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DEVELOPMENT OF PAPAIN PROTEASE EXTRACT CONJUGATED WITH ZINC OXIDE NANOPARTICLES TO EXTEND THE SHELF LIFE OF FRUITS

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ABSTRACT

An Enzyme found in the papaya species naturally is called papain, commonly known as papaya proteinase I. These days, papain is a necessary enzyme in the fields of wound healing, cosmetics, and meat tenderization. They can be used to treat jellyfish strings, parasite infections, and tooth caries. The processes used to make papain are synthetic, physical, and chemical processes. We would much rather make papain physically from papaya leaves. The zinc oxide nanoparticles are created afterwards. This was supported by FTIR, FESEM, and UV-visible. In order to create a coating solution, the synthesised zinc oxide nanoparticles were conjugated with papain protease. Sapota and grapes are coated with the produced coating solution, extending the fruit's shelf life.

Keywords : Papain, Physical method extraction, Production of Zinc oxide nanoparticles, Coating solution.

INTRODUCTION

Papain is a protease enzyme that is derived from papaya fruit (Carcia papaya) and is known for its broad specificity and high activity. It is a cysteine protease meaning that it cleaves peptide bonds within a protein by utilizing a cysteine residue at the active site. Papain is commonly used as a meat tenderizer due to its ability to break down muscle fibers but it also has a variety of other applications in food, pharmaceuticals and biotechnology industries.

In the food industry, papain is used for the production of protein hydrolysates, which are used as flavors enhancers and nutritional supplements. Papain can also be clarify beer an wine as well as to improve the texture and flavor of cheese. In the pharmaceutical industry, papain is used in the production of therapeutic enzymes, such as chymopapain, which is used to treat herniated discs. Papain is used in wound care products, as it has been shown to promote healing by removing dead tissue form wounds.

In biotechnology, papain is commonly used for protein digestion and purification. It can be used to cleave fusion tags from recombinant proteins, as well to digest proteins for mass spectrometry analysis. Papain has also been used in the production of bioactive peptides, which have a variety of potential therapeutic applications.

Research has also been conducted into the potential health benefits of papain. Studies have suggested that papain may have anti-inflammatory and anti-oxidant properties and may also have potential as a natural remedy for digestive issues..

ORIGIN AND MORPHOLOGY

A small botanical family of 31 species, the papaya is indigenous to tropical and subtropical America. The most commercially significant and frequently cultivated species of papaya is Carica. It is also known as papaw, paw paw, kapaya, lapaya, tapaya, papayao, papaya, papaia, papita, lechosa, fruta bomba, mamon, mamona, mamao, and tree melon. The majority of the time, it is grown between 32° North and 32° South in tropical and neotropical climates.

On papaya plants, there are huge monoaxial palms with leaves that resemble palms. Young plants have solitary stems during their first one to two years of growth.

Heavy lateral branch may also emerge early on juvenile plants in some extremely fertile orchards. The deep, palmate leaves of an adult papaya are held upright by smooth, hollow petioles. On the hollow, smooth, light brown stem, this petiole leaves smooth, highly textured leaf scars. As the plant gets bigger, the persistent leaf scar gets bigger. On the other hand, ripe papaya fruit are filled with vitamins A and C and resemble melons. Papain, a protease derived from unripe green fruit, is found in the latex.

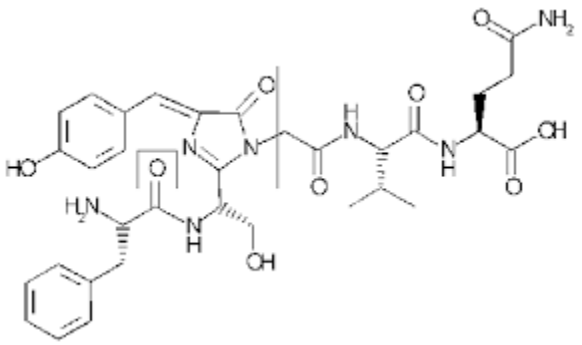
PAPAIN

Papain belongs to the family of cysteine proteases and has one polypeptide chain with three disulfide bridges. Crude papain is the name given to dried Carcia papaya latex in the industry (1). It belongs to the Caricaceae family and is often known as papaya. It is regarded as one of the most widely grown fruits in the Caricaceae family. Wurtz and Bouchut gave the papaya enzyme its initial name in the late 19th century(2). Papaya leaves contain large amounts of papain and chymopapain. They are a light, green tint and have a pH range of 6.5 to 9.0. The elimination of polypeptide sections is one of the many functions for papain, a member of a family of related proteins.

