Isolation identification and partial characterization of proteases in Carica papaya L. callus

Renta Magdalena Hutabarat
The University of Montana

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Isolation, Identification and Partial Characterization of Proteases in *Carica papaya* L. Callus.

by

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Two protease enzymes were purified from *Carica papaya* callus tissue by a procedure involving ammonium sulfate and acetone fractionation, CM-Cellulose chromatography, and Sephadex-G50(fine) filtration. The ammonium sulfate fractionation followed by the Sephadex-G50(fine) filtration, was the best method of isolating the enzymes. The fraction obtained by 45% ammonium sulfate saturation isolated one of the homogenous proteases with molecular weight of about 22,500 as determined by SDS-PAGE, and was recognized by antipapain IgG while the fraction obtained by 65% ammonium sulfate saturation contained the second protease enzyme with molecular weight of about 30,000 as determined by SDS-PAGE, was recognized by antichymopapain IgG. Both of these enzymes are most active at pH 7.0 and their activity decreases when heated at 75°C.
ACKNOWLEDGEMENT

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INTRODUCTION:

There are a variety of enzymes found in nature that are useful commercially. In industry, they are used to make cheese or bread and in the manufacture of paper or the desizing of textiles(63). Medically they have been used, in treating lymphoblastic leukemia, and in mitigating cardiovascular and thromboembolic diseases(13,46). Enzymes are defined as proteins that catalize the conversion of substrates to products. Proteolytic enzymes or proteases catalize the hydrolytic cleavage of peptide bonds. Endopeptidases act on proteins and peptides by hydrolizing "internal" peptide linkages, that is those situated away from the end of the peptide chains. The exopeptidases, on the other hand, catalize the hydrolysis of peptide bonds situated at the ends of the peptide chains. Because of their importance in industry and medicine large amounts of proteases such as papain and bromelain are imported into the U.S. every year. In industry, they are used to "chill proof" beer, tenderize meat and bate hides. In medicine, they are used as antiinflammatory agents, digestive aids, in the debridement of burns, removal of warts and chemonucleolysis(21,26,61). It would, therefore, be interesting to find other sources of the same proteolytic enzymes. Plant tissue culture techniques promise one method to produce such enzymes or other primary and secondary plant products of economic value(59).
There are many proteolytic enzymes found in nature, they comprise the most varied assortment of enzymes with regard to their origin. Often, these enzymes are classified into animal, plant and microbial proteinases or by their intracellular or extracellular origin. Often they are grouped according to their active center. Four different types can be distinguished:

1. Serine proteinases have a serine residue in their active center and are specifically inhibited by diisopropyl phosphofluoridated compounds and other organo phosphorous derivatives.

2. Thiol proteinases depend on an intact -SH group in their active center and are inhibited by heavy metals and their derivatives, alkylating agent, etc.

3. Metal proteinases depend for their activity on the presence of more or less tightly bound divalent cations. These can be inactivated sometimes by simple dialysis, or by the presence of chelating agents.

4. Acid proteinases contain one or more side chains of carboxyl groups in their active center.

Bromelain, ficin, papain, and chymopapain, are thiol proteinases obtained from plants. Bromelain is obtained from the fruit and stem of Ananas comosus L. merr. family Bromeliaceae(43,44), ficin from the latex of Ficus species family Moraceae(30) and papain and chymopapain are obtained from Carica papaya L. family Caricaceae.
The most studied of the proteinases from plants are the thiol proteinases from the papaya plant *Carica papaya* L. Papaya (figure 1) is a member of the small dicotyledenous family *Caricaceae*. There are only four genera within this family: *Carica*, *Jacaratia*, *Cyclicomorpha* and *Jarilla*. With the exception of *Cyclicomorpha*, which is indigenous to Africa, all are native to the tropics of North and South America. There are approximately twenty two species within the genus *Carica*. The papaya is believed to have originated in Central America. It is the only *Carica* species that is widely cultivated. Currently papaya are grown throughout the tropics and subtropics. The leading papaya producing countries are Zaire, Mexico, Brazil, India and Indonesia.

The papaya is a large and rapidly growing arborescent herb with a single straight hollow stem and crown of palmately lobed leaves. There are three primary sex types: pistillate, staminate, and hermaphroditic. Flowers are borne on cymose inflorescences that originate in the leaf axils. Laticifer occurs in all organs.

Papaya has a short juvenile stage of three to four months. Ripe fruits are ready for harvest ten to twelve months after germination of the seeds. Healthy trees bear fruit continuously there after for the lifetime of the plant, that is twenty to twenty-five years. Papaya trees can yield as much as 50,000 Kg of fresh fruit per hectare during the first year of production. Individual fruits can weigh 0.5 to 9.1 Kg. Because of the extraordinary productivity of this plant and its attractiveness not only as a fruit crop but also as a source
Figure 1: *Carica papaya* L. tree.

*Six months old.*
of biochemicals, it is one of the principle horticultural crops of the tropics. The annual worldwide production of papaya fruit is approximately equal to that of strawberries, apricots, or avocados(32).

Papaya cultivation is commercially very important as almost all the parts of the plant are utilized. The most important commercial product produced from the unripe papaya fruit is the papaya latex which contains a variety of proteolytic enzymes. This unripe fruit is composed of approximately 92% moisture, 4% to 5% carbohydrates, 1% pectic substances and 0.5% to 1% proteolytic enzymes which reach maximal concentration early in the development of the fruit. Beside this, papaya are also grown as a breakfast or dessert fruit, and closely resemble a melon in flavor and texture. Flesh color varies from pale yellow to red. The ripe fruit pulp contains primarily of 89% water, 0.5% protein, and 8.2% sugars. The fruit is rich in vitamins such as vitamin A, B1, B2, C and niacin(45).

Extracts from papaya fruit and leaves are used as purgative and vermifuge. The alkaloid carpaine, found in the leaves, has been used as a heart depressant, amoebicide, and diuretic properties. The seed which is rich in protein and oil has demonstrated insecticidal and contraceptive properties(32,45).

Large papaya plantations exist in Zaire and in the lake Manyara area of northern Tanzania which primarily produces the proteolytic enzyme papain. The product is derived from sun-dried latex tapped from immature papaya fruit. Just prior to ripening, many large latex vessels
are formed under the epidermal layers of the papaya fruit. When the epidermal layer of the pericarp is scratched, these latiferous vessels break and exude a clear fluid which rapidly becomes opaque on exposure to air. This material is scraped or allowed to dry in trays around the trunk. The average annual yield is about 100 G per tree and one pound of dried latex is obtained from about 800 pounds of fresh papaya latex. After drying, the latex is powdered, sieved and sold as papain. The United States is the principle importer(32).

The term "papain" was introduced in 1879 by Wurtz and Bochut to describe a proteolytic principle in papaya latex. Today the term applies to the crude latex as well as to the crystalline proteolytic enzyme isolated from the latex(55). Four major proteolytic fractions have been isolated from the latex of the fully grown unripe fruits of *Carica papaya* L., by chromatography on CM-Cellulose. These are papain, papaya peptidase A, and chymopapain which constitute 5%, 18%, and 27% respectively, of the soluble latex protein. Little information is available on the fourth component, representing 14% of the soluble protein, beyond the fact that like the other three it appears to be a sulfhydrl enzyme(50,51).

Papain is the most extensively studied thiol enzyme from the papaya fruit. In 1937, Balls, Lineweaver and Thompson crystallized papain from papaya latex. It is the first enzyme in which a free sulfhydrl group was implicated for catalytic activity and the first proteinase which was capable of hydrolizing a synthetic substrate. The crystalline enzyme
shows a high degree of stability in sodium chloride solution, and can be kept at 4 C for months without detectable loss in activity. The striking property of papain is its stability when exposed to high temperatures, organic solvents and reagents which cause denaturation of other enzymes. Papain powder resists dry heat at 100 C for 3 hours and even in solutions it shows a remarkable temperature stability. The latter is however pH dependent, and the enzyme is unstable in acidic solutions. Papain is unaffected by denaturing agents which are known to cause conformational changes in protein. The crystalline mercury derivative of papain, mercurypapain is a more desirable product than papain itself. This derivative, when activated, has a specific proteolytic activity about 10% higher than that of crystallized papain and does not autolyze on storage(3,56).

Papain has a molecular weight of approximately 23,000 and is activated by cysteine, sulfides, and sulfites. It is enhanced by chelating agents such as disodium ethylenediaminetetraacetic acid(Na2EDTA). Papain is reversibly inactivated in the presence of air and low concentration of cysteine. This inactivated enzyme is distinguishable from the native crystalline papain and can be reactivated in the presence of higher cysteine concentrations. Heavy metal ions such as mercury, zinc and lead are inhibitory to papain, but inhibition can be reversed in the presence of Na2EDTA and cysteine. Papain has a wide range of specificity regarding both proteins and small molecular weight substrates. Papain hydrolyzes proteins more extensively than
Studies of the amino acid sequence of this enzyme have been completed and, its three dimensional structure has been elucidated by X-Ray crystallography. Papain is a simple protein containing amino acids devoid of carbohydrate. All of the usual amino acids are present with the exception of methionine. Papain is a single peptide chain of two hundred and twelve residues folded into two parts that form a cleft. It has but one free sulfhydryl group which lies in that part of the molecule that forms a cleft. The structural conformation is stabilized by three disulfide bridges and their complete rupture results in a modification of the protein as indicated by the loss of biological activity, catalytic as well as immunological. Not all three disulfide bonds are required for biological activity(42,64).

