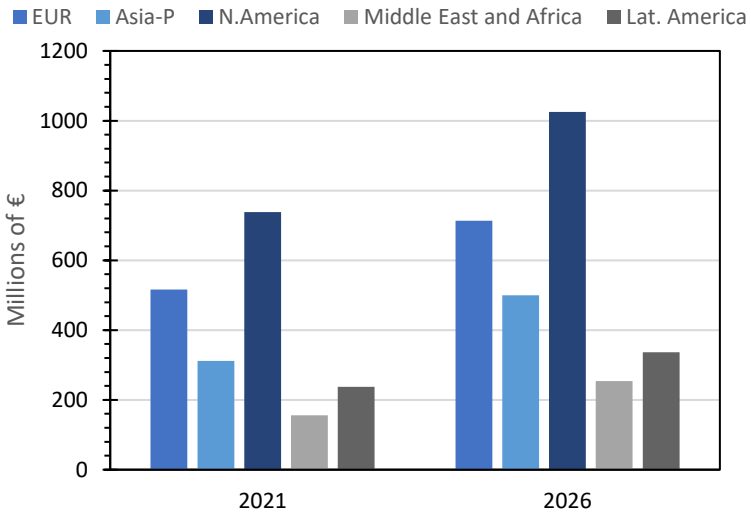


Figure 2. Market tendencies of food enzymes. (Data from Allied Market Research).

According to Industry ACR, by 2.019 papain market was valued at 139,04 millions of €. It is expected to grow at a CAGR (Compound Annual Growth Rate) of 4,67 % on the period of 2.020-2.025. North America represented the highest consumer with the 34% of the world papain market in 2.019. The food and beverage industry represented the highest papain consumer, with a CAGR at 4,4%.

1.3. **PROPERTIES**

Papain is sold as a powder. The Table 1 shows the properties of this powder of papain. Papain is sold as a powder for industry and medical uses. The colour of the powder depends on the level of purification and the methods used to dry it, it is a range between yellow and white, tending to be white. It is soluble in wate and insoluble in organic solvents. The level of solubility depends on the level of purification of the powder. The more purified it is, the least soluble it is.

Papain can also be sold as a crude latex for food industry with a lower level of purification. The storage of the latex of papaya must be stored frozen under 0 ºC to avoid the oxidation of the papain it has. Once purified, the papain enzyme can be stored under 25 ºC.

Table 1. Main properties of papain as powder.

Chemical name	Papain
Cas Number	9001-73-4
Molecular formula	$C_9H_{14}N_4O_3$
Formula weight [g/mol]	226,23
Physical Properties	
Density [mg/mL]	1,2
Form	Lyophilized powder
Colour	Almost white
Solubility	Partial solubility in water
Chemical Properties	
Enzyme Identification	EC 3.4.22.2
pH	3-11
Operation temperature [ºC]	30-60
Storage temperature [ºC]	< 25

On the Figure 3 it is shown the structure of the enzyme of papain.

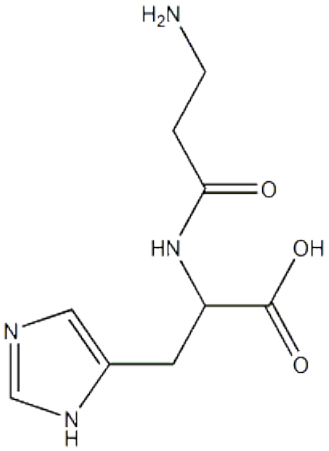


Figure 3. Structure of papain.

The European Chemicals Agency (ECHA) updated its labelling on November 9th, 2020, shown on Figure 4.

Figure 4

Danger! According to the harmonised classification and labelling (CLP00) approved by the EU, this substance causes serious eye irritation, causes skin irritation, may cause allergy or asthma symptoms or breathing difficulties if inhaled and may cause respiratory irritation.

1.4. **SIDE EFFECTS OF PAPAIN**

1.4.1. Excess of dosage

Papain supplements are for punctual situations or short-term periods of use. It is not recommend exceeding a dosage of 400 mg per day because it can cause stomach upset and throat irritation. It is recommended to divide the dosage between different meals to reduce the possible side effects. Due to a lack of research, there is no information about the consequences of a long-term use of papain and it is not recommended. It is neither recommended for children, pregnant women Adebiyi et al. (2.002) and nursing mothers.

1.4.2. Allergic reactions

There are evidences of the allergic reactions on human and animals because the use of papain. These allergic reactions come from lensing solutions, shampoos for people and animals and skin creams containing it. An allergic reaction can also be produced by the direct consumption of papain as a food supplement. These allergic reactions have been proved by different studies Quarre et al. (1.995) and Bernstein et al. (1.984).

The results carried on three patients are shown on the Table 2.The allergic reactions in people were all seen as urticaria and different irritations of the respiratory system. In the vast majority of cases, it was proved that papain allergy is related with other allergies to other fruits by a PST (Positive Skin Test).

Table 2. Results of the study. Quarre et al. (1.984)

Age [years]	Symptoms	IgE [U/ml]	Latex [kU/L]	Banana [kU/L]	Papain [kU/L]	Positive skin test results
28	Urticaria, dyspnea, angioedema	180	21	2,8	2,53	Banana, Latex Papain, Avocado
36	Dyspnea, urticaria	7	0,78	0	0,5	Banana, Kiwi, Avocado, Latex, Papain
45	Rhinitis, angioedema	187	5,9	1,43	0,48	Banana, Kiwi, Avocado, Latex, Papain

These reactions have been detected in just a little part of the population, it is still being used. The problem remains on the labelling of these products. As papain is a natural extract from fruits, some companies do not include it on the labelling information and consumers who are allergic are not informed to avoid it.

Allergic reactions of papain are common in people with allergies to kiwi fruit or latex.

In 2.008, the U.S. Food and Drugs Administration (FDA) ordered companies to stop marketing topical drugs containing papain because a lot of topical drugs containing it were sold and marketed without the approval of the FDA.