The precursor protein contains 345 amino acid residues. Papain only has 8 residues because there aren't enough cysteine residues to completely wrap the cluster. The various functional groups, such as -COOH and -NH2, grabbed the metal ions when they were added to the papain solution(5). The protein was employed as a potent reducing and encapsulating agent(4). With only one 212-residue chain, papain is a rather straightforward enzyme. Most papain residues have a hydrophobic nature.

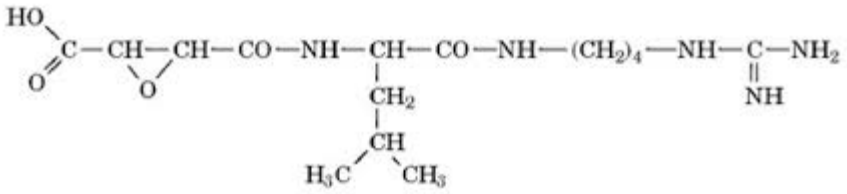
STRUCTURE OF PAPAIN

Papain takes on a globular shape mostly due to water's exclusion of these residues. Papain appears to be simple and straightforward, but it actually folds into two different domains of equal size, each with a separate hydrophobic core surface residues are transparent, hydrophobic-core residues are coloured and opaque, and the remaining residues are polar, non-surface residues(5).



Hydrophobic interactions and hydrogen bonds between sidechains of nearby structures hold secondary structures in place in the meantime. Papain has a broad selectivity for peptide bonds in its catalytic activity, although it prefers an amino acid with a long hydrophobic side chain at the P2 position while rejecting Val in the P1 position (5).

MOLECULAR FORMULA FOR PAPAIN



The substrate binding pocket is located between the two domains. The secondary structure of papain is made up of 25% alpha helices and 21% beta sheets (45 residues totaling 17 sheets and 51 residues totaling 7 helices). The remaining residues are organised non-repeating sequences, which make up more than 50% of the structure of the enzymes (6).

MATERILAS AND METHODS

Introduction

The papain protease is extracted from the papaya leaves by physical methods. The separated protease is estimated by using casein 1% and 0.1M of phosphate buffer pH of 5.5 which is incubated in a water bath for 15 minutes. Then Folin's C reagent is added to the sample. Then by using natural substrate method the papain is estimated. Then Zinc oxide nanoparticles are synthesized and conjugated with the papain protease in order to prepare coating solution. The Zinc oxide nanoparticles are characterized by SEM, FTIR, FESEM. Then the nutrient analysis, antimicrobial activity is noted.

Collection of sample

The freshly collected papaya leaves are collected and made to air dry. Then the dried leaves are grinded into fine powder by using electric mixer. The powder sample is stored for further use.

Extraction of papain crude enzyme

5.0 g of papaya leaves were weighed and then put into 50 mL of 0.1 M phosphate buffer, which is ice-cold and has a pH of 5.5. They were homogenised for two hours using a magnetic stirrer. The residue was removed after filtering the homogenate. The 500 mL of acquired iltrate was centrifuged in the cold-refrigerated centrifuge for 10 minutes at 4 °C at 3000 rpm. The collected sediment was washed with 1 mL of cold, 0.1 M phosphate buffer, pH 5.5, after the supernatant was discarded. For 10 minutes, the crude extract was maintained at 4 °C**.**

Precipitation of the papain enzyme

Two reagents, ammonium sulphate and acetone (70% saturation), were used for precipitation. Initially, 10 ml of the crude extract were combined with different mixtures of 70% acetone and ammonium sulphate. The supernatant was gently separated from the pellet using a cold centrifuge (4 °C) at 8000 rpm for 10 minutes, and this was employed for further research after the mixture was left overnight in the refrigerator at 4 °C.