Papaya peptidase A was first isolated by Shank from papaya latex and later by Robinson from commercial chymopapain. Recently, Lynn prepared papaya peptidase A from papaya latex and isolated another protease enzyme called papaya peptidase B. Papaya peptidase A, also a sulfhydryl protease, differs from papain and chymopapain in amino acid composition and activity toward protein substrates. It shows esterase activity toward p-toluenesulfonyl-L-arginine methyl ester and appears to be less active than papain toward denatured proteins. The structure of papaya peptidase A has not been deciphered yet. The enzyme has no methionine and isoleucine on its N-terminal region. Its molecular weight is approximately 24,000 by sodium dodecylsulfate polyacrylamide
gel electrophoresis (SDS-PAGE). Reduction is required for full activity, and it is less active than papain against substrates such as casein (50,51,52).

Chymopapain is a sulfhydryl protease first isolated from papaya latex by Jansen and Balls (24). Chymopapain was so named because of its higher ratio of milk clotting to proteolytic activity when compared to papain. Chymopapain closely resembles papain in its ability to hydrolyze a wide variety of substrates. Like papain it requires activation by reducing agents and is inhibited by sulfhydryl reagents. Chymopapain has a molecular weight of 34,000 with an optimum pH of 7.0 to 7.5. The quantity of chymopapain present in crude latex is considerably greater than that of papain. It is the main constituent of the soluble protein present in the crude latex. Chymopapain has greater heat stability than papain at neutral pH as well as greater stability to acid. It is soluble in saturated sodium chloride solution at pH 3.0 and unlike papain, it is stable at pH 1.8 at 10 C for several weeks. Its stability in acid may prove to be an important pharmaceutical and technical asset (3,12,15,27,28).

The thiol enzymes papain, chymopapain and papaya peptidase A in papaya latex differ in their activity to the oxidized B-chain of insulin. The bond leucine(17)-Valine(18) is cleaved by papain, while the bond Leucine(15)-Tyrosine(18) is cleaved by papaya peptidase A and the bond containing acidic amino acids (glutamic, cysteic, aspartic) and aromatic amino acids are cleaved by chymopapain (3).
The principles of tissue culture were contained in the cellular theory, expressed in 1838-1839 by Schleiden and Schwann. This theory postulated implicitly that the plant cell is totipotent. In 1902, Haberlandt began experiments in order to verify the cell theory using plant cells of Tradescantia, but failed. In 1908, Simon grew and observed the development of poplar stem segments which produced callus, roots, and buds, but the tissue died after a few weeks because the callus was not transferred. In 1922, Kotte, a student of Haberlandt, succeeded in cultivating excised root tips of pea and maize for a limited period. In 1924, two physicians, Blumenthalt and Meyer observed callus formation on carrot slices and finally in 1934, White succeeded in maintaining cultures of tomato roots for indefinite periods of time. Finally Gautheret in 1934, produced callus culture of cambial tissues of Acer psedoplatanus, and later Salix capraea and Sambucus nigra(20).

Tissue culture techniques have improved since then. Plant cell cultures are now used as a tool in the study of whole plants. The advantage of plant cell culture lie in its uniformity, reproducibility, possible control in suspension culture systems, the ready availability of large quantities of cells, and the decreased level of structural organization relative to that of the plant. Plant tissue culture techniques are profitably used today throughout the world. Academic, government and industrial laboratories are using the tissue culture techniques to rapidly and uniformly propagate many horticultural plants and to develop either improved or new plant varieties. In agriculture
plant tissue culture techniques are used for hybridization, for variety development and other genetic modification of crop plants, clonal propagation and to produce specific pathogen-free plants (14, 16, 17). Selections can be made from plant tissue cultures for cells, and ultimately plants, that are tolerant to stress, such as pathogens, drought, salinity, temperature, and herbicides or chemicals such as protein, oils, pyrethrins and alkaloids (22, 59). New plants have also been developed by fusing the protoplasts of the sunflower and frenchbeans, tomato and potato, and various Datura varieties. Bacterial vectors have introduced foreign genes for kanamycin into tobacco and for proteins into sunflower tissues and subsequently into their redifferentiated plants (59). Other major advantages from cell culture systems over conventional cultivation of whole plants include the production of useful compounds in a controlled sterile environment (41).

The success of producing useful metabolites such as shikonin from high yielding cultures of Lithospermum has been demonstrated by the Mitsui Petrochemical Industries (Tokyo) in 750-liter fermentors. Shikonin is a dye and an antibacterial agent valued at approximately $400.00 per kilogram. Production of thirty other compounds known to accumulate in plant cultures in concentrations equal to or higher than that of the mature plant have also been reported. Therefore, it is obvious that plant tissue culture biotechnology remains to be explored in the production of commercially important secondary plant products (57, 58, 59).
There are six general requirement for growing cultures with high yields of secondary plant products (22):

1. selection of high yielding plants,
2. establishment of cell cultures from the selected plant,
3. development of an optimum growth medium, without consideration of secondary natural product production,
4. development of methods to induce primary and secondary natural product formation,
5. clonal selection of highly inducible cell strains, and
6. development of an optimum production medium.

Medora et. al (36,37,38) have shown the presence of proteolytic enzymes in the Carica papaya L. callus tissue cultures. They have also shown that the protein and enzyme activity can be increased by decreasing the amount of macroelements and/or microelements in the medium. Further, addition of peptone to the medium has been shown to induces protease production in papaya cultures. Thus the second, third and fourth requirements mentioned above have more or less been fulfilled. Therefore, it would be very interesting now to identify the proteases present in the papaya callus or determine if they are similar to the proteases present in the papaya latex. After identifying the proteases present in the culture, it will be easier to select a high yielding clone which produces a particular protease. The purpose of this work is to identify and partly characterize proteases such that steps five and six are facilitated.
MATERIALS

The following chemicals and special materials were purchased from the listed companies and their addresses. Chymopapain, papain, TritonX-100 and other chemicals, Sigma Chemical Company(St. Louis, Mo.63178); Sodium dodecylsulfate, British Drug House(England); Freund Complete Adjuvant, Difco(Detroit, Michigan); Immuno-Blot kit, Protein analysis kit, molecular weight marker, nitrocellulose paper, Bio-Rad(Richmond, Ca.94804); CM-Cellulose, Sephadex-G50(fine), Pharmacia(Piscataway, N.J.08854).

METHODS

STERILIZATION OF EQUIPMENT

All glassware and surgical instruments were sterilized in a drying oven at 140 C for two to three hours. Solutions, water and media were sterilized for 20 minutes in an autoclave at 121 C and 15 pounds pressure per square inch. All aseptic procedures were carried out in a Laminar Flow Hood using sterile techniques(19).

ESTABLISHMENT OF CARICA PAPAYA L. CALLUS CULTURES

Carica papaya L. seeds of the solo variety were obtained from the fruits purchased at a local store. The seeds were surface sterilized by immersing them in 0.1%(w/v) of sterile mercuric chloride solution for 5 minutes and followed by 3 washes with sterile double distilled water. The seeds were then washed with 1%(w/v) sodium hypochlorite for 3
minutes and washed again 3 times with sterile distilled water (vacuum was applied on and off every minute to ensure penetration of the solution). The washed seeds were then transferred to sterile petri dishes containing two Whatman number(No.) 1 filter papers soaked in 10 ml of sterile distilled water and allowed to germinate. Seeds were germinated and cultures maintained in the dark in a walk-in growth chamber at 27 C and 50% humidity. After germination, 1 to 2 inch long, seedlings were transferred into modified White's medium(table 1) containing 2 parts per million(2 ppm) of 2,4-dichlorophenoxyacetic acid(2,4-D) and adjusted to pH 5.7. Calluses formed on the seedlings were excised and transferred to modified White's medium containing 0.4 mg/l of 2,4-D and subcultured every 4 weeks. Four week old callus was harvested lyophilized and stored in a freezer for future use(19,36,37,38).