1.4.3. Papain and other medicines or illnesses

Papain can not be used in people who are taking anticoagulants, specially Coumadin and Plavix, as it slows blood clotting.

Papain is not recommended for people with diabetes medication. Papain lowers sugar blood levels and it can cause hypoglycaemia.

People must stop consuming papain at least two weeks before surgery to avoid bleeding excess.

2. OBJECTIVES

The enzyme of papain is still being studied by research centres and universities around the world because there are properties and side effects of its use that have not been discovered yet. Therefore, this work is going to be a bibliographic research including the different methods of extracting papain enzyme from papaya fruit.

The object of this bibliographic research will be to have a guide for all the researchers who need it, easy to understand and easy to follow, to achieve an extraction and purification of papain and determine its enzymatic activity on a laboratory to obtain a high quality enzyme. This guide is going to be based on publications of previous research and studies carried on by other scientists.

3. PAPAIN EXTRACTION AND PURIFICATION PROCESS

There are two processes for the extraction of papain, depending on the use of the enzyme. For industrial objectives, the raw papain is used, i.e. the process consists on the latex extraction and the subsequent drying.

Further, when the purification standard increases, papain is obtained after the latex extraction, purification and then drying. This purified papain does not have a high demand on the market, it is mostly used for medicines preparation and laboratory studies.

On the Figure 5 we can see that the only difference is the middle operations required on the purified papain obtention, which make this process more complex.

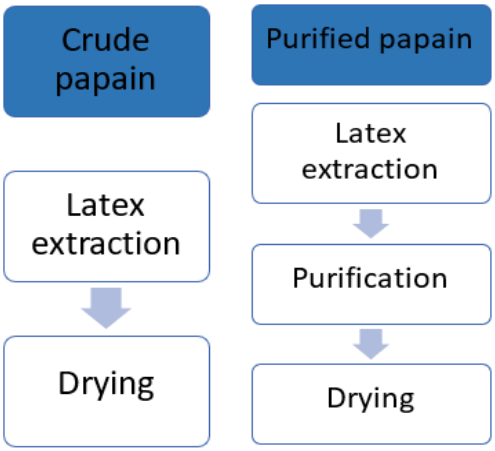


Figure 5.Types of papain.

3.1. **LATEX EXTRACTION**

The latex extraction is the same for each of the two processes of papain extraction.

Latex is extracted from the skin of papaya fruit. To extract the latex, it is necessary to use stainless steel tools. Nirmal et al. (2.012) suggests that other metals could inhibit the enzyme. In addition, papain in solution is easily oxidized by temperatures of 70ºC or higher, sunlight or exposure to air.

The extraction of latex is based on Nitsawang et al. (2.006) and consists on different longitudinal incisions on the surface of the skin. The amount and length of the incisions will depend on the size of the fruit, but it is recommended to make around 4 incisions and leave it hanging and dripping. After the extraction of latex, it should be homogenized in an agitation system.

Before moving to the purification or drying process, it is recommended to use a chemical agent for the stabilization of the papain enzyme. There are different stabilizers that can be used on this process. The stabilizers have a maximal operation temperature, and it is important not to go over this temperature during the following steps of the process, especially on the drying. After adding this stabilizer, if the latex is not going to be immediately used for the purification or drying process, it must be stored in temperatures under 0ºC.

Some of the stabilizers recommended are shown on the Table 3. These stabilizers and their optimum concentrations have been proven by two studies: Dam et al (1.990) and Andrade-Mahecha et al. (2.011). These studies also show that a combination of two of these stabilizers does not have a substantial increase on the enzymatic activity of the enzyme.

Table 3. Stabilizers for extraction and purification of papain.

Stabilizer	Concentration [% w/w]
Potassium metabisulphites	0,5
Cysteine	0,1
EDTA	0,2
Sodium metabisulphite	0,5

3.2. **PURIFICATION**

This process is only used when a more purified papain is needed. There are two main methods to purify the enzyme. If the aim is to make an analysis of the enzyme, then more sophisticated methods should be used. However, if the aim is to determine a process of purification for industry, the priority must be on the escalation of the laboratory process to an industrial one. Figure 6 shows the two more used purification processes. The method of crystallisation of papain is not very used because the yield of enzyme obtained by this method is not very high. However, a recent study by Monti et al. (2.000) has shown that with a modification of the method good yields of papain can also be obtained. Another new method for papain extraction is also reported by Limin et al. (2.009) based on ultrasonic waves, but this method has not still escalated to industry.

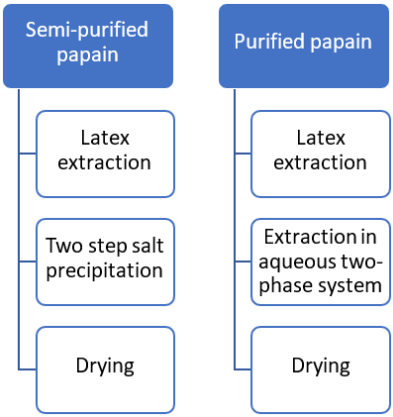


Figure 6. Purification methods.

3.2.1. Purification by two step salt precipitation

This method consists on a two-step precipitation adding salts or organic solvents, usually ammonium sulphate at 45% of saturation or ethanol. The different separation processes can be adapted to the material and infrastructure of the laboratory. Here are two examples of this method based on different studies. All the examples are carried on at 4ºC. Between the different separation processes, it is recommended to leave the sample with the enzyme between 15 and 30 minutes at this temperature to avoid a high increase of temperature during next phases.

Example 1: This method is based on Nitsawang et al. (2.006) and Baines et al. (1.979). . It is used ammonium sulphate and sodium chloride as salts for the precipitation.

