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Isolation of anticoagulant from *carica papaya* [papain enzyme] and its separation using SDS-PAGE

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Abstract:

The present study aims to the isolation of anticoagulant from carica papaya (papain) and its separation using SDS-PAGE. The aim is to prove that papain is an anticoagulant and to determine its molecular weight using SDS-PAGE and to prove that it is a blood thinning agent using gravimetry method.

Keywords: Carica papaya (papain), anticoagulant, SDS-PAGE, gravimetry

Introduction:

Papain:

Papain is a globular cysteine-protease family consisting of a single polypeptide chain with three disulfide bridges and a sulfhydryl group necessary for proteolytic activity. Its application is extensive to the fields of medicine and food. The aim of this study is to extract, purify and characterize papain enzymes from the leaves of *Carica papaya*. Crude extracts containing the enzymes were extracted from the leaves of papaya, and purified

Papaya enzyme was first named in the late 19th century by Wurtz and Bouchut who partially purified the product from the sap of papaya and recognized it as a constituent in the latex of tropical papaya fruit . It is basically obtained by making incisions on the epicarp of unripe papaya, collecting and drying the latex which flows out. More active papain can also be obtained from a greener or unripe fruit . Papain which is a member of the papain superfamily possessed inherent proteolytic properties which are of vital importance.

Botanical description of *carica papaya:*

The papaya or pawpaw is the plant species Carica papaya, one of the 21 accepted species in the genus Carica of the family Caricaceae. It was first domesticated in Mesoamerica, within modern-day Southern Mexico and Central America. Southern Mexico and Central America. It is grown in several countries

***Carica papaya* (fig 1)**

Health Benefits of Papaya Leaves

1: Treats dengue fever.

2: Prevents malarial infection.

3: Aids digestion.

4: Prevents cancer.

5: Boosts immunity.

6: Helps in diabetes.

7:Helps in weight loss.

8: Ease menstrual pain

MATERIALS AND METHODS:

Methods involved in the process of isolation of papain from carica papaya are:

Extraction of enzyme.

Ammonium sulphate precipitation (method used to salt out protein) SDS-PAGE (separation of papain protein)

Materials involved in enzyme extraction:

- Papaya leaf
- Ice cold PBS (pH:5.5)
- Mortar and pestle
- Whatman filter paper
- Centrifuge

Materials involved in ammonium sulphate precipitation:

- Ammonium sulphate (70% saturation)
- Centrifuge
- Magnetic stirrer

Materials involved in SDS-PAGE:

- Compositions and separation properties of SDS-PAGE gels*
- 30% acrylamide/0.8% bis-acrylamide stock solution.
- 2.5x separating gel buffer.
- 5x stacking gel buffer.
- 5x electrophoresis buffer.

Coomassie staining solution.

Destaining solution.

SDS-PAGE for separation of papain protein :

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE):

SDS-Poly acrylamide gel electrophoresis was performed on slab gel with separating and stacking gels (10 & 5 % w/v) by the method of (Laemmli, 1970).

Reagents:

Stock solutions:

Solution A	1.5 M Tris HCl buffer (pH 8.8)	Tris HCl -18.171g DD.H2O -100ml Adjust pH to 8.8 with HCl
Solution B	0.5 M Tris HCl buffer (pH 6.8)	Tris HCl -9.61g DD.H2O -100ml Adjust pH to6.8 with HCl
Solution C	30 % Acrylamide mix	Acrylamide-29.2g N,N'-methylene-bisAcrylamide-0.8g DD.H2O -100ml

Solution D	10 % Ammonium per sulphate	Aps-100mg DD.H2O -1ml
Solution E	10% SDS	SDS-100mg DD.H2O -1ml
Solution F	N,N,N,N' tetramethyl ethylene diamine (TEMED)	packed

Electrophoresis procedure:

❖ Polymerization of separating gel was carried out on the glass plates.

❖ Stacking gel was polymerized over the separating gel after inserting a comb.

❖ The known amount of enzyme sample mixed with sample buffer with bromophenol blue was loaded in to the wells.

❖ And then the power supply was connected with cathode in the upper tank and anode in the lower tank.

❖ Electrophoresis was carried out at 4°C with constant voltage and 20 mA current supply for 2 hr until the tracer dye reached 0.5 cm above the lower end .

STAINING OF SEPARATED PROTEINS:

At the end of electrophoresis, gel was removed and stained with silver staining method of Blum et al (1987). After staining, the gels were stored in 7 % (v/v) acetic acid.

Silver nitrate staining protocol: (Blums et al., 1976)

Fixation: (1 hr)

Dehydration: (20min × 3)

Re hydration: (60 sec)

Washing: (20 sec x 2)

Silver nitrate: (6min)

Washing: (20 sec*2)

Developing: (until bands are seen)

CBB Staining:

Take out the gel from the glass plate carefully and transferred to a plastic tray.

Pour the CBB R-250 staining solution into the gel tray and place it on the rocker for overnight.

After overnight incubation remove the excess amount of stain by using destaining solution.

Transfer the gel on the Transilluminator and visualize the protein bands.

Preparation of Destaining solution:

Methanol – 50ml

Acetic acid – 10ml

Dis. Water – 40m.

The destaining solution and the gel prepared was left in the rotary shaker over night and destained until thebands are seen.