Papain estimation

1 mL of 0.1 M phosphate buffer pH 5.5, 1 mL of the extracted papain, and 4 mL of the substrate (1% casein) were added. The mixture was then incubated at 37 °C in a water bath for 15 minutes. With the addition of 1.0 mL of 4.2% perchloric acid, the process was halted. The water bath was then filled with 1 mL of copper reagent, and it was left there for exactly 20 minutes. After the mixture had cooled, 1 mL of Folin-C reagent and 7 mL of distilled water were added. After thoroughly mixing the mixture, the absorbance at 280 nm was measured against a blank. utilising a natural substrate. At 37 °C, one unit of enzyme hydrolyzes one mole of casein per minute.

Preparation of zinc oxide nanoparticle

In order to create zinc oxide nanoparticles, the sol-gel process was used. A weighting balance was used to weigh 0.1M of zinc acetate dihydrate in order to prepare a sol gel (25ml). The solutions and papain extract were swirled continuously for two to three hours at 400°C in a magnetic stirrer. Following thorough mixing, PVA (1ml) was added to the zinc acetate solution while being continuously stirred by a magnetic stirrer for approximately one hour. White precipitate that was produced after the reaction was used for more research.

Characterization of synthesized nanoparticle

1) **FTIR**

Using an FT-IR spectrometer (Shimadzu), Fourier transform infrared (FT-IR) spectra were performed for the study of functional groups. In order to analyse the sample's infrared spectrum of absorption, a range of 4000cm-1 to 400 cm-1 was used.

2) **FESEM**

The synthesised sample was ground into a fine powder by heating it on a hot plate to 100°C for the duration of the study, which was used to complete the morphological analysis of the size and form of the synthesised nanoparticles.

Coating on the fruits

A sterilised cotton swab (50–100 l) was used to coat fresh sapotta and grapes, which were then air-dried for one hour. The fruits were divided into coated and uncoated groups, and they were left at room temperature for 10 days without any additional wrapping. The fruit samples were examined every three days, and microbial load was likewise measured every three days. These tests were preserved until the fruit samples were spoiled.

Microbial load

Nutrient broth (Himedia, Mumbai, India) was used to test the microbial burden after sterilise. The medium was made by combining 13g with 1000ml of distilled water, then sterilising it in an autoclave for 15 minutes at 1210C. A single swab was completed and put to the broth after the sterilised media had been cooled to room temperature under aseptic conditions. The broth was then incubated at 370C for 24 hours. After incubation, the microbial load was assessed using a spectrophotometer to measure the OD at 600 nm.

Nutrient analysis

Protein estimation using Lowrys et al, method

Reagents

A. 2% Na_2CO_3 in 0.1 N NaOH

B. 1% NaK Tartrate in H_2O

C. 0.5% $CuSO_4.5H_2O$ in H_2O

D. Reagent I: 48 ml of A, 1 ml of B, 1 ml C

E. Reagent II- 1 part Folin-Phenol [2 N]: 1 part water

BSA Standard - 1 mg/ ml

Procedure

- 0.2 ml of BSA working standard in 5 test tubes and make up to 1ml using distilled water.
- The test tube with 1 ml distilled water serve as blank.
- Add 4.5 ml of Reagent I and incubate for 10 minutes.
- After incubation add 0.5 ml of reagent II and incubate for 30 minutes
- Measure the absorbance at 660 nm and plot the standard graph .
- Estimate the amount of protein present in the given sample from the standard graph.

Using the above said method protein content of the coated and non coated sample was analysed and calculated in mg/g.

Estimation of total carbohydrate by anthrone method

Reagents:

(i) Anthrone reagent: Dissolve 2g of Anthrone in 1 litre of concentrated H2SO4. Use freshly prepared reagent for the assay

(ii) Glucose stock solution: 200µg glucose per mL distilled water.

