International Journal of Scientific Research in Biological Sciences **Research Paper**
Vol.6, Special Issue.1, pp.89-92, May (2019) **E-ISSN:** 2347-7520
DOI: https://doi.org/10.26438/ijsrbs/v6si1.8992

Isolation of Papain from leaf & latex of Papaya (*Carica Papaya)* and study of various factors affecting enzyme activity

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Abstract-- Papain (EC 3.4.22.2) is an endolytic plant cysteine protease enzyme which is present in papaya (*Carica papaya*). Papain shows extensive proteolytic activity towards proteins, amino acid esters and amide links and its applications in the fields of food and medicine are ever emerging .The objective of this study was to extract papain enzyme from leaf & latex of Papaya plant by grinding and salt extraction methods using ammonium sulfate. The identification of papain was done using casein as substrate and enzyme concentration was determined using Lowry method. The enzyme activity from both sources of leaf and latex was shown optimum at 50 mM concentration of Casein and at pH 7.0 for leaf and pH 6.0 for latex papain. The optimum temperature for both leaf and latex papain was shown to be as 60^{o} C. The leaf and latex papain have shown only marginal differences in their activities at various factors and this study provides an easy procedure for isolation and characterization of papain for various other purposes.

Key words: Papaya, latex, leaf, salt precipitation, casein

I. INTRODUCTION

Papain enzyme is used extensively in the fields of food and medicine [1]. Papain enzyme plays an important medicinal role, such as used to treat sports injuries, other causes of trauma and allergies. The mechanism of biochemical removal of caries involves cleavage of polypeptide chains and hydrolysis of collagen cross linkages. These cross-linkages give stability to the collagen fibrils, which become weaker and thus more prone to be removed when exposed to the papain gel [2]. Papain has been used to overcome the allergies associated with leaky gut syndrome, hypochlorhydria (insufficient stomach acid) and gluten intolerance. Papain has been reported to have significant therapeutic activity against inflammation and symptoms of acute allergic sinusitis like headache and toothache pain without side effects [3]. Papain show nearly identical folding patterns with mammalian cysteine proteases especially around the active site which has been useful for drug design [4]. Papain is reported as an experimental model structure to understand the inhibition mechanism of newly developed specific inhibitors of cathepsin L, the papain super family and the antioxidant properties of papain can be useful in preventing certain types of illnesses [5].Papain is used as a clarifying agent and meat tenderizer in food industry and also used in reducing dyspepsia, other digestive disorders and disturbances of the gastrointestinal tract [6]. Papain finds its applications in the tanning industry for uniform dyeing of leather.

The objective of the proposed work was to develop an easy extraction method of papain from latex and leaf of Papaya and to measure the concentration in the extracted material. Various factors affecting the activity of Papain were also under study to provide optimum conditions for the use of this enzyme.

II. MATERIALS AND METHODS

Papaya leaf and latex were collected from local cultivar. 6M HCl , 6M NaOH, 20 mM and 40 mM Cystein, 10% sodium chloride, 50 mM casein, 20 mM EDTA, 50 mM Tris-HCl buffer are prepared with analytica grade reagents. Trichloro acetic acid (TCA), ammonium sulphate, acetic acid are of analytical grade.

Enzymes extraction from papaya leaf

Fresh leaves of Papaya were cut and washed with glass distilled water. The leaves were then dried in the normal atmospheric conditions of the lab for seven days. The leaves were ground using a mechanical grinder. Accurately 5g of grinded papaya leaf powder was dissolved in accurately measured 20 ml double distilled water. The extract was filtered through filter paper [7].