Blood coagulation study

Principle

.Tissue thromboplastin, in the presence of calcium ions and Factor VII, activates the extrinsic pathway of coagulation. When a mixture of tissue thromboplastin and calcium ions is added to normal anticoagulant plasma, the clotting mechanism is initiated and a clot will form within a specified time period. If a deficiency exists within the extrinsic pathway, the time required for clot formation will be prolonged. The degree of prolongation is proportional to the severity of single factor deficiency, or in a cumulative deficiency of all the factors involved.

1. Bring all reagents, controls and sample to room temperature 15 minutes prior to testing.

2. Pre-warm PT reagent at 37°C for 5 minutes.

3. Pipette 100µl of PT reagent to each tube.

4. Add 50 µl of sample, controls to the tubes prepared in step 3, start stop watch mix in a water bath(37°C)for 8 seconds , then record the time required for clot formation .

$$\text{Prothrombin Time Ratio (PTR)} = \frac{\text{Clot time of the test plasma}}{\text{Clot time of the control plasma}}$$

Reagents used in prevention of blood coagulation

Results and discussion:

SDS PAGE:

An increasing number of enzymes are recently introduced into pharmaceutical industry for their therapeutic potentials . This fact increases the demand for reliable quantification tools for such products. SDS-PAGE is widely used for proteins separation according to their mass but literature lacks any application for its use in quantitative analyses of proteins or enzymes. The proposed method describes the first application of SDS-PAGE for quantification of papain. Standard solutions of different concentrations of papain were prepared, mixed with SDS sample buffer and denatured at 95 °C for 5 min prior to application to the gel. Broad range protein standard was applied with standard papain to ensure the molecular weight range. The chromatogram run was carried out at 20 mA and 200 V. The gel was stained in Coomassie blue and distained in 5% methanol and 10% acetic acid. Distained gels were imaged and analyzed using the ChemiDoc™ XRS + System Bands corresponding to papain were sharp, symmetrical, and well resolved at R_f value 0.78 ± 0.03 corresponding molecular weight 23.406 KDa Calibration plot was obtained between absorbance of the bands and concentrations and was linear in the range 5–50 µg/spot . Linear regression data for the plot confirmed the good linear relationship .The correlation coefficient was 0.9909 which was highly significant .

Separated papain protein at 23.406KDa using SDS-PAGE

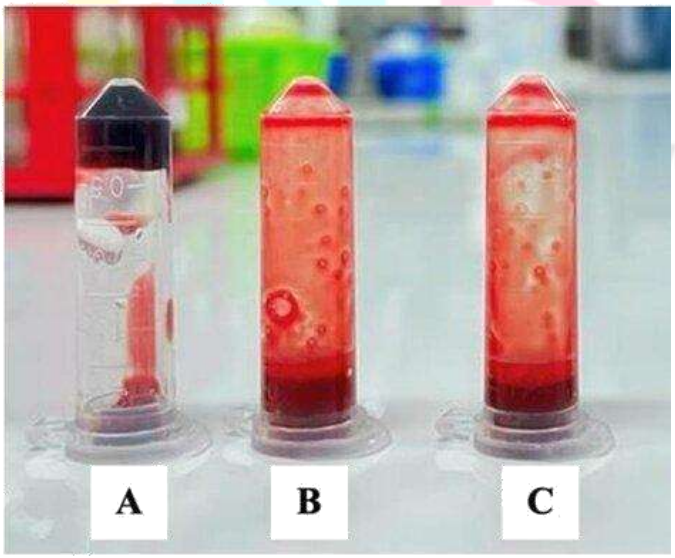
The separated papain protein forming bands at 18 KDa to 23 KDa

SDS-PAGE analysis of crude papain extract.

- lane A, B,C the crude papain protein extract
- lane D: protein molecular markers

Anticoagulant:

Blood was used to assess the aPTT and PT levels to examine the impact of papain on anticoagulant properties. In comparison to individuals who were not treated with papain, those who received treatment saw an improvement in their PT and aPTT values (Table 1). The PT and aPTT were extended over 35 sec and 200 sec at the indicated papain concentration (0.8 U/mL). Due to the modulation of the intrinsic and extrinsic coagulant pathways, papain possesses components that function as anticoagulants.



Blood clot-lysis (gravimetry) test results of crude enzyme of *carica papaya* (papain)

A. No addition of enzyme (negative control).

B. 100 uL commercial papain enzyme (positive control)

C. 100 uL crude enzyme 100% from CS-2 isolate

Conclusion:

The papain protein present in the latex of *carica papaya* can be simply extracted using phosphate buffer at pH 5.5 and can be filtered using whatmans filter paper No.7 on filtration the sediment obtained contain papain enzyme that is isolated from the papaya leaf latex.

Ammonium sulphate precipitation was done to salt out the protein contain in the extract. This process is done by using 70% saturation of ammonium sulphate in 100 ml of distilled water with the isolated protein and stirred using a magnetic stirrer . after attaining the saturation level,the mixture was centrifuged at 12,000 Rpm for 5 mins. The sediment obtain contain the papain protein was isolated .

The isolated papain protein is subjected to SDS-PAGE for the process of separation of protein and molecular weight determination.

The SDS-PAGE was ruined at 20 V to 200 V and the protein loaded was separated by the electrophoresis. The molecular weight of the protein (papain) was found to be around 16KDa to 23KDa.

The blood coagulation studies was done by collecting the blood in 3.8% tri sodium citrate polypropylen tube (ESR TUBE) and centrifuged at 4000Rpm. The plasma obtained was mixed with seiman's reagent. Pre-warmed calcium cholride and extracted papain protein was added to the mixture in different concentration. The clotting time was noted. At the concentration of 0.8 ml of papain protein the blood doesn't get coagulated.

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