Procedure:

1. Pipette out into a series of test tubes different volumes of glucose solution (follow up Table 1) from the supplied stock solution(200µg /ml) and make up the volume to 1 mL with distilled water.
2. Consider tube 1 as blank and tubes 2 through 9 for construction of a standard curve. Tubes 10-15 are for the unknown samples.
3. To each tube add 5 mL of the anthrone reagent (supplied) and mix well by vortexing.
4. Cool the tubes.
5. Cover the tubes with marbles/ Caps on top and incubate at 90^0C for 17 minutes or boiling water bath for 10 minutes.
6. Cool to room temperature and measure the optical density at 620 nm against a blank.
7. Prepare a standard curve of absorbance vs. µg glucose

RESULTS AND DISCUSSION

The result of the study showed that the papain form the papaya leaves proved the extend of the shelf life of fruits. They also prevent the microbial growth and the prevent the breakdown of protein molecules inside the fruits. Synthesis of zinc oxide nanoparticles are achieved and thus the coating solution is prepared by above procedure. Sapotta and grapes are used as test samples. The prepared coating solution is coated on fruits and microbial activity is noted. Due to the prevention of protein molecule the decaying process is delayed. The results of extend of shelf life in fruits and prevention of microbial activity is imaged below.

COLLECTION OF PAPAYA LEAF

Carica Papaya Leaves will collect from nearby garden, Erode. It was ensured that the plant was healthy and uninfected. The leaves were washed under running tap water to eliminate dust and other foreign particles and to

cleanse the leaves thoroughly and dried. Then the dried leaves are grinded into fine powder by using electric mixer. The powder sample is stored for further use.

Figure 4.1(a) DRIED PAPAYA LEAF

Figure 4.1(b) PAPAYA LEAF POWDER

4.2 PREPARATION OF EXTRACT

Figure 4.2 PAPAYA EXTRACT

The sample is prepared and kept at room temperature. The prepared sample contains Papain protease enzyme. Later it is double filter to separate the presence of solute particles from the extract.

4.3 PRECIPITATION OF PAPAIN ENZYME

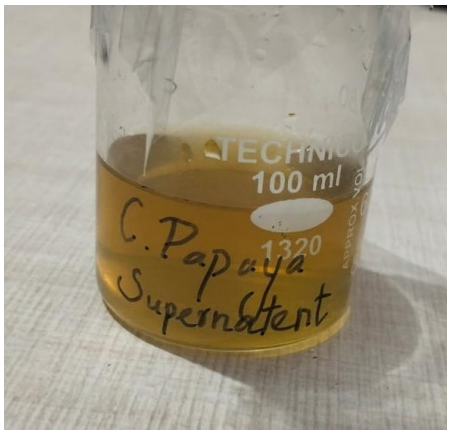


Figure 4.3 Papain Precipitation

Precipitation was done for the sample to isolate Papain protease. After centrifugation was carried out at 800rpm for 10 minutes. The supernatent contains the Papain protease which is collected gently and stored at refrigerated at 4 °C.

4.4 SYNTHESIS OF ZINC OXIDE NANOPARTICLES

The zinc oxide nanoparticles are synthesized by using sol gel method. By mixing the extract with the zinc acetate (0.1M), zinc oxide nanoparticles are synthesized.

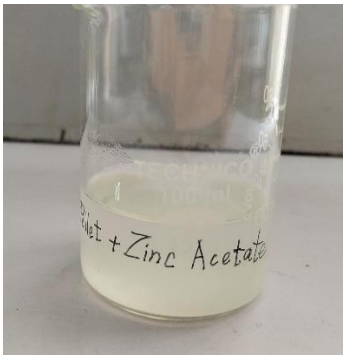


Figure 4.4 (a) Pellet with Zinc Acetate

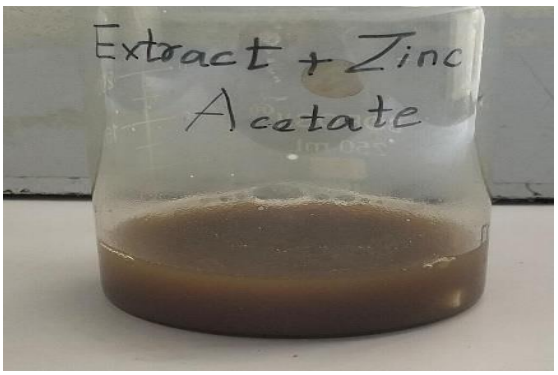


Figure 4.4(b) Extract with zinc Acetate

4.5 COATING ON FRUITS

4.5.1 Coating on Sapota

The fruits are coated with the prepared coating solution. It is viewed that the coated fruits are in extend shelf-life period. The differences are noted and tabulated.