EXTRACTIONS AND PRECIPITATIONS OF PROTEOLYTIC ENZYME FROM LYOPHILIZED CALLUS

1) Extraction from callus

For every one gram of lyophilized callus 20 ml of 0.1 M sodium phosphate buffer(with or without TritonX-100) pH 6.0 was used as a solvent(39). Callus was ground in a waring Blender(with a circulating cold water jacket) at room temperature for 15 minutes, with intermittent stopping every 5 minutes. Table 2 summarizes the steps that follow. TritonX-100 extractives are indicated by(X) in table 2.
### MINERAL SALTS (mg/l)

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<table>
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<tbody>
<tr>
<td>KNO3</td>
<td>80.0</td>
<td>KI</td>
</tr>
<tr>
<td>MgSO₄.7H₂O</td>
<td>720.0</td>
<td>H₃BO₃</td>
</tr>
<tr>
<td>NaH₂PO₄.H₂O</td>
<td>16.5</td>
<td>MnSO₄·H₂O</td>
</tr>
<tr>
<td>Ca(NO₃)₂·4H₂O</td>
<td>300.0</td>
<td>ZnSO₄·7H₂O</td>
</tr>
<tr>
<td>KCl</td>
<td>65.0</td>
<td>Na₂MoO₄·2H₂O</td>
</tr>
<tr>
<td>Na₂SO₄</td>
<td>200.0</td>
<td>CuSO₄·5H₂O</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CoCl₂·6H₂O</td>
</tr>
</tbody>
</table>

### VITAMINS (mg/l)

- Calcium Pantothenate: 2.0
- Biotin: 2.0
- Nicotinamide: 2.0
- Nicotinic Acid: 0.8
- Pyridoxal Hydrochloride: 2.0
- Riboflavin-5'-P₂₄: 0.4
- Thiamine hydrochloride: 4.0
- Vitamin B₁₂: 0.006
- Folic acid: 4.0
- Ascorbic acid: 200.0

### AMINO ACID (mg/l)

- Glycine: 2.0
- L-Glutamine: 100.0
- L-Glutamic acid: 50.0
- L-Aspartic acid: 50.0
- L-Asparagine: 25.0

### MISCELLENEOUS

- Myo-inositol: 200.0 mg/l
- Adenine sulfate: 5.0 mg/l
- Choline chloride: 1.0 mg/l
- Urea: 50.0 mg/l
- FeSO₄·7H₂O: 37.3 mg/l
- Na₂EDTA: 27.8 mg/l
- Agar: 8.0 g/l
- Sucrose: 20.0 g/l
- 2,4-Dichlorophenoxyacetic acid: 2.0 ppm for callus initiation/
  0.4 ppm for callus maintenance
- Coconut water: 150.0 ml/l

pH was adjusted to 5.7, autoclaved for 15 minutes at 121 °C and 15 psi.

Table 1: Modified White's medium
The brown slurry was then centrifuged at 10,000 RPM for 30 minutes. After centrifugation, CE1 samples were taken, for SDS-PAGE analyses and the rest of the extractive was adjusted to pH 9.0 (from pH 5.3) with 1.0 N NaOH and stirred for 15 minutes at room temperature (3). This basic CE1 extractive was centrifuged again at 10,000 RPM for 15 minutes, the precipitate discarded and the basic supernatant (CE2) fluid saved for future use. Some of this crude extract was dialyzed against 800 ml of water at 4°C for two days, while changing water thrice (this procedure was followed for all dialysis work). The rest of the crude extract was used for further fractionation with ammonium sulfate, acetone or hydrochloric acid.

2) Ammonium Sulfate Precipitation

The CE2 was adjusted to 45% saturation with ammonium sulfate by slowly adding 0.258 g/ml of ammonium sulfate while stirring at room temperature. The pH of the saturated solution was adjusted to 5.5 with 1.0 N hydrochloric acid (HCl). The solution was left stirring at 4°C for 30 minutes, followed by centrifugation at 5000 RPM for 15 minutes. The white precipitate isolated (ASp0-45), was dissolved in a minimum amount of 0.02 M sodium acetate buffer, pH 5.5, and then dialyzed. The dialyzed ASp0-45 solution was then frozen for further analysis (3). The supernatant collected (ASs0-45) from this treatment was adjusted to 65% saturation with ammonium sulfate, by adding 0.123 g/ml of solid ammonium sulfate slowly while stirring at 4°C, for 30 minutes.
Table 2: Fractionation of Extracts.
The mixture was again centrifuged at 5000 RPM for 15 minutes. The supernatant (ASs45-65) was discarded while the small amount of white precipitate (ASp45-65) collected was dissolved in a minimum volume of 0.02 M sodium acetate buffer, pH 5.5 and was dialyzed. The ASp45-65 solution was then stored in a freezer for future use (12, 15).

3) Acetone Precipitation

The CE2 was adjusted to pH 5.5 by the addition of 1.0 N HCL. Acetone was added to make a 40% solution and the mixture stirred at 4°C for 30 minutes followed by centrifugation at 5000 RPM for 15 minutes. The light brown colored precipitate (ACp0-40) was dissolved in a minimum volume of 0.02 M sodium acetate pH 5.5 and was dialyzed. The ACp0-40 or ACp0-40X solution was then frozen for further analysis. The supernatant (ACs0-40) fluid was adjusted to 80% acetone and was stirred for 30 minutes at 4°C. At the end of the 30 minutes the mixture was centrifuged at 5000 RPM for 15 minutes. The supernatant (ACs40-80) was discarded while the precipitate (ACp40-80) was collected and dissolved in a minimum volume of 0.02M sodium acetate pH 5.5 and was dialyzed. The ACp40-80 solution was then stored in a freezer, for further analysis (2, 39, 66).
4) Hydrochloric Acid Precipitation

The CE2 fraction was adjusted to pH 2.00 by slowly adding 1.0 N HCl while stirring at room temperature. After stirring for 1 hour at 4°C, the mixture was then centrifuged at 5000 RPM for 15 minutes. The supernatant (Hs2) fluid was discarded while the precipitate (Hp2) collected was dissolved in a minimum amount of 0.02 M sodium acetate buffer, pH 5.5 and was dialyzed (15, 24).

CHROMATOGRAPHY OF ISOLATED FRACTIONS

1) CM-Cellulose Cationic Exchange Chromatography

CM-Cellulose cationic exchanger was swollen in 0.02 M sodium acetate buffer pH 7.0, in a boiling water bath for 2 hours. During the swelling period, the supernatant was removed by suction and replaced with fresh buffer every 30 minutes. The swollen CM-Sephadex G-50 was then washed 3 times, 15 minutes each in 300 ml of 0.1 M of sodium chloride. The white slurry was then poured into a 2 X 25 cm column, which settled to give a bed height of 22 cm. The CM-Sephadex column was then equilibrated with the starting buffer of 0.02 M sodium acetate, pH 5.5 by passing the buffer through the column at a rate of 18 ml/hour. After equilibration the bed height was 18 cm. The ammonium sulfate fraction or acetone fraction (5 to 50 mg of protein) was placed at the top of the column. Stepwise elution was carried out with 0.02 M, 0.1 M, and 0.7 M sodium acetate pH 5.5, 100 ml each, followed by a final
elution of 1.0 M NaCl-1.0 M sodium acetate pH 5.5 of 100 ml. The emerging solution was monitored for protein by reading the absorbance at 280 nm. Four milliliter fractions were collected per tube at the rate of 18 ml per hour. The pooled fractions from peak areas were then dialyzed. These pooled fractions were then stored in a freezer for future use(54).

2) Gel Filtration with Sephadex G-50(fine)

Twenty gram of Sephadex G-50(fine) was added to 300 ml of 0.2 M sodium acetate buffer, pH 4.8 and heated in a boiling water bath for 1 hour. The gel was then washed 4 times, 15 minutes each with 200 ml of 0.1 M sodium acetate pH 4.8 and the supernatant was removed by suction. The white slurry was then poured into a column, 70 X 1.5 cm, to make a bed height of 56 cm. The column was then equilibrated with the starting buffer of 0.1 M sodium acetate, pH 4.8. After equilibration the bed height of the column was 45 cm. From 5-50 mg of protein from the ammonium sulfate fractions or acetone fractions were applied to the column. Elution of protein was done with 250 ml of 0.1 M sodium acetate buffer, pH 4.8 at the rate of 18 ml per hour. The emerging solution was monitored for protein by reading the absorbance at 280 nm. Three milliliters fractions were collected per tube. Selected fractions were pooled representing separated proteins and dialyzed. The pooled fractions were then stored in a freezer(18,33).
Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The gel (table 3) was run at constant power of 5 watts until the sample entered the stacking gel and then the power was increased to 10 watts. The electrophoresis was carried out for approximately 3 hours and the protein was fixed in the gel with 40% methanol-7% acetic acid for 2 hours or overnight. Finally, the gel was treated with 0.25% coomassie brilliant blue in 40% methanol-7% acetic acid and destained with 40% methanol-7% acetic acid (29).

The molecular weight of the protein band obtained by SDS-PAGE was determined by comparing the relative mobility of the protein with that of marker proteins (molecular weight range of 10,000 to 100,000 daltons). The molecular weights of the marker proteins are as follows: phosphorylase B, 92,500; bovine serum albumin, 66,200; ovalbulmin, 45,00; Carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500; and lysozyme 14,400 (34, 65).