- For this method the stabilizer used after the latex extraction is cysteine 0,33 % (w/v). The pH was fixed to 5,6 with HCl.
- A first filtration is done to retain particles in suspension on the latex.
- The pH of the filtrate is adjusted to 9 using NaOH. The pH of nine is the isoelectric point of the enzyme of papain (8.75-9.55), and in this point the electric charge of the enzyme is zero. A centrifugation is carried on at 9.000 x g for 30 minutes.
- The supernatant of the centrifugation is mixed with water and it is added a solution of $(NH_4)_2SO_4$ at 45% of saturation.
- Another centrifugation as the first one is done. This time, the precipitate containing the enzyme is collected and dissolved again in cysteine.
- Addition of sodium chloride 10% (w/v). Another centrifugation as the first one is done to separate the purified enzyme.
- The enzyme is then dissolved in water and dialyzed the whole night at 4ºC to be ready for the drying.

On the Table 4, extracted from Nitsawang et al. (2.006), it is shown the recovery of the enzyme on each separation process for three experiments A, B and C. In case a lower recovery is needed, some of the steps can be missed. As it is shown, the first step gives the highest percentage of papain recovery.

Table 4. Extracted from Nitsawang et al. (2.006).

Influence of protein concentration on purification of papain from 30 g papaya latex by two-step salt precipitation

Experiment	Steps	Initial protein concentration (mg/ml)	Total protease activity[a] (Units)	Recovery of protease activity (%)	Relative papain amount[b] (%)	Papain recovery[b] (%)
Latex	Clarified solution	43.5	30534	100	8.2	100
A	1st step[c]	20	3511	11.5	36.2	51
	2nd step[d]	6	824	2.7	89.8	30
		14	1374	4.5	78	43
B	1st step[c]	30	7359	24.1	30.8	91
	2nd step[d]	6	1099	3.6	86.3	38
		12	1496	4.9	75.8	45
		14	1191	3.9	87.5	42
C	1st step[c]	40	11969	39	20	96
	2nd step[d]	6	1344	4.4	89.6	49
		14	1618	5.3	83.1	53

[a] The amount of protease activity recovered in the precipitate was measured by assaying with casein.
[b] Determined by FPLC.
[c] Precipitation by 45% saturated ammonium sulfate.
[d] Precipitation by 10% (w/v) sodium chloride.

Figure 7 shows a scheme of the Example 1.

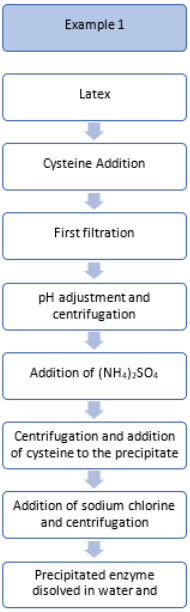


Figure 7. Scheme of Example 1.

Example 2: This method is based on Andrade-Mahecha et al. (2.011). This example uses an organic solvent and ammonium sulphate for the precipitation. The study compared two different ratios of alcohol concentration to determine which one was better using different drying methods foreach experiment. The results are shown on Table 5. As it can be seen, a ratio of 1:3 latex/alcohol gives the highest yield of papain for both drying methods.

Table 5. Results of latex/alcohol ratio.

Experiment	Ratio of latex/alcohol	Yield [g of papain /100g of latex]
Vacuum drying	1:2,1	10,83
	1:3	11,53
Refractance Window drying	1:2,1	7,80
	1:3	11,10

Steps:

- The stabilizer used on this case is sodium metabisulphite (0,5 % w/w). The latex is cooled at -5ºC until it is going to be used for the next phase of this process.
- EDTA is added as a stabilizer after the cooling and ammonium sulphate is also added.
- The latex is first solubilized and then diluted on ethanol on 10% concentration.
- The particles formed are filtrated and eliminated on a diatomaceous earth filter.
- The liquid obtained is diluted again on alcohol. To carry out this dilution, it is needed a 1:3 latex/alcohol concentration.
- The precipitated enzyme is recovered using a vacuum filtration. On this study the vacuum filter used was the Wathman paper N1. The enzyme is then already for the drying.

Khag Dam et al. (1.990) shows that the highest purification level is obtained by using ethanol as precipitant. On the Table 6 the differences between the two examples given for this method of purification are shown.

Table 6. Differences between examples.

Differences	Example 1	Example 2
Stabilizer	Cysteine	Sodium metabisulphite and EDTA
Precipitant	Ammonium sulphite and sodium chloride	Ethanol and ammonium sulphite
Precipitation	Centrifugation is needed	Centrifugation is not needed
Purification level	Lower	Higher

On the figure 8 there are all the steps of the example 2.

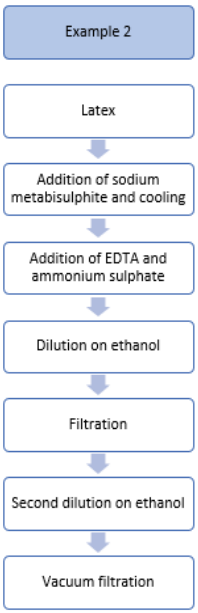


Figure 8. Scheme of Example 2.

3.2.2. Purification by extraction in aqueous two-phase system

This method is also based on the studies of Nitsawang et al. (2.006) and Li et al. (2.010). The method consists on a two-phase system composed by PEG and ammonium sulphate. On the Table 7 shows the results of the experiments carried out. By these experiments, the optimum amounts of PEG based on Salabat et al. (2.001), ammonium sulphate and the optimum pH are determined. The study shows that the highest levels of protease activity where obtained by a pH of 5, a PEG concentration of 8% (w/w) and an ammonium sulphate concentration of 15% (w/w).

Table 7. Results of the optimal conditions for aqueous two-phase purification. Extracted from Nitsawang et al. (2.006).