DAY	CONTROL	COATED
Day 1	271.18g	279.57g
Day 7	261.58g	270.52g
Day 8	256.67g	269.37g
Day 9	250.81g	268.15g

It is figured out that the coated sapota have extend shelflife period when compared to the uncoated fruit. The decaying is not controlled but postponded.

Figure4.5.1(a) Before Coating Figure4.5.1(b) After Coating

4.5.2 Coating on Grapes

With the sample coating procedure for sapota the grapes are coated and weighed, the results are tabulated down.

DAY	CONTROL	COATED
Day 1	264.16g	289.47g
Day 7	260.39g	283.34g
Day 8	259.73g	282.52g
Day 9	259.09g	281.76g

From the above tabulation, it is found out that the coated fruits have extend shelf-life period when compared to uncoated fruits.

Figure 4.5.2(a) Before Coating Figure 4.5.2(b) After Coating

4.6 FTIR ANALYSIS

The sample is positioned in a holder in the IR source's path. The analogue signal is read by a detector, who then transforms it into a spectrum. The signals are analysed by a computer, which also locates the peaks. A mirror that has been partially silvered splits an IR beam into two equal-intensity beams.

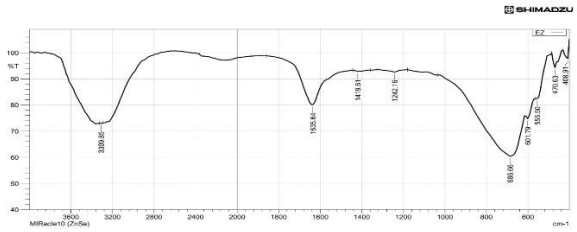


Figure4.6(a) FTIR for EZ

The results of FTIR for EZ drawn between transparence and wavelength is visualized at the spectrum region of 3363.86, 1635.64, 1242.16, 686.66, 601.79, 563.21, 470.63, 416.62.

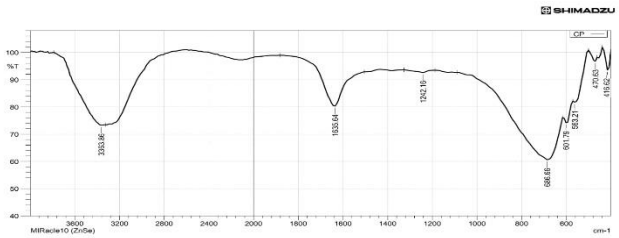


Figure4.6(b) FTIR for CP

The results of FTIR for CP drawn between transparence and wavelength is visualized at the spectrum region of 3309.85, 1635.64, 1419.61, 1242.16, 686.66, 601.79, 555.50, 470.63, 408.91.

4.7 SEM ANALYSIS

The SEM is a device that forms a picture using electrons rather than light, resulting in a significantly magnified image. An electron gun at the top of the microscope produces an electron beam. The microscope is maintained in a vacuum and the electron beam travels through it in a vertical path.