Purification of papain from fresh leaf by two-step salt precipitation

Filtered sample was mixed with 40mM cysteine at a ratio of 3:1(w/v) and the pH of the suspension was adjusted to 5.6 using 6M HCl. The contents were stirred for 15 min at 4^oC and filtered and again the pH of the filtrate was adjusted to 9.0 using 6M NaOH. Centrifugation at 10,000rpm for 30 min at 4^0C was performed to remove any insoluble material.The supernatant was precipitated with ammonium sulphate at 45% saturation. The salt–enriched solution was incubated at 4^0C 30 min. The precipitation was collected by centrifugation at 10,000rpm for 30 min, and dissolved using 20 mM Cysteine. The solution was kept at 4^0C and sodium chloride (10% w/v) was added. The mixture was slowly stirred for 30 min, followed by centrifugation at 10,000rpm for 30 min. The enzyme papain (pellet) was dissolved in water and stored at 4 ^0C [8].

Isolation of Papain from latex

The latex from papaya tree was collected, dried and ground at room temperature using mortar and pestle to form a homogenous mixture. 200 ml of 0.04M cysteine solution was added and continued grinding and the suspension was filtered using filter paper. The pH of the suspension was found to be between pH 5 to 7. The extraction methods was carried out at 0-4^0C. The pH of the filtrate was adjusted to 9.0 by 1N sodium hydroxide and the solution was centrifuged at 3500 rpm for 20min. The supernatant was collected and 40% ammonium sulphate was added and kept at 4 ^0C for 20min. The precipitate formed is removed by centrifugation at 3500rpm and the supernatant was discarded. The collected precipitate was washed with 40% ammonium sulphate solution. The precipitate was dissolved in 0.02ml of cysteine (pH-7). To the above solution 15g of sodium chloride was added and stirred continuously for 20min and incubated for 15min at 4 ^0C. The precipitate was collected by centrifugation at 2500rpm for 20min. The precipitate was suspended in 0.02M cysteine (pH6.5) at room temperature and stored at 4 ^0C.

Identification of papain

Three drops of papain extract was added to 10ml of 20% powdered skim milk and the pH was adjusted to 5.5 using acetic acid and it was incubated at 37^0C [9].

Determination of papain content

The protein content in the samples during purification was determined by folin's lowry method [10].The standard BSA in the range of 200µg/ml was used.

Protease activity determination

The proteolytic activity of the enzyme was determined by using 200µl of 50mM casein, 200 µLof 20 mM EDTA (disodium salt), pH 8.0, 700µL 50 mM Tris-HCl buffers, pH 8.0 and1000µl enzyme solutions in a tube named reaction mixture. The mixture was incubated at 37^o C for15min and followed by 3ml of 50% (v/v) trichloroacetic acid (TCA) and then cooled. Similarly another tube was made (reaction blank) with 200µl of 50mM casein, 20mM EDTA (disodium salt), pH 8.0, 700µL 50 Mm Tris-HCl buffer, pH 8.0 followed by addition of TCA and finally enzyme was added. Similarly a tube was made without enzyme. The contents were centrifuged and the supernatant was collected When protease acts on casein in the sample the amount of tyrosine liberated was estimated.by nitrosonapthol method using standard tyrosine (150**µ**g/ml). To the Different aliquots (0.2, 0.4, 0.6, 0.8, 1.0 ml), 1 ml of nitrosonapthol reagent and 2ml of acid base reagent were added and mixed. The tubes were kept in boiling water bath for 10min then cooled. 4 ml of sulphuric acid was added to all the tubes and vortexed. The color developed was measured at 520 nm and the graph was constructed.

Enzyme activity=Weight /molecular weight of Tyrosine X 1/Time X volume of enzyme

Effect of pH on papain

Tris buffers of various pH (4, 5, 6, 7, 8, 9) were prepared separately and the enzyme assay was carried out as follows: 200µl of 50mM casein, 200 µLof 20 mM EDTA (disodium salt), pH 8.0, 700µL 50 mM Tris-HCl buffers of various pH were taken in six different tubes and 200 µl of enzyme was added to all test tubes. The above test tubes were incubated 37^o C for15 min .An appropriate reagent blanks at various pH were also run. 3ml of trichloroacetic acid was added to all the tubes to stop the reaction and the mixtures were centrifuged for 15min at 10,000rpm and the supernatants were collected. 200 µl aliquots of all the supernatants were taken from each tube and the volume was made to 1ml using distilled water. To this 1ml of nitrosonapthol reagent was added and vortexed followed by the addition of 2ml acid-base reagent. The tubes were incubated for 10min in boiling water bath and concentrated Sulphuric acid was added followed by cooling of the tubes. The absorbance was measured at 520nm and by using the standard graph of tyrosine, the amount of tyrosine liberated by the papain enzyme activity at various pH values was determined.