Effect of (a) concentration of phase components and (b) protein concentration and pH, on the total protease and papain recovery from 30 g papaya latex[a] by aqueous two-phase extraction in PEG–ammonium sulfate system

X% PEG–15% $(NH_4)_2SO_4$[b]			12% PEG–*X*% $(NH_4)_2SO_4$[b]		
PEG (*X*%)	Protease activity in top phase (U)	Papain recovery in top phase[c] (%)	$(NH_4)_2SO_4$ (*X*%)	Protease activity in top phase (U)	Papain recovery in top phase[c] (%)
Part (a)					
4	1515	78.8	9	1708	35.5
6	1611	83.7	12	1627	84.6
8	1708	88.8	15	1642	85.3
10	1653	85.9			
12	1659	86.2			

12% PEG–15% $(NH_4)_2SO_4$

Protein concentration[d] (mg/ml)	Protease activity in top phase (U)	Papain recovery in top phase[c] (%)	pH[e]	Protease activity in top phase (U)	Papain recovery in top phase[c] (%)
Part (b)					
10	1443	75.0	3	313	16.3
20	1515	78.7	4	1154	60.0
30	1443	75.0	5	1659	86.2
40	1515	78.7	6	1563	81.2
50	1347	70.0	7	1563	81.2
60	794	41.3	8	1515	78.7
70	625	32.5	9	1419	73.8

[a] The protease activity in 30 g latex was 24,050 units, 8% of which was papain.
[b] Two-phase extraction was done at pH 5 and 40 mg protein/ml.
[c] Papain recovery was estimated by FPLC. The protease activity recovered in the top phase of all the two-phase systems tested was constituted by papain only, with the exception of the 12% PEG–9% $(NH_4)_2SO_4$ where papain was 40% of the protease activity.
[d] The two-phase system was maintained at pH 5.
[e] Protein concentration in the latex was 40 mg/ml.

The temperature of this method is also 4 ºC. This system is more used on laboratory scales and for obtaining a high purified industrial enzyme.

- The first step after the latex extraction is the addition of PEG and ammonium sulphate. Then the pH must be adjusted using HCl or NaOH.
- Latex is dissolved with water on 3:5 ratio latex/water.
- The two phases are formed after centrifugation (9.000 x g for 30 minutes). Even though there could be rests of papain on lower phase, the top phase, the one of PEG, is the one which contains the enzyme.
- The top phase of the two-phase system is dialyzed at 4º and a buffer solution of 50 mM sodium acetate at pH 5 should be added.
- To separate the enzyme from the PEG, an ion-exchange chromatography is carried out with the method of Azarkan et al. (2.003).
- A CM-cellulose column (1,5 x 2 cm) with another 50 mM of sodium acetate are used to wash the solution. The enzyme is loaded to the column, and then eluted from column with the buffer containing NaCl.
- The enzyme of papain is eluted because of the use of the two buffer solutions, but it is ready for the drying.

3.2.3. New method of purification based on ultrasonic grinding

This method has been recently patented by Limin et al. (2.009) and is based on new technologies to extract and purify the enzyme. The aim of this method is to achieve a higher purity if the enzyme due to the higher demands of the enzyme on the pharmaceutical industry. In addition, this method uses an aqueous two-phase extraction, but without producing any organic solvent residue.

- Ultrasonic grinding

On this method there is no latex extraction. The papaya fruit is cleaned by peeling the skin and extracting the seeds. Then the papaya flesh should be cut up and some distilled water must be added to create a slurry solution with the pulp of the fruit. This solution must be kept under ultrasonic waves between 40 and 59 kHz between 30 minutes and one and a half hour. Then the solution is centrifugated and impurities are eliminated by an ultrafiltration. The temperature during this part of the process should be around 2 ºC.

- Chromatography

An air-dry process is carried out on the liquid solution obtained from the grinding, until the liquid has completely been evaporated. 100 g from the powder obtained is added to distilled water. A suction filtration is done with a G5 molten sand funnel. The filtrate can be separated on a Sephadex G100 chromatography column. A solution with the enzyme is obtained.

- Double aqueous phase extraction

This last part of the purification process consists on creating a two-phase system with polyethylene glycol and potassium phosphate. The mass ratio of both of them is 2,3:1. The pH of the two-phase system has to be adjusted to 3,4. When this aqueous two phase system is created, the sample with the enzyme can be added an all the system has to be mixed. Finally, a centrifugation is needed to obtain the enzyme, and the enzyme is ready for the drying.

The results of this method are shown on Table 8. For this study, no drying method has been used, the enzyme recovered for the centrifugation was directly analysed.

Table 8. Results of the study.

Enzyme activity at 37 ºC [million u/g]	3,2
Enzyme purity [%]	99
Enzyme activity recovery rate [%]	117

Even though the enzyme purity and the enzyme recovery rate are high, this method has not been escalated to industry, but there is the possibility to do it. In addition, the results of the study are shown without the drying carried out, and the method of drying also has a high influence on the final enzyme activity.

3.2.4. Crystallizing papain

This was one of the first methods used for papain purification. After the methods of two step salt precipitation and the aqueous extraction in two phases and drying where designed, which obtained a higher purification and enzyme activity, the crystallization was not used. However, the study of Monti et al. (2.000) has shown that with a modification of the crystallization method, also high-quality enzyme can be obtained. The entire procedure has to be carried out under nitrogen bubbling for protection against atmospheric oxygen. This new method is based on the following steps:

- After the latex extraction,1 mM of EDTA at pH 7 is added and the sample is kept in constant shaking during one hour under nitrogen at room temperature.
- The suspension must be centrifugated at 12.000 x g for 30 minutes at room temperature. On this study a Sorvall RC-2B centrifuge with an SS-34 rotor were used.
- The precipitant resulted of the centrifugation is discarded. The supernatant must have a yellow-greenish colour. The pH of the supernatant has to be adjusted to 9 by the addition of NaOH 0,1M. This addition should be slowly done and with agitation because an excess of pH will denaturalize the enzyme.
- Another centrifugation is carried out at 12.000 x g for 10 minutes at room temperature. The clear supernatant has to be placed on a flask and kept in an ice bath and in an ice box.
- After 72 hours in the ice bath, the first crystallization happened by spontaneously precipitation. The enzyme is collected by another centrifugation at 12.000 x g for 20 minutes at 0 ºC.
- The supernatant is washed three times with 0,1 mM of EDTA at pH 7 and at 4 ºC to carry out another centrifugation at 12.100 x g for 20 minutes at 0 ºC.