PREPARATION OF ANTISERA AGAINST C-PAPAIN AND C-CHYMOPAPAIN

1) Immunization of New Zealand Rabbits

Sigma papain and chymopapain were used for this part of the experiment. For convenience the papain and chymopapain from Sigma will henceforth be referred to as crude papain and chymopapain and abbreviated as C-papain and C-chymopapain.
Running Gel:

Acrylamide: bis-acrylamide (30%-0.8%) 12.5 ml  
Tris-HCl 1 M, pH 8.8 6.0 ml  
Na4EDTA 0.2M 0.3 ml  
Double distilled water 10.9 ml  
N,N,N',N'-tetramethylenediamine (Temed) 0.015 ml  
Ammonium persulfate (0.1 mg/ml) 0.3 ml

Stacking Gel:

Acrylamide: bis-acrylamide (30%-0.8%) 2.5 ml  
Tris-HCl 1 M, pH 6.8 1.88 ml  
Na2EDTA 0.2 M 0.15 ml  
Double distilled water 10.3 ml  
Temed 0.008 ml  
Ammonium persulfate 0.3 ml

Table 3: Sodium dodecylsulfate polyacrylamide gel electrophoresis.
Ten milliliter of Freund Complete Adjuvant was added to 10 ml of 3 mg/ml of C-papain or C-chymopapain and mixed between two 25 ml glass syringes connected with PVL tubing to form a thick consistency. This mixture was kept at 4 C. New Zealand rabbits were injected in four sites (two axillary and two inguinal) subcutaneously with a total of 6 mg of C-papain or C-chymopapain. At 2, 4 and 6 weeks after the first immunization the rabbits were sensitized by injecting 3 mg of C-papain or C-chymopapain at 2 inguinal sites subcutaneously. The rabbits were bled at 0, 2, 4, 6, 8, 10, 12, 14, and 16 weeks after immunization, to obtain the antisera. The blood collected in a conical centrifuge tube was left at room temperature for 30 minutes, the clot was rung with a wooden applicator. The serum was then left overnight at 4 C and centrifuged at 1000 RPM for 30 minutes. The antisera, that is, supernatant fluids were stored at -20 C (4, 23, 35, 53).

2) Sensitization of Rabbits Against Purified Papain and Chymopapain from SDS-PAGE Electrophoresis

Twenty ug of C-papain or C-chymopapain, were applied to each of the sixteen wells of a 12.5% SDS-PAGE prepared and run according to Laemmli's method (29). One of the lanes, was cut and stained with 0.25% coomassie brilliant blue. The stained gel was placed next to the unstained gel and the bands containing purified papain or chymopapain (P-papain or P-chymopapain) were cut off and left to dry for 2 days. This experiment was repeated 10 times to give a total of 3 mg
of P-papain or P-chymopapain from the gel. The dry gel was then ground in 6 ml of 0.02 M sodium phosphate buffer containing 0.85% NaCl, at pH 7.2 (0.02 M PBS), to give a concentration of 0.5 mg/ml of P-papain or P-chymopapain in the solution. Rabbits that were previously immunized with C-papain or C-chymopapain, were then sensitized by injecting 1 ml, that is 1 mg, of P-papain or P-chymopapain subcutaneously at two sites on the hind leg, 18 weeks after immunization. At the 22 and 24 weeks, the rabbit were again injected intravenously with 0.5 ml, that is 0.5 mg of P-papain (or P-chymopapain). The rabbits were bled at 18, 20, 22, 24, 26, 28, 30, 32, 34 and 36 weeks after immunization. Antisera was collected and stored at -20°C (18, 60, 62).

IMMUNOCHEMICAL METHODS:

1) Double Immunodiffusion (Ouchterlony)

Ten milliter of 2% agar in 0.85% sodium chloride (physiological saline) with 1:10,000 merthiolate was poured into a petri dishes and allowed to solidify. Then 10 ml of 1% agar in physiological saline with 1:10,000 merthiolate was poured on top of the solidified 2% agar. Wells with diameter of 0.5 cm were made in agar by cutting through the 1% agar layer. Agar plugs were removed by sunction. The center wells of different plates was filled with P-papain or P-chymopapain antisera while the surrounding wells were filled with antigens. Plates were incubated at 37°C for 3 days, and the wells were refilled daily with the appropriate solution. At the end of the third day, the plates were
placed at 4 C. On the fourth day, plates were flooded with physiological saline solution to remove non precipitated proteins. This was repeated on the fifth and sixth days with fresh solution of physiological saline and the gel observed for precipitation bands. The plates were then stained with coomassie brilliant blue, destained and photographed(6,47).

2) Precipitation Test for Antibody Titer

Fifty microliter of physiological saline was added to wells number 2-11 of row 1 in a microtiter plate. In well number 12, 50 ul of control serum was added. Then to wells number 1 and 2, 50 ul of P-papain or P-chymopapain antiserum was added. Using a 50 ul diluter, serial 2-fold dilutions of antiserum starting on well number 2 and ending at well number 10 were made. In row number 2, 50 ul of antigen was added to all twelve wells. A capillary tube was filled with 50 ul of antiserum from well-1 of row-1, followed by the antigen in well-1 of row-2. The capillary tube was then inverted, and placed in a clay plate to enable the tube to stand. This procedure was repeated with each corresponding well, that is well-2 of row-1 with well-2 of row-2 and so on. The tubes were then incubated for 1 hour at 37 C. After the end of an hour the tube were observed for precipitation and were placed in a cold room overnight. The next day the capillary tubes were observed again for precipitation(25).
3) **Passive Hemaglutination**

A) **Coating Human Red Blood Cells With Antigen**

Twenty milliliter of human blood was centrifuged at 3000 RPM for 15 minutes to separate serum from human red blood cells (HRBC). The HRBC obtained were washed 3 times in a mixture of 0.15 M sodium phosphate buffer and 0.15 M sodium chloride, pH 7.2 (0.15 M PBS) adjusted to their original volume, and centrifuged at the end of each wash. The supernatant fluid was tested for the presence of protein by adding 1 drop of concentrated nitric acid to it. After the last wash, the HRBC were resuspended with 0.15 M PBS to yield a 5%(v/v) suspension. A fresh solution of tannic acid 1:20,000(w/v) was prepared in 0.15 M PBS. Equal volumes of tannic acid solution and 5% HRBC were mixed slowly in a test tube and then incubated for 15 minutes in a water bath at 37 C. The tanned cells were centrifuged for 15 minutes at 3000 RPM. The supernatant was discarded and the centrifuge tubes were inverted over a filter paper. The harvested tanned cells were then resuspended in 0.15 M PBS to make a 5%(v/v) suspension. Then 1 volume of tanned cell suspension was added to 4 volumes of antigen solution and the mixture incubated in a water bath for 15 minutes at 37 C, with occasional agitation. Cells were then centrifuged thrice for 15 minutes at 3000 RPM and washed in between each centrifugation with 0.15 M PBS containing 1% Normal Rabbit Serum (NRS) which had been heated at 56 C for 30 minutes. After the last wash, the cells were made into a 5% suspension.
in the 0.15 M PBS + 1% NRS medium. The antigen coated cells (Ag-HRBC) were then stored at 4°C (1).

B) Passive Hemaglutination Assay

Fifty microliter of 0.15 M PBS + 1% NRS were added to wells number 2 to 11 in row 1 and 2 of a microtiter plate. To both rows of wells number 1 and 2, of row 1 and 2, 50 ul of P-papain or P-chymopapain antiserum which had been heated at 56°C for 30 minutes were added and to well number 12, 50 ul of control serum was added. Using a 50 ul diluter, 2-fold serial dilutions were done from well No. 2 to well No. 10 in both rows. Then 50 ul of prepared Ag-HRBC were added to all 12 wells of row 1 and 50 ul of uncoated HRBC were added to all 12 wells of row 2. The plate was rotated gently for mixing, and then was placed in an incubator at 37°C for 1 hour. Agglutination was observed at the end of the hour and again after 16 hours at 4°C (1).

4) Isolation of Antipapain-IgG and Antichymopapain-IgG

ProteinA-Sepharose Column

Four hundred milligram of proteinA-Sepharose was rehydrated in 10 ml of 0.02 M PBS. ProteinA-sepharose was then placed in a 3 ml plastic syringe and equilibrated with 0.02 M PBS. The column was then connected to a U.V spectrophotometer with recorder and monitored at 280 nm. The base line of the recorder was obtained before the antiserum was applied to the column. As soon as the serum was applied to the column, the
first peak was observed on the chart, and the column was washed with the PBS buffer until this peak came down to the base line. Then 0.58 M acetic acid was added to the column, and a second peak was observed. The acetic acid elutant was collected until the peak came down to base line. The acetic acid elutant was then dialyzed. The dialyzed anti-IgG was then lyophilized and stored at -20 C(49).