Effect of Temperature on papain

The assay procedure followed was same as described above except that the incubation temperatures were maintained at 20°C,40°C,60°C,80°C and other parameters were kept constant.

Substrate specificity of papain

0.2 ml of 50mM egg albumin, casein, bovine serum albumin were taken in three different tubes followed by the addition of 0.2ml of 20 mM EDTA (disodium salt), pH 8.0, 700µL 50 mM Tris-HCl buffer, pH 8.0 and incubated for 20 min at 37^0C. The rest of the procedure was as described earlier.

Effect of substrate concentration on papain activity

Substrate solution of casein at various concentration of 10mM,20mM,30 mM, 40 mM, 50 mM,60 mM,70 mM were prepared separately. The assay procedure followed was exactly as above but at various substrate concentrations.

III. RESULTS

Identification of Papain

Presence of papain in papaya leaf and latex was confirmed by the coagulation of milk (Fig.1).The protein content in leaf extract of papain was found to be 47µg/ml whereas the protein content in latex extract of papain was 52 µg/ml. Hence the protein content of papain was more in case of latex as compared to leaf.

Fig.1. Identification of papain from leaf and latex

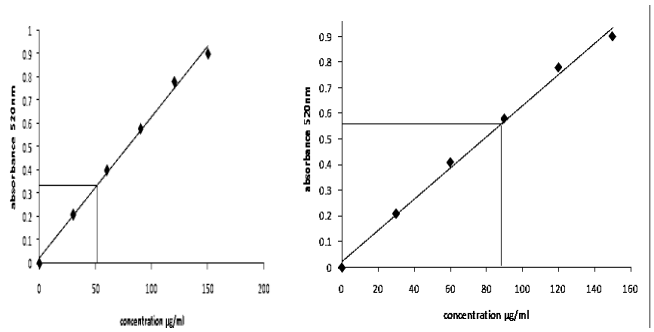

Fig.2 Enzyme activity of papain from a) leaf and b) latex

Enzyme activity

The activity of papain from leaf was found to be 24.2×10^{-3}µmoles/min and the activity of papain from latex was 42.4×10^{-3}µmoles/min (Fig.2). Thus the activity of papain from latex was comparatively high to the activity of papain from leaf.

Effect of pH on Papain

Papain from leaf exhibits its maximum activity of 1.7×10^{-3}µmoles/min at the optimum pH 7.0 but the papain from latex shows its maximum activity of 2.7×10^{-3} µmoles/min at optimum pH 6 (Fig.3).