- The precipitant is now collected to be dissolved again in 0,1 mM of EDTA at pH 7 at 37 ºC for 30 minutes at the proportion of 25 mg of protein / ml of EDTA.
- The solution is placed on an ice bath at 0 ºC and the recrystallization happens by spontaneously precipitation. This precipitated has to be dissolved again in 1 mM of EDTA and stored at 4 ºC and is already for the drying.

The results of this study show that a papain can be obtained with similar purity properties than other methods. However, this study concludes that a natural inhibitor of papain was released during the process. Table 9 shows the esterase activity of papain measured in $Kcat \cdot s^{-1}$ per mol of protein by different concentrations. As it can be seen, when a higher concentration of the enzyme was reached, it had a lower esterase activity.

Table 9. Results of esterase activity. extracted from Monti et al. (2.000).

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

The following conditions were used: kcat was determined using 2×10^{-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, 25ºC

The authors suggest that further has to be researched in order to avoid the releasing of this inhibitor during the process. As this method has not given the expected results, it is not going to be compared to the other methods of purification on the next sections of this bibliographic research.

3.3. **DRYING**

The drying is the last separation process to obtain the enzyme. The method of drying is determinant on the quality of the final product. Drying is an operation that tends to require a high level of energy, but it will depend on the type of drying.

3.3.1. Sun drying

This is the traditional method. This method is viable in countries where room temperatures are high. This method produces the lowest enzyme quality. It is hard to control the temperature of the enzyme during the process. Therefore, usually the enzyme burns and it turns brown. This method is still used in many countries because of the low costs, it just consists on spreading over a surface under the sun to dry. The time will depend on the temperature and the sunlight.

3.3.2. Oven drying

This method consists on laying the solution with papain on an oven during 4 to 5 hours and a temperature between 35ºC and 40ºC. The drying finishes when the latex or solution with the enzyme is crumbly and not sticky. This method should not be carried on a metal container because the enzyme could be denaturalized. This method has an easy scalability to industry, but the problem it has is that the enzyme activity is not as high as other methods. Lambri et al. (2.014) reports that due to the long period of exposure to high temperatures the enzyme could also lose enzyme activity. This method suggests decreasing the drying time by increasing the temperature, but because of the risk of denaturalization of the enzyme further has to be researched.

3.3.3. Spray drying

This method is the most expensive. The enzyme obtained by this method is the most pure and active, and it must be handled with care because it can cause allergic reactions when inhaled.

This method consists on the following steps. The concentrations of reactants, temperatures and the steps are based on Sin et al. (2.020). This study determined the optimal conditions to carry out the spray drying.

The Table 10, extracted from the study, shows the optimal relation of maltodextrin concentration fixing the feed flow and the temperature (based on Fang et al. (2.011)). The optimal maltodextrin concentration has been proven to be at 20 % (w/w). Chang et al. (2.018) suggests that higher concentrations of maltodextrin give more solid powder, but the yield of papain is smaller due to a higher concentration of maltodextrin in the powder.

Table 10. Results process yield at different maltodextrin concentration. Extracted from Sin et al. (2.020).

Maltodextrin concentration (% w/w papaya puree)	Process yield (%)	Feed flow rate (g/min)	Outlet temperature (°C)
10	63.09 ± 7.38	2.87 ± 0.17	92-95
20	74.91 ± 9.15	2.71 ± 0.23	95-99
30	60.48 ± 6.55	3.00 ± 0.17	94-99
40	59.61 ± 4.61	3.15 ± 0.04	94-97
50	53.07 ± 8.53	3.30 ± 0.04	94-98

The Table 11 shows the result of different inlet temperatures. The inlet temperature with the highest yield is at 160 ºC.

Table 11. Results of process yield at different temperatures. Extracted from Sin et al. (2.020).

Inlet Temperature (°C)	Process yield (%)	Feed flow rate (g/min)	Outlet temperature (°C)
140	42.28 ± 4.72	2.93 ± 0.07	84-87
150	74.01 ± 7.69	2.78 ± 0.15	89-91
160	80.67 ± 3.74	2.78 ± 0.10	96-98
170	72.60 ± 9.64	2.91 ± 0.81	100-102
180	34.32 ± 7.88	2.93 ± 0.07	106-114

The steps to carry out a spray drying are the following ones:

- The enzyme puree that comes from the purification process must be dissolved on a maltodextrin solution of a 20% of concentration. The solution should be prepared dissolving maltodextrin DE-10 in warm water. The ratio of the dissolution must be 0,4 g of enzyme puree / ml of maltodextrin solution.
- It is important for the solution to be stirred to guarantee the homogenization of the solution for the spraying.

- To warm up the spry dryer it is recommended to first spray distilled water for 5 minutes. A mini spray dryer (in the case of the study, the Büchi Labortechnik AG, B-290) is used in an aspiration rate of 90%. The nozzle speed is set to 5 and the air compressor to 40 nm. The inlet temperature of the spry dryer must be set to 150 ºC.
- Then the sample can be pumped. After the spraying of the sample, a spry-dried papain powder can be recovered from the collection vessel of the spry dryer.

This method is more used on a laboratory or if the enzyme obtained requirements must have a very high quality. The main problems of this process are the difficulty of the scalability of the process and the security measures due to the effects that the high purified enzyme have to people. On the Figure 9 it is shown the scheme for the spry drying method.

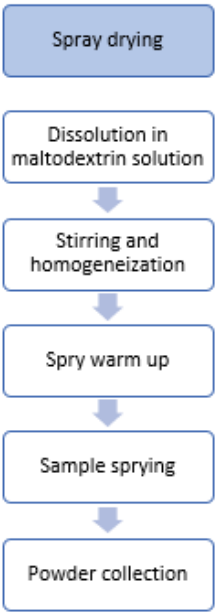


Figure 9. Scheme of the Spry drying.

3.3.4. Refractance window drying

It consists on leaving the liquid or puree with the enzyme on a thin transparent film (the window). This film usually is from Pyrex glass or polyethylene. This film rests over a surface of hot water (95 ºC) where the heat transfer occurs.