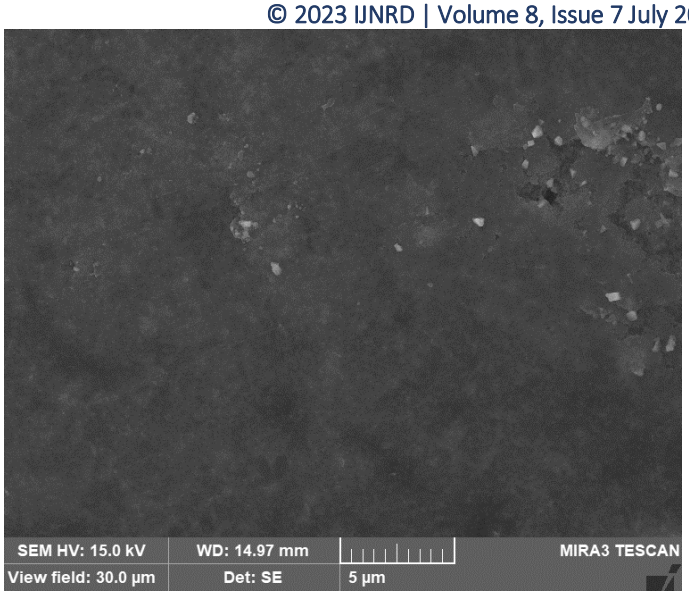


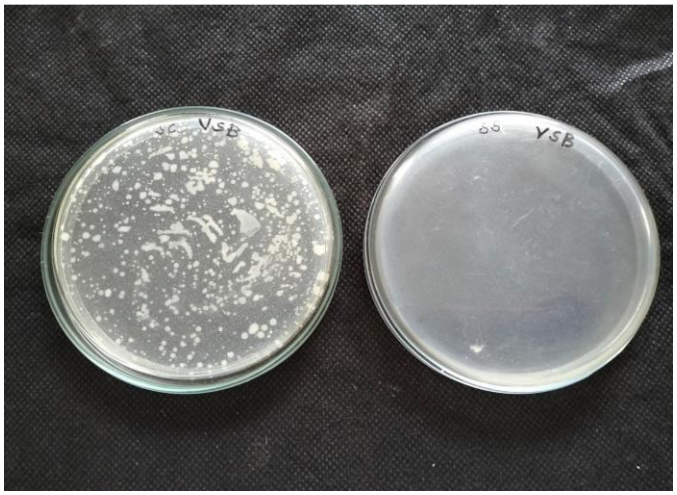
Figure4.7 SEM result

The SEM is carried to figured out the presence zinc oxide nanoparticles. The SEM results reveal that zinc oxide nanoparticles were spherical in shape and having the particles size range of 15 to 35nm.

MICROBIAL GROWTH

Microbial growth for sapota

The microbial growth is noted by using cotton swabbing both on coated and non-coated fruits. The solution is tested for microbial activity by UV-VIS spectrum at 600nm.



BLANK	UNCOATED	COATED
0	0.203	0.014

By evaluation the above table it is confirmed that the coated fruits have very low microbial activity when compared with the uncoated.

Microbial growth for grapes

BLANK	UNCOATED	COATED
0	0.192	0.00

The procedure employed to check the microbial is same as for sapota. From the above tabulation, it is found out that the microbial growth is vey low when compared with uncoated.

DISCUSSION

Papain, a proteolytic enzyme extracted from papaya leaves used to tenderize the meat, alcohols etc. Several studies have shown that the papain can extend the shelf life of fruits. For example, a study published in the Journal of Food Science and Technology in 2015, (Sarker, M. R., *et al*,2015) found that papain treated fresh-cut cucumber has a longer shelf-life period and maintained better quality attributes such as firmness, colour and microbial count. Papain has been shown to be effective against a wide range of bacteria, including Escherichia coli, Salmonella enterica, Staphylococcus aureus, etc.

And in other study published in the Journal of Food Science in 2008, demonstrated that papain could effectively inhibit the spoilage bacteria in fresh pork sausages, thereby extending their shelf life (Daniela istrati,2008).

Zinc oxide nanoparticles have been investigated for their potential to extend the shelf life of food products due to their antimicrobial properties. Zinc oxide nanoparticles are known to exhibit broad spectrum antimicrobial activity against bacteria, fungi, and viruses. Several studies have investigated the use of zinc oxide nanoparticles as food preservative. The journal of Food and Technology in 2017 found that the addition of ZnO-NPs to fresh cut lettuce reduced the growth of spoilage micro-organisms and extended the shelf life of the lettuce (Wang, L., *et al,* 2017).

So, by using all the above said conclusion we prepared the coating solution by conjugating the papain protease extracted from papaya leaves with zinc oxide nanoparticles to extend the shelf life of fruits. And the extention of shelf life of fruits is effectively seen.

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