5) Immunoblotting

Strips of 1.5 X 9.0 cm of nitrocellulose paper (NCP) with grid were immersed in 20 mM Tris-HCl containing 500 mM NaCl at pH 7.5 (TBS) for 10 minutes. Papers were removed from TBS and air dried on filter paper. One ul of antigen was applied to the NCP. This application was repeated 3 times allowing the antigen to dry between each application. The dry NCP containing antigens was immersed into blocking solution (3% Gelatin-TBS) and was gently agitated for 1 hour. The NCP was removed from the blocking solution and transferred to the first antibody solution (1 in 50 dilutions of a monospecific antibody in 1% Gelatin-TBS) and allowed to incubate with gentle agitation overnight. The NCP from the first antibody solution was then rinsed with double distilled water twice for 10 minutes each with gentle agitation. Then the papers were placed in the second antibody solution (1:3000 dilution of Goat anti-rabbit IgG-horseradish peroxidase conjugate in 1% gelatin-TBS) for 1 hour. The NCP was removed from the second antibody solution and rinsed with double distilled water, then washed twice for 10 minutes.
each with Tween-TBS (0.05% Tween-TBS). Finally, the membranes were transferred to horseradish peroxidase (HRP) color development solutions which contain 4-chloro-1-naphtol (60 mg HRP dissolved in 20 ml of ice cold ethanol added to a mixture of 60 ml of ice cold 30% hydrogen peroxide in 100 ml TBS) with gentle agitation, bluish-purple dots or bands appeared in 15 to 30 minutes (8,9).

6) Western Blotting

SDS-PAGE was run in triplicate (1 for control serum, 1 for monospecific antibody and 1 for amido black staining) on samples that had proteolytic activity. Two Whatman No. 1 filter papers, 19 X 11 cm NCP and two scotchbrite pads were soaked in 50 mM of degassed sodium phosphate buffer, pH 7.5 (PB). The SDS-PAGE was placed on top of one of the wetted filter paper, followed by NCP which was placed on top of the SDS-PAGE, and finally the second filter paper. Air bubbles that were present between the gel and filter papers were removed. The whole preparation was then sandwiched between the wetted scotchbrite pads and placed in a plastic sandwich rig. The sandwich rig was placed in the transblot chamber which was filled two-thirds with PB. The nitrocellulose side of the sandwich rig was connected to the positive electrode. The electrotransfer was carried out at 2 amperes for 2 hours. After the transfer, the NCP was marked and cut according to the gel lane. Two of the papers were then soaked for ten minutes in TBS, while the third paper was stained in amido black. The papers which were
soaked in TBS were transferred to a blocking solution with gentle agitation for 1 hour. The rest of the procedure was the same as for immuno blotting\(^5\,9\,31\).

**Spectrophotometric Caseinolytic Assay**

One milliliter of casein solution (1 g of casein dissolved in 100 ml of Tris-HCl, pH 8.0, and heated in a 37 °C water bath) was added to a 20 ml tube, and placed in the water bath at 37 °C. Enzyme samples of 0.1 to 0.8 ml (0.05 mg/ml of C-papain and 0.05-0.20 mg/ml of C-chymopapain were used as standards) and 0.2 ml of activating agent (freshly prepared 0.05 M cysteine + 0.02 M Na\(_2\)EDTA, pH 8.0) were added to the tube, with 0.05 M Tris-HCl buffer, to bring up the volume to 1 ml. The tubes were then incubated for 10 minutes at 37 °C, and the reaction was stopped by adding 3 ml of 5% TCA. The precipitate was removed by centrifugation at 5000 RPM for 15 minutes. The supernatant was removed carefully, and the absorbance was read at 280 nm against a blank which did not contain the enzyme.

One unit of enzyme activity is defined as the activity which will give rise, under the conditions described, to an increase of 0.001 unit of absorbancy at 280 nm per minute of digestion. The specific activity is the number of units per milligram of protein\(^3\).
Proteolytic Enzyme Assay at Different pH

The experiment was carried out by the same procedure as the spectrophotometric caseinolytic assay except that the callus extract was substituted for standard enzymes. The buffer used was 0.05 M sodium phosphate buffer with a range of pH 3 to 7 and 0.05 M Tris-HCl buffer with a range of pH 8 to 10 (15).

Effect of Temperature on Proteolysis

The experiment was carried out by the same procedure as the spectrophotometric caseinolytic assay. The proteolytic enzyme samples were heated at 75°C for 30, 60, 90 and 120 minutes before the assay(15).

Caseinolytic Agar Plate Assay

Fifteen milliliter of 2% agar in 0.05 M dibasic sodium phosphate, was poured into a petri dish and allowed to solidify. One gram of casein dissolved in 50 ml of 0.05 M dibasic sodium phosphate, brought up to pH 6 by the addition of 0.05 M citric acid and 1 ml of PC-EDTA(0.038 M cysteine + 0.0376 M Na2EDTA was dissolved in 0.05 M dibasic sodium phosphate) was mixed, and its volume brought to 100 ml with 0.05 M dibasic sodium phosphate. Ten milliliters of this casein solution was added to 5 ml of 3% agar solution and poured on top of the 2% agar in the petri dish. After it solidified, wells were cut in the top casein layer and 50 ul proteolytic enzymes samples were added to the wells. Petri plates were incubated for 10 minutes at 37°C and then left
overnight at room temperature. The next day plates were flooded with 5% trichloroacetic acid for one hour. The clear white rings around the wells were observed.

**Bio-Rad Protein Assay**

Five milliliter of diluted dye reagent of Bio-Rad protein kit (1 volume of concentrated dye reagent diluted with 4 volume of double distilled water) were added to 0.1 ml of protein sample in a 10 ml test tube. The tubes were agitated slowly to prevent frothing and were read at 595 nm spectrophotometrically 5 minutes to 1 hour after the mixing. A solution of 0.02 M sodium acetate buffer, at pH 5.5 was used as a blank.

The total protein content of the callus extracts were compared to the total protein content of Bio-Rad protein standard of lyophylized bovine gamma globulin.
RESULTS AND DISCUSSION

Establishment of Callus from Carica Papaya L Seeds

Papaya seeds germinated within 5 days and grew approximately 2 inches long in 9 days. The seedlings which were grown on modified White's medium containing 2 ppm of 2,4-D, began to swell after 3 days. It took 2 more weeks for the seedlings to show callus. The subcultured callus tissue grew rapidly after three months on modified White's medium containing 0.4 ppm 2,4-D (figure 2 and 3). The callus tissue was pooled, lyophilized, powdered and stored in the freezer for analysis.

Production of Papain and Chymopapain Monospecific Antibody

The C-papain and C-chymopapain antisera were collected from rabbits, after immunization with C-papain (or C-chymopapain). The antibody production profile in figure 4 and 5, showed the highest titer of 512 for both, obtained after eight weeks for C-papain and six weeks for C-chymopapain. The titer number was obtained by the precipitation test of C-papain antiserum against C-papain as antigen and C-chymopapain antiserum against C-chymopapain as antigen. The C-papain (figure 6) and C-chymopapain antisera did not show cross reactivity with the Ouchterlony double immunodiffusion test, as only one precipitating band was observed for both antisera. With immuno blotting using these antisera, however, cross reactivity was observed (figure 7). Therefore, C-papain and C-chymopapain from Sigma do seem to produce nonspecific antibodies.
Figure 2: Callus of papaya. One month old after third transfer.

Figure 3: Callus of papaya maintained in modified White's medium.
Figure 4: Papain antibody production.

Figure 5: Chymopapain antibody production.
Figure 6: Ouchterlony double immunodiffusion precipitation band against C-papain antiserum. Well 1, bromelain; well 2, C-chymopapain; well 3, control serum; well 4, C-papain; and well 5, C-papain antiserum.

Figure 7: Immuno blotting of antigens as labelled. Lane 1 was blot against C-papain antiserum; lane 2 was blot against C-chymopapain antiserum; and lane 3 was blot against control serum.
The cross reactivity of C-papain and C-chymopapain antibodies observed in immuno blotting and not in Ouchterlony plates is because immuno blotting is more sensitive. The nonspecificity of the C-papain and C-chymopapain antisera could be the result of the protein band with molecular weight of 19,500 as shown in figure 8 which both C-papain and C-chymopapain have in common. Therefore, purification of the C-papain and C-chymopapain was carried out by SDS-PAGE to obtain monospecific antibodies.

Thus C-papain and C-chymopapain were purified by SDS-PAGE(Fig.8), according to Laemmli's method. The P-papain and P-chymopapain were then used for the preparation of monospecific antiserum. The antibody production profile(figure 4 and 5) was determined by the precipitation assay. The highest titer for P-papain antiserum was 512 at week No. 26 and for P-chymopapain antiserum the highest titer was 512 at week No. 24. Ouchterlony and immuno blot test on P-papain antiserum and P-chymopapain antiserum obtained did not show cross reactivity(figure 9 and 10). Therefore, the antisera obtained from this sensitization was used in the analyses of all the callus extractives.