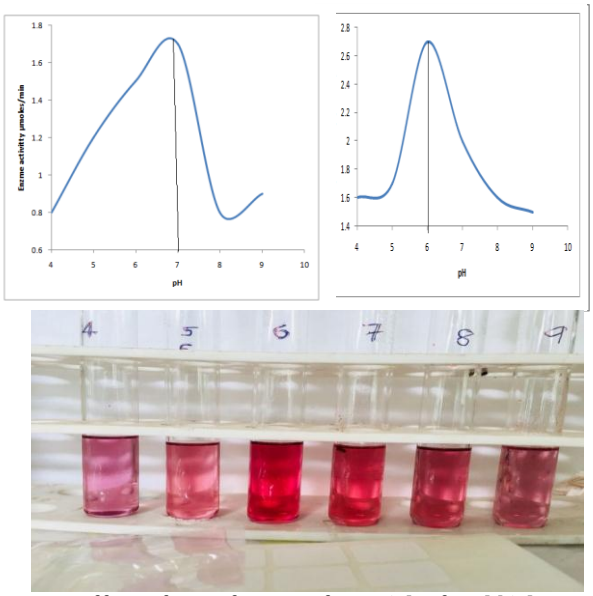

Fig.3 Effect of pH of papain from a) leaf and b) latex

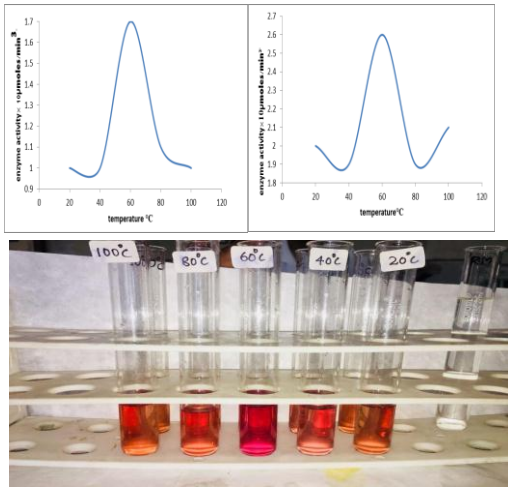

Fig.4. Effect of temperature on Papain from leaf

Effect of temperature on papain

Papain from leaf and latex exhibits its maximum activity at optimum temperature of 60˚C (Fig.4).

Effect of substrate specificity and substrate concentration on Papain

The effect of substrate on enzyme activity was studied at various concentrations. It was found that with increase substrate concentration, the activity of enzyme was also increased. At the maximum concentration of 50 mM the enzyme was found to be saturated. Hence the specific substrate for papain from leaf and latex was found to be 50mM casein (Fig.5).

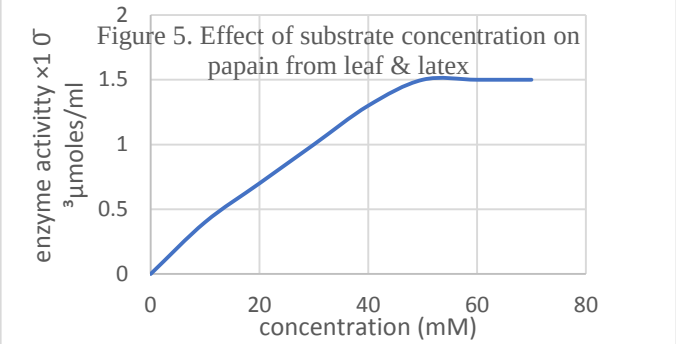

Figure 5. Effect of substrate concentration on papain from leaf & latex

IV. DISCUSSION

The isolation procedure described here was found to be easier and reproducible for both leaves and for latex. Once extracted, both the enzymes were suitable to study for further aspects with same ease. The temperature optima for both leaf and latex enzyme was found to be at 60^{o}C and this is in range of reported range of 60^{0}C to 70^{0}C. The pH optima reported here also correlating with the reported range of 6 to 7, though our results have reported different values for leaf and latex. The best suitable substrate in our studies was found to be 50mM Casein when compared with other substrates of egg albumin and bovine serum albumin. Both leaf and latex papain have shown same affinity towards substrates. The high activity in latex fraction could be due to high concentration of papain compared to one present in leaf extract. At high saturating concentrations of substrates both enzymes behaved same.

V. CONCLUSION

The present study provides a detailed procedure for isolation and precipitation of papain from leaf and latex extracts of papaya plant. Since papain has multiple uses, one may use this study for further scaling up procedures and uses in commercial aspects.

ACKNOWLEDGEMENT

Authors are thankful to the management and Principal of Bhavan's Vivekananda College of Science, Humanities and Commerce for providing necessary financial and infrastructural facilities for carrying out this study in the department of Biochemistry.

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