In an industrial process, the film is moved in a co-currently with hot water. In a laboratory scale, the film tends to be stationary. This method has the benefits of an easy scalability from laboratory to industry, not very high costs, and a high quality on the final product. However, vacuum drying has a similar cost and it is industrial scalability is also viable, and a higher enzyme activity is obtained. The Table 5 shows that vacuum drying gives a higher enzyme activity by a same purification method.

3.3.5. Lyophilization

The lyophilization is based on leaving the sample with the enzyme on a freeze-dryer during several hours and at very low temperatures. Before the lyophilization, the samples or enzyme-puree must be frozen. To obtain the enzyme powder, the conditions of the freeze dryer have to be set on -80 ºC and 9,8 Pa for 17 hours. This method is the slowest one.

3.3.6. Vacuum drying

This method is based on patent of Steiner et al. (2.006). The method consists on creating a vacuum. By this vacuum, the evaporation temperature of the water decreases and the enzyme is more protected from a denaturalization because of the high temperatures. The drying is carried out at 40 ºC.

The drying takes place in a non-metallic recipient. This recipient must have a doble wall, where in the space between walls the heat sources are installed. There is not a specific heating source, it can be electric, hot water, etc. Pumps are used to create the vacuum inside the recipient. To increase the surface of transfer, an air swirling is generated on the surface of the sample with the enzyme by injected air curtains.

A variant of this process consists on reducing the pressure inside the recipient. By this variant the time of drying is reduced, but more pumps are needed to reduce the pressure. In addition, to have a large transfer surface, slide sheets are also needed inside the recipient.

This method is the most used in industry because the risks of denaturalizing the enzyme are low. Moreover, the enzyme obtained has a high purity and the enzyme activity is also high. This method is easy to escalate to an industrial scale. On Table 5 the comparison with Refractance Window Drying is shown. This method obtains a higher yield of papain per latex.

3.4. **METHODS COMPARISON AND SELECTION**

The selection of the method of extraction and purification of the enzyme should be based on the object of the experiment, the purification level of the enzyme and the viability of scaling it to an industrial level if needed.

The Tables 12, 13 and 14 show the differences between the type of papain because of the methodology used to obtain it, and the differences between the purification and drying methods to obtain the papain.

The idea of these tables is to have all the information to compare and choose the best method to extract, purify and dry the enzyme of papain from papaya fruit.

Table 12. Differences between types of papain.

Differences	Crude papain	Semi-Purified papain	Purified papain
Purification	Low	Medium	High
Enzymatic activity	Medium	High	Very high
Need of stabilizer agents	No	Yes	Yes
Costs	Low	Medium	High
Industrial scalability	Easy	Complex but viable	Complex, will depend on the method of drying
Use of the enzyme	Meat tenderization and beverages clarification	Food industry (Juices) and some pharmaceutical and cosmetic commodities	Laboratory analysis and medicines

Table 13. Differences between the purification methods.

Purification methods			
Differences	Two step salt precipitation	Extraction in aqueous two-phase system	Ultrasonic grinding purification
Purification level	Low	High	Very high
Method complexity	Medium	High	High
Enzymatic activity	High	High	High
Industrial scalability	Yes	Yes	Possible but not done

Table 14. Differences between drying methods.

Drying methods						
Differences	Sun drying	Oven drying	Spray drying	Refractance window drying	Lyophilisation	Vacuum drying
Complexity level	Very low	Low	Very high	Medium	Medium	Medium
Industrial scalability	Easy	Easy	Viable, complex	Easy	Easy	Easy
Time	Depends on the weather	4 to 5 hours	Quickly, but it will depend on workers and automatization of the process	Depends on the forced contact between the water and the window	17 hours	Quickly, but it will depend on the heating system
Purification level	Very low	Medium	Very high	High	Medium	High
Enzyme activity	Low	Medium	Very high	High	Medium	High

The Table 15 shows an example for each type of papain, which methods of purification and drying will be the most appropriate. In the case of crude papain, the combination of methods chosen are based on the low costs and industrial scalability because of the industrial uses it has. In the case of the purified papain, the methods chosen are based on the purification level and enzyme activity of the extracted papain, as it is required for this type of papain. For the semi purified papain, the methods chosen are based on a relation between the costs, the industrial scalability, the enzyme activity and the purification level, as it is required for this type of enzyme.

Table 15. Examples of methodology.

Type of papain	Crude papain	Semi purified papain	Purified papain
Latex extraction	There is just one method of latex extraction		
Purification	-	Two step salt precipitation	Extraction in aqueous two-phase system
Drying	Sun drying	Vacuum drying	Spray drying

4. STORAGE OF PAPAIN

Sometimes, the determination of the enzymatic activity cannot be directly done after the purification, and then a storage of the enzyme is needed until the next use. Paul et al. (2.013) shows which methods are possible and how much time can the enzyme been stored with the same or a similar enzyme activity.

On the Table 16 there are shown the results of the study. As it can be seen, if papain is directly stored, the enzyme activity has a big decrease in just few days. Therefore, it is recommended to use chemical agents to store it. The three methods of storage studied are:

- Papain with a pectin film over the sample.
- Papain with a pectin film and a 0,75% of glycerine.
- Papain with a 0,25% of pectin.

As it is shown on Table 16, the most efficient storages are the ones which use the pectin film over the sample. The addition of Glycerine on the sample does not improve the activity of the enzyme after some days, so the best method will be with just the pectin film.

Table 16. Results of different storage methods.

Method of storage	Storage time [Days]	Activity	Related Activity [%]
Papain		Abs [min]	
	0	0,022 ± 0,004	100
	3	0,0022 ± 0,004	9,9
	7	0	0
Papain with pectin film		Abs [min x g of film]	
	0	6,409 ± 0,628	100
	30	6,345 ± 0,32	99
	180	6,28 ± 1,696	98
Papain with pectin film and 0,75% of Glycerine		Abs [min x g of film]	
	0	3,974 ± 0,168	100
	30	3,925 ± 0,175	98
	180	3,568 ± 1,103	89
Papain with 0,25% of Pectin		Abs [min x g of film]	
	0	6,585 ± 0,657	100
	30	1,904 ± 0,13	29
	180	1,097 ± 0,411	16

5. DETERMINATION OF THE ENZYME QUALITY

5.1. AMOUNT OF LATEX AND PROTEOLYTIC ACTIVITY DEPENDANCE

The proteolytic activity and the amount produced from a papaya fruit will depend a lot on the procedure, the fruit age and the sex of the tree (female and hermaphrodite). Madrigal et al. (1.980) makes an analysis to all these patterns to determine which fruit has the highest yield of latex and the highest proteolytic activity. On the Table 17 there are shown the differences depending on the sex of the tree and the age of the fruit.

Table 17. Results of the research.

Yield of latex	Between female and hermaphrodite were similar.
Proteolytic activity	For hermaphrodite trees, the proteolytic activity of the enzyme was lower than for female trees, and the proteolytic activity changed depending on the time of the day the latex was extracted.
Yields of crude papain	In both sexes, the yields of crude papain were higher when the fruit was older, from 2,5 to 3 months old. However, for fruits from female trees the activity was higher in younger fruits, from 1,5 to 2 months old.
Incisions	There have not been seen differences between yields of papain depending on the number of incisions.

Hulikere M. et al. (2.014) shows the differences of latex extraction and enzyme activity between the different papaya fruits. This study analyses the four main types of papaya, the carica papaya, the red lady, the arca surya and the prabath. The study showed which of these varieties had the highest enzyme activity on different temperatures, pH and substrate concentrations. The results are shown in Figure 10 from the lowest enzyme activity to the highest.

Figure 10. Level of enzyme activity on different varieties of papaya fruit.

5.2. **METHODS OF DETERMINING THE ENZYME ACTIVITY**

There is not a correct method for determining the enzyme activity, all the methods are valid. The difference between methods is the units of measurement of the enzyme activity.

5.2.1. Tyrosine method

On this method based on Andrade-Mahecha et al. (2.011), Paul et al. (2.013) and Afaq et al. (2.001), casein is used as a substrate at 40 ºC and pH=8,2. Tyrosine is released and can be measured by spectrophotometry at 280 nm. The activity can be determined using the Equation 1.

$$AU = Sampling\ absorbance \cdot \frac{Tyrosine\ [\mu mol]}{Absorbance\ of\ 1\ \mu mol\ of\ Tyrosine} \cdot T_{incubation} \qquad (1)$$

For this method, a known amount of papain has to be mixed with a fixed amount of casein. The reaction happens at 40 ºC. After 60 minutes, the reaction must be stop with a change of pH by a strong acid addition. Then the tyrosine formed can be read on the spectrophotometer.

5.2.2. Milk coagulation method

This method from Andrade-Mahecha et al. (2.011) and Ming et al. (2.002) is based on the break of the protein structure of milk, which is going to be the substrate of the enzyme. In this method 10 mg of a solution with papain are going to be analysed.

This solution must be prepared with 1 g of enzyme in 10 g of acetic acid (0,01%). These 10 mg of the solution were added to 10 ml of milk. The solution of milk must be prepared with 2,5 g of milk powder in 100g of water and heated in a water bath at 50 ºC.

At this point, the time must be registered while the tube is agitated until the phenomena of coagulation can be seen. Using the Equation 2, the Upe (Units of potency to coagulate milk per gram enzyme) can be determined.

$$Upe = \frac{1.000}{E \cdot t} \qquad (2)$$

5.2.3. N-CBZPHE-ARG-7-MCA Method

This method based on Paul et al. (2.013) consists on using N-CBZ-PHEARG-7-MCA as a substrate. For this method, 20 µL of the sample with the enzyme are mixed with 120 µL of N-CBZ-PHEARG-7-MCA, acetic acid (30% v/v) and 40 µL of EDTA cysteine buffer.

The experiment is carried on a micro-plate. The micro-plate and all the solutions should be kept in ice bath before the experiment. To carry out the experiment, the micro-plate must be heated in water et 40 ºC for 45 minutes with the solution. Every 15 minutes, the reaction has to be interrupted with the acetic acid 30% (v/v) addition. A curve with four time values can be done. Fluorescence has to be registered with filters giving a wavelength at 360 and 460 nm, determining the concentration of the enzyme with the increase of the velocity of fluorescence.

5.2.4. Z-Gly-pNP Method

This method is also based on Paul et al. (2.013) and Monti et al. (2.000). It consists on a reaction of hydrolysis between N-benzoyl-L-argininethyl ester (BAEE) and papain (Kirsch et al., 1.966). This reaction must be monitored at 400 nm and a temperature of 25 ºC. The reaction must be done with a buffer solution of 0,1 M of sodium phosphate, 6,7% of acetonitrile and 1 mM of EDTA in order to have a pH of 7 during the experiment. On this study it is recommended to add HCl to adjust the ionic strength to 0,3 M.

On this case, the specific activity is defined as the relation between the µmol of N-Carbobenzoxiglycil P-nitro Phenyl Ester (Z-Gly-pNP) produced per minute and the mg of protein.

5.3. METHODS FOR DETERMINIG ENZYME PURITY

The purity of the enzyme is also important to determine the quality of the final product. As it happens with the enzyme activity, there is no right or wrong method to analyse the purity. These are the two methods more used to determine the purification level of the enzyme.

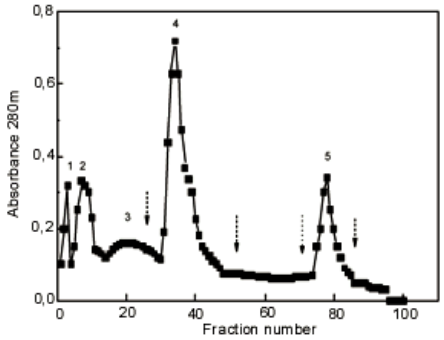
5.3.1. Fast Protein Liquid Chromatography (FPLC)

This is one of the most used methods to determine the purity of the enzyme. This method is based on Nitsawang et al. (2.006) and consists on an ion-exchange chromatography on FPLC. The steps to carry out this analysis of purification are the next ones:

- 25 µL of the sample are mixed with 20 mM of glycine-NaOH buffer at pH 10,6.
- This solution was introduced into a column (Mono Q HR 5/5) already equilibrated with the same buffer. The column should be washed before with 5 ml of the buffer.
- Using a programmed linear gradient of sodium chloride from 0 to 0,5 M allows the elution of papain and other proteins at a flow rate of 1 ml/min.
- Fractions of 1 ml have to be collected.
- Chromatographic plots at A280 and the gradient composition versus the elution volumes must be recorded. The elution peak of papain can be obtained with an automatic integrator.

As there are peaks of other proteins, it is recommended to analyse before the experiment standard papain to compare the peak with the experimental one.

On Figure 11 extracted from Monti et al. (2.000) the result of a chromatography is shown. This study carried out the chromatography directly to the latex extracted without purification. Therefore, other peaks can be seen corresponding to other substances. The second peak is the one that corresponds to papain.



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 11. Results of the study Monti et al. (2.000).

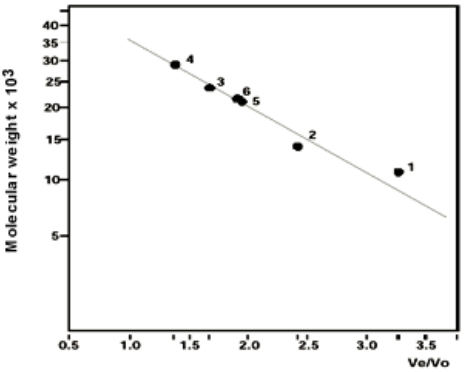
5.3.2. PAGE method

This method is based on Monti et al. (2.000) and Dhivya et al. (2.018) and consists on a Dodecyl Sulphate -Polyacrylamide Gel Electrophoresis (PAGE).. The electrophoresis is carried out like in Reisfield et al. (1962) with a gel made of 12% of polyacrylamide described by Moutim et al. (1.998), a buffer of 34 mM b-alanine at pH 4,3 and a constant current of 4 mA per tube. The samples are prepared in Tris-glycerol-b-mercaptoetanol and placed in boiling water for 60 seconds. Gels are stained with Coomassie-Blue R-250 Brilliant Blue G-colloidal concentrated. This two Gels are based on the preparation of Neuhoff et al. (1.988).

The molecular weight of the enzyme obtained is determined with a Sephadex G-75 column (1,1 x 100 cm). This column has to be previously equilibrated with a buffer of 0,1 M of sodium phosphate and 1 mM of EDTA at pH 7.

For the determination of the molecular weight, a pattern with different substances has to be carried out to obtain a calibrate of the column. It is recommended to use a standardized papain enzyme between the substances of the pattern to compare it with the result of the enzyme obtained in the laboratory.

The calibration using different substances obtained on the study is shown on Figure 12. On the study, the standard papain enzyme gave a molecular weight of 21,3.



- Molecular weight estimation of papain by Sephadex G - 75 gel filtration column (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 6) Papain from our methodology

Figure 12. Calibration extracted from Monti et al. (2.000).

6. METHOD PROPOSAL

As the aim of this research is to compare and find an extraction and purification method of papain in order to carry out other experiments or further research with the enzyme, the highest purification level of the final product is the main criteria for this proposal.

The variety of papaya suggested to use for an experiment is the Arka Surya from a female tree, as it is reported in section 5.1. it gives the highest proteolytic activity.

For the latex extraction the method proposed is the only one viable now and explained in section 3.1. After the latex extraction, as reported in Nitsawang et al. (2.006), it must be stored at -20 ºC. However, if it is not possible to reach this temperature, one of the stabilizers proposed by Dam et al. (1.990) can be used, e.g. cysteine 0,1 % (w/w), and the stored around 0 ºC.

A purification by extraction in aqueous two-phase system as explained in 3.2.2. is proposed. Furthermore, as it is suggested in Monti et al. (2.000), a nitrogen bubbling is proposed on this research for the purification whenever it is possible in order to improve the quality of the final product by protecting the enzyme from atmosphere oxygen.

The drying method proposed is the spry drying explained in section 3.3.3., as it is reported as the method with the highest quality enzyme obtention.

Figure 13 shows the scheme of this method proposal.

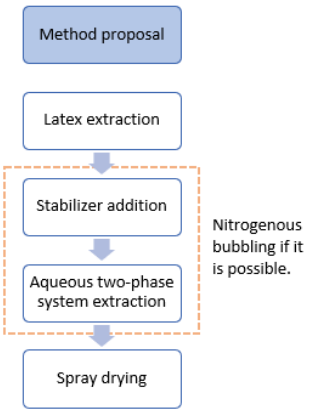


Figure 13. Scheme of the method proposal.

7. CONCLUSIONS

There are different methods that can be used to obtain purified papain depending to the aim of the experiment. Moreover, these methods depend on different separation processes and can be adapted to different laboratory instruments and materials. These separation processes are improved every day and it is important to be up to date to assure the most updated method for the extraction and purification of papain.

An extraction and purifying method has been proposed based on the bibliographic research carried out in order to achieve an enzyme with the highest quality. This method is based on the latex extraction, the addition of a stabilizer, e.g. cysteine 0,1 % (w/w), an aqueous two-phase system extraction and a spray drying.

Enzyme activity is not the only important factor to determine the quality of an enzyme, also purification is important. Different methods of purification and of determination of the enzyme activity are viable, and there is not one better than the other. It is important to take into account that units of the enzyme activity can be different depending on the method used to determine it.

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ACRONYMS

ECHA: European Chemicals Agency

EU: European Union

PST: Positive Skin Test

U.S.: United States

FDA: Food and Drugs Administration

CAGR: Compound Annual Growth Rate

EDTA: Ethylenediaminetetraacetic acid

PEG: Polyethylene glycol

Z-Gly-pNP: N-Carbobenzoxiglycil P-nitro Phenyl Ester

BAEE: N-benzoyl-L-argininethyl ester

FPLC: Fast Protein Liquid Chromatography

PAGE: Polyacrylamide Gel Electrophoresis