The P-papain and P-chymopapain antisera from week numbers 26 and 24 respectively were used to isolate antiP-papain and antiP-chymopapain IgG. From 12 ml of P-papain antiserum, 73 mg of antiP-papain IgG was collected and from 12 ml of P-chymopapain antiserum, 81 mg of antiP-chymopapain IgG was collected.
C-chymopapain
C-papain
19,500

Figure 8: SDS-PAGE.
Lane 1, C-chymopapain; lane 2, C-papain; and lane 3, molecular weight marker.

Figure 9: Ouchterlony double immunodiffusion precipitation band against P-papain antiserum. Well 1, bromelain; well 2, C-chymopapain; well 3, control serum; well 4, C-papain; and well 5, P-papain antiserum.
Figure 10: Immuno blotting of antigens as labelled. Lane 1 was blot against control serum; lane 2 was blot against P-papain antiserum; and lane 3, was blot against P-chymopapain antiserum.
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<th>Volume (ml)</th>
<th>Activity (unit)</th>
<th>Total Activity (unit)</th>
<th>Protein (µg/ml)</th>
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Table 4: Specific activity and total protein contents of callus extracts without TritonX-100.
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Table 5: Specific activity and total protein contents of TritonX-100 containing callus extracts.
Purification and Isolation of Proteolytic Enzymes Present in Papaya Callus

1) Extraction of Protein from Callus

A sample of CE1 or CE1X obtained from callus was used for SDS-PAGE analysis. Then the CE2 or CE2X extractive was divided into 4 parts. A portion was dialyzed for qualitative and quantitative analyses, while other portions were used for fractionation with hydrochloric acid, ammonium sulfate or acetone.

The presence of 0.05% TritonX-100 in the buffer increases the total protein content and specific activity of the crude extract to some extent (see table 2, 4 and 5). The increase in protein content isolated from the callus tissue treated with TritonX-100 is probably a result of better extraction of the protein due to the decrease in surface tension on the lyophilized powdered tissue caused by the surfactant. Therefore, it is possible that the callus powder dispersed better and the extraction of proteins from it was more complete. The increase in specific activity of the sample treated with TritonX-100 may also be due to improved extraction of total proteins. Similar results have also been obtained during extraction of protein from mitochondria (48).
2) **Hydrochloric Acid Fractionation:**

The precipitates Hp2 and Hp2X obtained from the hydrochloric acid fractionation of 110 ml of CE2 and CE2X respectively were dissolved in 50 ml of 0.02 M sodium acetate, pH 5.5. There was no proteolytic activity in these fractions. The amount of total protein isolated from Hp2X was slightly higher than that in Hp2 (tables 4 and 5).

The above procedure was suggested earlier by Jansen and Balls (24) and used by Ebata for isolating chymopapain from the 45-65% ammonium sulfate fraction of papaya latex which is active at pH 2.0 (15). But since there was no activity at all in the Hp2 and Hp2X fractions, chymopapain or a similar enzyme does not seem to be present. It appears that the proteolytic activity identified earlier was destroyed or inactivated by the drastic acid treatment.

3) **Ammonium Sulfate Fractionation:**

The CE2 and CE2X obtained from callus extraction was treated with ammonium sulfate. To the 110 ml of crude extracts was added 28.38 gram of ammonium sulfate to give a 45% saturation at 0 C. The precipitates (ASp0-45 or ASp0-45X) obtained were dissolved in 84 ml of 0.02 M sodium acetate and dialyzed against water. The total protein present and the specific activity was slightly higher in the ASp0-45X than that of the ASp0-45 (tables 4 and 5).
Figure 11: Chromatograph of fraction ASp0-45X on CM-Cellulose.
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<th>Sample</th>
<th>Volume (ml)</th>
<th>Activity (unit)</th>
<th>Total Activity (unit)</th>
<th>Protein (ug/ml)</th>
<th>Total Protein (mg)</th>
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Table 6: Specific activity and total protein contents of callus extracts prepared from ammonium sulfate fractions separated by chromatography on CM-Cellulose and Sephadex-G50(fine) columns.
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<th>Activity (unit)</th>
<th>Total Activity (unit)</th>
<th>Protein (ug/ml)</th>
<th>Total Protein (mg)</th>
<th>Specific Activity</th>
<th>Protein % Yield</th>
<th>Purification Fold</th>
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<td>100</td>
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<td></td>
<td>84</td>
<td>210</td>
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<td>1246</td>
<td>105</td>
<td>169</td>
<td>50</td>
<td>0.99</td>
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</tbody>
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1) ASP0-45X:

| C' cellulose: | Fr1 36 | 76 | 2745 | 240 | 8.6 | 318 | 18 | 1.86 |
| Fr2 40 | ---- | ---- | ---- | 181 | 7.3 | ---- | 15 | ---- |
| Fr3 24 | ---- | ---- | ---- | 338 | 8.1 | ---- | 16 | ---- |

Sephadex-G50:

| Fr1 30 | 99 | 2963 | 292 | 8.8 | 339 | 18 | 1.99 |
| Fr2 30 | ---- | ---- | ---- | 269 | 8.1 | ---- | 16 | ---- |
| Fr3 30 | ---- | ---- | ---- | 245 | 7.4 | ---- | 15 | ---- |

2) ASP-45-65X:

| C' cellulose: | Fr1 28 | 70 | 1960 | 212 | 5.9 | 330 | 9 | 1.93 |
| Fr2 32 | ---- | ---- | ---- | 102 | 3.3 | ---- | 5 | ---- |
| Fr3 32 | ---- | ---- | ---- | 160 | 5.1 | ---- | 8 | ---- |

Sephadex-G50:

| Fr1 30 | ---- | ---- | 271 | 8.1 | ---- | 13 | ---- |
| Fr2 24 | 93 | 2220 | 255 | 6.1 | 362 | 10 | 2.12 |

Table 7: Specific activity and total proteins contents of callus extracts containing TritonX-100 prepared from ammonium sulfate fractions separated by chromatography on CM-Cellulose and Sephadex-G50(fine) columns.
The ASs0-45 (table 2) was increased to 65% saturation by adding 13.53 gram of ammonium sulfate. The precipitates (ASp45-65 or ASp45-65X) were dissolved in 66 ml of 0.02 M sodium acetate buffer pH 5.5 and dialyzed against water. The total protein present and specific activity in ASp45-65X was slightly higher than that in ASp45-65 (tables 4 and 5).

3A) CM-cellulose Fractionation of Ammonium Sulfate Fraction:

Twenty milliliter of the dialyzed solution of ASp0-45 or ASp0-45X, was applied to the CM-cellulose column. A typical chromatograph is shown in figure 11. Three major protein fractions were obtained, but only fraction No. 1 (ASp0-45 Fr1-CM or Asp0-45X Fr1-CM) in both ASp0-45 and ASp0-45X had proteolytic activity. The total protein and specific activity of ASp0-45X Fr1-CM was slightly higher than that in ASp0-45 (tables 6 and 7).

Twenty milliliter of the dialyzed solution of ASp45-65 or ASp45-65X was applied to the CM-cellulose column. A typical CM-Cellulose chromatograph is shown in figure 12. Three protein fractions were obtained, and the first peak (ASp45-65 Fr1-CM or ASp45-65X Fr1-CM) was found to have proteolytic activity while the other 2 peaks did not. The total protein and specific activity of ASp45-65X Fr1-CM is slightly higher than that in ASp45-65 Fr1-CM (tables 6 and 7).
Figure 12: Chromatograph of fraction ASp45-65X on CM-Cellulose.

Figure 13: Filtration of fraction ASp0-45X on Sephadex-G50(fine).
Figure 14: Filtration of fraction ASp45-65X on Sephadex-G50(fine).
3B) Sephadex G-50(fine) Filtration of Ammonium Sulfate Fraction:

Twenty milliliter of the dialyzed solution of ASp0-45 or ASp0-45X was applied to a sephadex-G50(fine) column. A typical chromatograph is shown in figure 13. Three protein fractions were obtained but only the first fraction (ASp0-45 Fr1-S or ASp0-45X Fr1-S) had proteolytic activity. The total protein contents and specific activity of ASp0-45X Fr1-S is slightly higher than that in ASp0-45 Fr1-S (tables 6 and 7).

Twenty milliliter of the dialyzed solution of ASp45-65 or ASp45-65X was applied to a Sephadex-G50(fine) column. A typical chromatograph is shown in figure 14. The first fraction had no proteolytic activity while the second was active. The total protein content and specific activity of ASp45-65X Fr2-S is slightly higher than that in ASp45-65 Fr2-S (tables 6 and 7).

Chromatography of callus extractives on Sephadex-G50(fine) appears to give better resolution than chromatography on CM-Cellulose. This seems to support Fukal's claim that pure papain can be isolated in a single step by Sephadex-G50(fine) chromatography from crude papain(18).

4) Fractionation with Acetone:

One hundred and ten milliliter of CE2 or CE2X callus extract was added to 183 ml ice-cold acetone to give 40% saturation. The precipitates (ACp0-40 or ACp0-40X) obtained from both extracts were dissolved in 80 ml of 0.02 M sodium acetate buffer, pH 5.5 and dialyzed against water. The total protein content and specific activity of
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<th>Total Activity (unit)</th>
<th>Protein (ug/ml)</th>
<th>Total Protein (mg)</th>
<th>Specific Activity</th>
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Table 8: Specific activity and total protein contents of callus extracts prepared from acetone fractions separated by chromatography on CM-Cellulose and Sephadex-G50(fine) columns.
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<th>Total Activity (unit)</th>
<th>Protein (ug/ml)</th>
<th>Total Protein (mg)</th>
<th>Specific Activity Protein % Yield</th>
<th>Purification Fold</th>
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<td>1892</td>
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<td>170</td>
<td>100</td>
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<td>2363</td>
<td>276</td>
<td>8.3</td>
<td>286</td>
<td>16</td>
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</table>

Table 9: Specific activity and total protein contents of callus extracts prepared from acetone fractions with TritonX-100 separated by chromatography on CM-Cellulose and Sephadex-G50(fine) columns.
Figure 15: Chromatograph of fraction ACp0-40X on CM-Cellulose.

Figure 16: Filtration of fraction ACp0-40X on Sephadex-G50(fine).
ACp0-45X was slightly higher than that of ACp0-40 (tables 3 and 4).

The ACs0-40 or ACs0-40X (see table 2) was further treated with 367 ml of ice-cold acetone to give 80% saturation. The precipitate (ACp40-80 or ACp40-80X) obtained was dissolved in 50 ml of 0.02 M sodium acetate buffer pH 5.5 and dialyzed against water. This ACp40-80 and ACp40-80X solutions did not have any proteolytic activity (tables 3 and 4).

It is difficult to comprehend why only one fraction showed proteolytic activity. Perhaps the large amount of acetone added to increase saturation to 80% may have caused irreversible denaturation of the proteins or the excessive dilution of the liquid may have caused dispersion of protein particles which did not settle during centrifugation.

4A) CM-cellulose Fractionation of Acetone Fraction:

Twenty milliliter of the dialyzed ACp0-40 or ACp0-40X were applied to the CM-cellulose column. Three protein fractions were obtained but only fraction NO. 1 (ACp0-40 Fr1-CM or ACp0-40X Fr1-CM) had proteolytic activity (see figure 15). The total protein contents and specific activity of ACp0-40X Fr1-CM is slightly higher than in AC0-40 Fr1-CM (tables 8 and 9).
4B) Sephadex G-50 Filtration of Acetone Fraction:

Twenty milliliter of dialyzed ACp0-40 or ACp0-40X were applied to the sephadex column. Two protein fractions were obtained, as shown in figure 16. The first protein peak did not have proteolytic activity, but the second fraction had proteolytic activity. The total protein content and specific activity of ACp0-40X Fr2-S is slightly higher than in ACp0-40 Fr2-S (tables 8 and 9).

Casein-Agar Plates Assay:

Callus extracts after each step of purification were assayed for their proteolytic activity against casein-agar plates. The assays showed that CE2, CE2X, ASp0-45, ASp0-45X, ASp0-45 Fr1-CM, ASp0-45X Fr1-CM, ASp0-45 Fr1-S, ASp0-45X Fr1-S, ASp45-65, ASp45-65X, ASp45-65 Fr1-CM, ASp45-65X Fr1-CM, ASp45-65 Fr2-S, ASp45-65X Fr2-S, ACp0-40, ACp0-40X, ACp0-40 Fr1-CM, ACp0-40X Fr1-CM, ACp0-40 Fr2-S and ACp0-40X Fr2-S had proteolytic activity as shown by clearing zone on the casein-agar plates in figures 17 and 18. These assays cannot be quantified because of the discrepancy in diameters obtained when the experiments were repeated, but they gave a good qualitative visual method to identify fractions containing proteolytic activity.
Figure 17: Caseinolytic agar plate assay.
Well 1, CE2X; well 2, ASp0-45X; well 3, ASp0-45X Fr1-CM; well 4, ASp0-45X Fr1-S; well 5, ASp45-65X; well 6, ASp45-65X Fr1-CM; and well 7, C-papain.

Figure 18: Caseinolytic agar plate assay.
Well 1, ASp45-65X Fr2-S; well 2, ACp0-40X; well 3, ACp0-40X Fr1-CM; well 4, Hp2X; well 5, ACp40-80X; well 6, Hp2; and well 7, ACp0-40 Fr2-S.
Ouchterlony Double Immunodiffusion:

Callus extracts from each purification step were tested against P-papain and P-chymopapain antiserum. These assays showed that both CE2 and CE2X reacted with P-papain (figure 19) and P-chymopapain antiserum (figure 20). P-Papain antiserum produces three precipitating bands when reacted with the CE2X and only 2 precipitating bands when reacted with the CE2 (figure 19) while the P-chymopapain antiserum, produces only one precipitating band with either CE2 or CE2X (figure 20). Therefore, callus extracts containing triton-x seems to produce an extra papain like substance that P-papain antiserum recognized.

The fractions ASp0-45, ASp0-45X, ASp0-45 Fr1-CM, ASp0-45X Fr1-CM, ASp0-45 Fr1-S, and ASp0-45X Fr1-S have affinity for P-papain antiserum but not for P-chymopapain antiserum, because they all produced precipitation bands against P-papain antiserum only as shown in figure 21. P-chymopapain antiserum had an affinity for ASp45-65, ASp45-65X, ASp45-65 Fr1-CM, ASp45-65X Fr1-CM, ASp45-65 Fr2-S, ASp45-65X Fr2-S, ACp0-40, ACp0-40X, ACp0-40 Fr1-CM, ACp0-40C Fr1-CM, ACp0-40 Fr2-S, and ACp0-40X Fr2-S producing precipitation bands as shown in figure 22, while P-papain antiserum did not.
Figure 19: Ouchterlony double immunodiffusion precipitation band against P-papain antiserum. Well 1, CE2; well 2, CE2X; well 3, P-papain antiserum.

Figure 20: Ouchterlony double immunodiffusion precipitation band against P-Chymopapain antiserum. Well 1, CE2; well 2 CE2X; and well 3, P-chymopapain antiserum.
Figure 21: Ouchterlony double immunodiffusion precipitation band against P-papain antiserum. Well 1, ASp0-45X; well 2, ASp0-45X Fr1-CM; well 3, ASp0-45X Fr1-S; well 4, ASp0-45 Fr1-CM; well 5, ASp0-45 Fr1-S; well 6, ASp45-65X and well 7, P-papain antiserum.

Figure 22: Ouchterlony double immunodiffusion precipitation band against P-chymopapain antiserum. Well 1, ASp45-65X; well 2, ASp45-65X Fr1-CM; well 3, ASp45-65X Fr2-S; well 4, ACp0-40X; well 5, ACp0-40X Fr1-CM; well 6, ACp0-40X Fr2-S; and well 7, P-chymopapain antiserum.
**Passive Hemaglutination:**

All of the different fractions from each purification step that had proteolytic activity were used as antigens, were coated on uncoated tanned-human red blood cells, to test for the presence of papain or chymopapain like substances. The results of the passive hemaglutination test is summarized in table 10 for P-papain antiserum and table 11 for P-chymopapain antiserum.

Table 10 demonstrates that P-papain antiserum recognizes CE2 and CE2X giving a titer of 256 for both. The ASp0-45 and ASp0-45X gave titer of 128, ASp0-45 Fr1-CM and ASp0-45 Fr1-S fraction gave a titer of 64 while ASp0-45X Fr1-CM and ASp0-45X Fr1-S gave a titer of 128 for both with P-papain antiserum.

Table 11 demonstrates that antigens that reacted with P-chymopapain antiserum were found in CE2 and CE2X giving a titer of 64 and 128 respectively. Fraction ASp45-65 and ASp45-65X recognizes P-chymopapain antiserum giving a titer of 64 and 128 respectively while ASp45-65 Fr1-CM and ASp45-65X Fr1-CM gave a titer of 32 and 64 respectively. Also ASp45-65 Fr2-S and ASp45-65X Fr2-S recognizes P-chymopapain antiserum giving a titer of 32 and 64 respectively. The ACp0-40 and ACp0-40X gave a titer of 64 and 128 respectively while ACp0-40 Fr1-CM gave a titer of 16 and ACp0-40X Fr1-CM gave a titer of 32 with P-chymopapain antiserum. The ACp0-40 Fr2-S gave a titer of 16 while the ACp0-40X Fr2-S gave a titer of 32 with P-chymopapain antiserum.
Table 10: Passive Hemagglutination reaction of different callus extracts against P-papain antiserum.
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Table 11: Passive hemaglutination reaction of different callus extracts against P-chymopapain antiserum.
There were no agglutination reactions to P-papain antiserum when reacted with ASp45-65, ASp45-65X, ASp45-65 Fr1-CM, ASp45-65X Fr1-CM, ASp45-65 Fr2-S, ASp45-65X Fr2-S, ACp0-40, ACp0-40X, ACp0-40 Fr1-CM, ACp0-40X Fr1-CM, ACp0-40 Fr2-S and ACp0-40X Fr2-S fractions. Similarly there were no agglutination reactions to P-chymopapain antiserum when reacted with ASp0-45, ASp0-45X, ASp0-45 Fr1-CM, ASp0-45X Fr1-CM, ASp0-45 Fr1-S and ASp0-45X Fr1-S.

**Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis**

The SDS-PAGE shows that CE1 and CE1X of the callus gave 10 protein bands (figure 23 lane 4 and 6). The CE2 and CE2X gave 8 protein bands (figure 23 lane 5 and 7). The hydrochloric acid treatment of CE2 and CE2X produced Hp2 and Hp2X both of which gave one protein band only with molecular weight of 31,500 (figure 24 lane 9 and 16) and these fractions do not have any proteolytic activity as indicated in table 3 and 4.

The fractions obtained by ammonium sulfate precipitation, ASp0-45 and ASp0-45X each gave 3 protein bands with molecular weight of 22,500, 18,500, and 15,000, respectively as shown in figure 25 lane 6 and 7. The band corresponding to a molecular weight of 22,500 has proteolytic activity (tables 6 and 7) and is identical with ASp0-45 (or ASp0-45X) as shown in figure 25 lane 6, 7 and figure 26 lane 3 and 6, ASp0-45 Fr1-CM (or ASp0-45X Fr1-CM), shown in figure 26 lane 4 and with ASp0-45 Fr1-S (or ASp0-45X Fr1-S) as shown in figure 26 lane 7.
The fraction ASp45-65 and ASp45-65X also each gave three protein bands with molecular of 38,000, 35,000, and 32,000 respectively as shown in figure 25 lanes 8 and 9. The band with molecular weight of 30,000 has proteolytic activity (tables 6 and 7) and is identical to ASp45-65 (or ASp45-65X) shown in figure 25 lane 8,9 and figure 26 lane 8 and 10, ASp0-45 Fr1-CM (or ASp0-45X Fr1-CM) shown in figure 26 lane 9, and ASp45-65 Fr2-S (or ASp45-65X Fr2-S) as shown in figure 26 lane 11.

The fractions obtained by acetone precipitation ACp0-40 and ACp0-40X gave 4 protein bands with molecular weights of 38,000, 23,000, 32,000 and 30,000 respectively as shown in figure 25 lanes 10 and 11. The band with molecular weight of 30,000 has proteolytic activity (tables 8 and 9) and is identical to ACp0-40 (or ACp0-40X) see figure 25 lanes 10 and 11 and figure 26 lane 12, ACp0-40 Fr1-CM (or ACp0-40X Fr1-CM), and ACp0-40 Fr2-S (or ACp0-40X Fr2-S) as shown in figure 26 lane 13.

The acetone fraction of ACp40-80 and ACp40-80X gave one protein band (figure 24 lane 7 and 15) with molecular weight of 43,000 and did not have proteolytic activity (tables 8 and 9).

The proteases present in the callus obtained by SDS-PAGE have molecular weight of 22,500 and 30,000 while standard P-papain and P-chymopapain molecular weights were 24,000 and 29,000 respectively obtained by the same method.
Figure 23: SDS-PAGE of callus extracts.
Lane 1, P-chymopapain; lane 2, molecular weight marker; lane 3, P-papain; lane 4, CE1; lane 5, CE2; lane 6, CE1X; lane 7, CE2X.

Figure 24: SDS-PAGE of callus extracts.
Lane 1, P-chymopapain; lane 2, P-papain; lane 3, molecular weight marker; lane 4, CE2; lane 5, ASpO-45; lane 6, ASp45-65; lane 7, ACpO-40; lane 8, ACp40-80; lane 9, Hp2; lane 10, molecular weight marker; lane 11, CE2X; lane 12, ASpO-45X; lane 13, ASp45-65X; lane 14, ACpO-40X; lane 15, ACp40-80X; and lane 16, Hp2X.
Figure 25: SDS-PAGE of callus extracts.
Lane 1, molecular weight marker; lane 2, P-papain; lane 3, P-chymopapain; lane 4, CE2; lane 5, CE2X; lane 6, ASpO-45X; lane 7, ASpO-45X; lane 8, ASp45-65; lane 9, ASp45-65X; lane 10, ACpO-40; lane 11; ACpO-40X.

Figure 26: SDS-PAGE of callus extracts.
Lane 1, P-papain; lane 2, P-chymopapain; lane 3, ASpO-45X; lane 4, ASpO-45X Fr1-CM; lane 5, molecular weight marker; lane 6, ASpO-45; lane 7, ASpO-45X Fr1-S; lane 8, ASp45-65X; lane 9, ASp45-65X Fr1-CM; lane 10, ASp45-65; lane 11, ASp45-65X Fr2-S; lane 12, ACpO-40X; and lane 13, ACpO-40X Fr2-S.
Immuno Blotting of Callus Extracts Against P-papain and P-chymopapain

IgG


Western Blotting

Western blotting also shows that protein band with molecular weight of 22,500 of ASpO-45, ASpO-45X, ASpO-45X Fr1-CM, and ASpO-45X Fr1-S, were recognized by the anti-P-papain IgG while protein band with molecular weight 30,000 of ASp45-65X, ASp45-65X Fr1-CM, ASp45-65X Fr2-S, ACp0-40X, ACp0-40X Fr1-CM and ACp0-40X Fr2-S were recognized by the anti-P-chymopapain IgG.

pH and Temperature Effects

The ASp0-45X Fr1-CM, ASp0-45X Fr1-S, ASp45-65X Fr1-CM and ASp45-65X Fr2-S fractions were tested for their pH and temperature stability. The result are shown in figure 32 and figure 33 respectively. Both fractions were most active at pH 7.0 and least active at pH 3 and show a decrease in their proteolytic activity when heated at 75 C.
Figure 27: Immuno blotting of antigens from callus extracts as labelled. Lane 1 was blot against antiP-papain IgG and lane 2 was blot against antiP-chymopapain IgG.
Figure 28: Immuno blotting of antigens from callus extracts as labelled. Lane 1, 2 and 3 was blot against antiP-papain IgG.

Figure 29: Immuno blotting of antigens from callus extracts as labelled. Lane 1, 2 and 3 was blot against antiP-chymopapain IgG.
Figure 30: Caseinolytic activity of proteolytic fractions from CM-Cellulose and Sephadex-G50(fine) fractionation at varying pH. Graph 1, ASp0-45X Fr1-CM; graph 2, ASp45-65X Fr1-CM; graph 3, ASp0-45X Fr1-S; and graph 4, ASp45-65X Fr2-S.

Figure 31: Caseinolytic activity of proteolytic fractions from CM-Cellulose and Sephadex-G50(fine) fractionation heated at 75°C from 0 to 30 minutes. Graph 1, ASp0-45X Fr1-CM; graph 2, ASp45-65X Fr1-CM; graph 3, ASp0-45X Fr1-S; and graph 4, ASp45-65X Fr2-S.
CONCLUSION

The *Carica papaya* L. callus tissue seems to contain two distinct proteolytic enzymes. One of them has a molecular weight of 22,500 while the other 30,000 as determined by SDS-PAGE. Both enzymes are most active at pH 7.0 and their proteolytic activity decreases when heated at 75°C. The amount of enzyme with molecular weight of 22,500 represents 18.51% of the total protein content of CE2X, isolated by Sephadex-G50(fine) column chromatography from the 45% ammonium sulfate fraction. The amount of enzyme with molecular weight of 30,000 present is 9.7% of the total protein contents of CE2X, isolated by Sephadex-G50(fine) column filtration from the 65% ammonium sulfate fraction. All of the immunological tests carried out to identify the two enzymes showed that the enzyme with molecular weight of 22,500 was recognized by anti-P-papain IgG while the enzyme with molecular weight of 30,000 was recognized by anti-P-chymopapain IgG. The SDS-PAGE of these two enzymes compared to the standard P-papain or C-papain and P-chymopapain or C-chymopapain were not identical. Therefore, these two enzymes present in the callus tissue are neither papain or chymopapain, as indicated by immunological tests but more likely to be papain and chymopapain like substances. That is, they have sites which are identical to standard papain and chymopapain which are recognized by anti-P-papain and anti-P-chymopapain IgG. It has been reported that the papain enzymes in the papaya latex have a catalytically inactive precursor or isomer called propapain(10). Therefore, these enzymes in
callus extractives may be like the precursors of papain. It is also possible that because the callus was grown in the absence of light, only the precursors of papain or chymopapain were produced. More work needs to be done to study the properties of these two enzymes and compare them with papain and chymopapain. The precursor theory also needs to be investigated by studying the enzymes produced in chlorophyll containing callus cells or by labelling the enzymes in the callus and studying the generation of other enzymes as the callus cells differentiate into entire plants. It would also be interesting to find out if these proteolytic enzymes (molecular weight 22,500 and 30,000) are present in papaya latex obtained from a full grown plant.
REFERENCES:


