CHAPTER III

RESULTS

3.1 Screening of chitinase in some local crops

Bean seeds, leaves, and pericarp of some local crops were screened for chitinase activities using agar plate assay, colorimetric assay and activity staining on polyacrylamide gel.

3.1.1 Chitinase in leaves of some local crops.

Fresh leaves of seven plants, ivygourd (*Coccinia indica*), tamarind (*Tamarindus indica* Linn.), papaya (*Carica papaya* Linn.), jackfruit (*Artocarpus heterophyllus* Lamk.), star gooseberry (*Cicca acida*), roseapple (*Eugenia Linn.*) and aztec kuamochill (*Pithecellobium dulce*) were extracted with 100 mM soduim citrate buffer pH 5.0 as described in section 2.2.2, and screened for chitinase activity by agar plate assay (section 2.3.1). Only the extract of leaves from papaya (*Carica papaya* Linn.) showed clear zone on the plate (Figure 3).

The leaf extracts were also subjected to SDS polyacrylamide gel electropheresis and stained for chitinase activity according to the method described in section 2.2.3. No chitinase band was visible in star gooseberry leaves (lane 1, Figure 4) but similar chitinase band were detected near the top of the gel in papaya leaves and aztec kuamochill leaves (lane 2, 3, Figure 4).

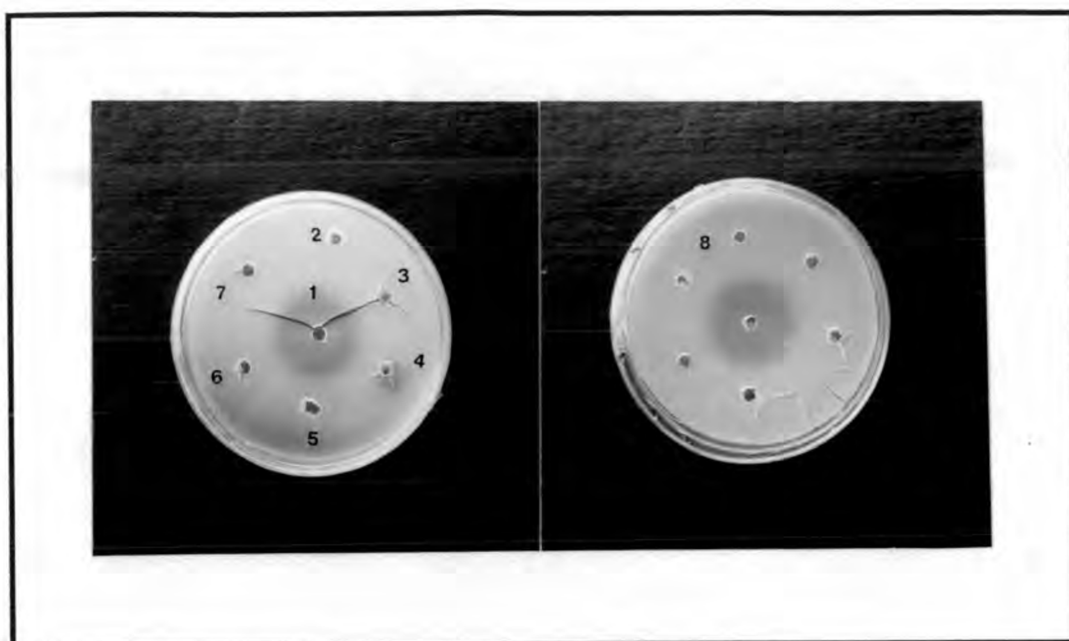

Figure 3 Chitinase screening on agar plate of leave extracts of local crops (36 μg protein).

Well 1 Commercial chitinase from *Serratia marcescens*

Well 2 leaves of star gooseberry

Well 3 leaves of ivygourd

Well 4 leaves of papaya

Well 5 leaves of jackfruit

Well 6 leaves of tamarind

Well 7 leaves of roseapple

Well 8 leaves of aztec kuamochill

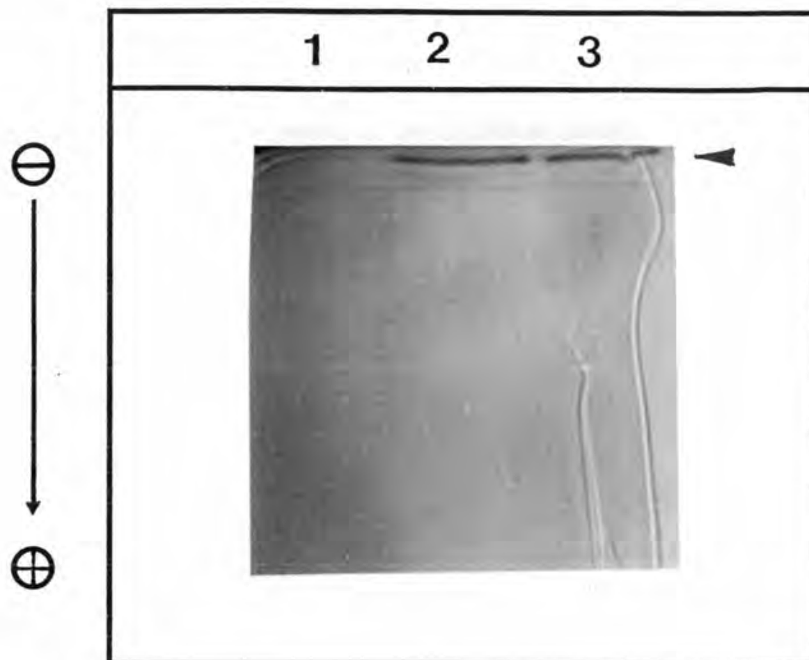

Figure 4 SDS-PAGE (12%) staining for chitinase of leaves extracts of local crops.

Lane 1 leaves of star gooseberry

Lane 2 leaves of papaya

Lane 3 leaves of aztec kuamochill

3.1.2 Chitinase in Bean seeds

There were reports on the presence of chitinase in bean seeds induced with ethylene. In this study, bean seeds were treated separately with colloidal chitin as an inducer of the enzyme since it was reported to occur only by induction using the procedure described in section 2.2.3.

Chitinase in bean seeds can be induced after incubation with 1% colloidal chitin for at least 30 hours or 2% colloidal chitin for at least 24 hours (figure 5(a), 5(b) respectively). Moreover , when detected by activity staining in ND-PAGE, chitinase band also appeared in the induction of enzyme by 0.5% colloidal chitin for 36 hours (Figure 6, lane 1). Therefore, chitinase can be said to be inducible in bean seeds by 0.5% collidal chitin for 36 hours.

3.1.3 Chitinase in angled loofah fruit pericarp

Pongdontri (1993) reported the presence of a lectin in the pericarp of angled loofah fruit which is specific to chitin. It is interesting to investigate if this lectin also contains chitinase activity. Experiment was carried out to prepare extract of fruit pericarp of angled loofah following the method of Pongdontri (1993) and assay if there is any chitinase activity presence.

Pericarp of angled loofah fruit was extracted with 0.02M sodium phosphate buffer (pH 7.4) for chitinase activity as described in section 2.2.1. Crude extract (protein 0.3 mg) and ammonium sulfate precipitation fraction were tested for chitinase activity by agar plate

(a)

Figure 5 (a) Chitinase screening on agar plate of bean seeds extract induced with 1% colloidal chitin at various times.

Well 1 Commercial chitinase from *Serratia marcescens*

Well 2 at 6 hours

Well 3 at 12 hours

Well 4 at 18 hours

Well 5 at 24 hours

Well 6 at 30 hours

Well 7 at 36 hours

(b)

Figure 5 (b) Chitinase screening on agar plate of bean seeds extract induced with 2% colloidal chitin at various time.

Well 1 Commercial chitinase from *Serratia marcescens*

Well 2 at 6 hours

Well 3 at 12 hours

Well 4 at 18 hours

Well 5 at 24 hours

Well 6 at 30 hours

Well 7 at 36 hours

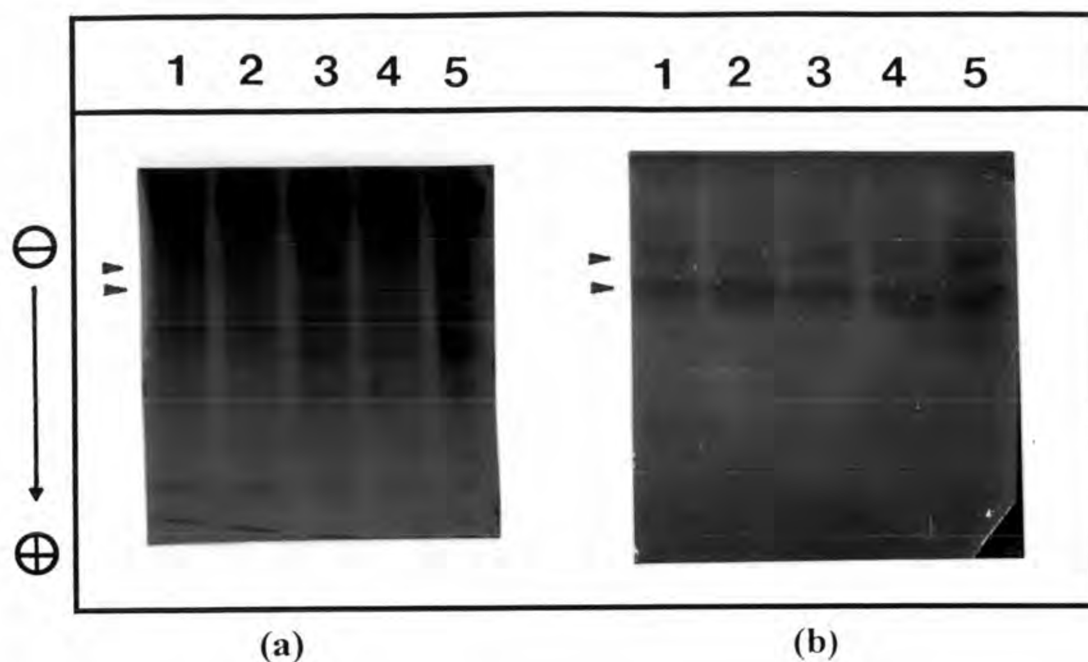

Figure 6 Native PAGE (12%) of the extract from bean seeds induced with 0.5, 1 and 2% colloidal chitin (a) protein pattern, (b) staining for chitinase activity.

Lane 1 0.5% colloidal chitin, at 36 hours

Lane 2 1.0% colloidal chitin, at 30 hours

Lane 3 1.0% colloidal chitin, at 36 hours

Lane 4 2.0% colloidal chitin, at 30 hours

Lane 5 2.0% colloidal chitin, at 36 hours

assay as mentioned in section 2.3.1. Figure 7 shows clear zone of chitinase activity in the crude extract of the pericarp.

3.2 Purification of chitinase from angled loofah pericarp extract

From all the samples screened for chitinase activity in section 3.1, it was considered that the pericarp of angled loofah be selected for future study since it was easily prepared and can be obtained in large amount. Furthermore, it would also answer the question to Pongdontri's work (1993) whether the chitin-specific lectin reported is the same protein as the chitinase detected in 3.1.3. The crude extract was detected for chitinase activity on agar plate assay (Figure 7) and subjected to purification for further study.

3.2.1Ammonium sulfate fractionation

Purification of chitinase from fruit pericarp of angled loofah started with conventional step of ammonium sulfate precipitation. To determine the appropriate concentration for precipitating chitinase from the crude extract, serial precipitation of proteins by increasing concentration of ammonium sulfate was performed as described in section 2.5.1. The crude extract was obtained as described in section 2.2.1 and fractionated by gradual addition of solid ammonium sulfate with stepwise increase of percent saturation from 0-100 %. The precipitate at each step was collected by centrifugation at 7,800 g or 7,300 rpm for 30 minutes, resuspended in 0.02M sodium phosphate

Figure 7 Chitinase screening on agar plate of pericarp of angled loofah fruit.

Well 1 commercial chitinase from *Serratia marcescens*

Well 2 crude extract (0.1 mg protein)

Well 3 crude extract (0.3 mg protein)

Well 4 0-30% ammonium sulfate precipitation

Well 5 30-70% ammonium sulfate precipitation (0.1 mg protein)

Well 6 70-100% ammonium sulfate precipitation

Well 7 30-70% ammonium sulfate precipitation (0.2 mg protein)

buffer (pH 7.4) and dialysed overnight in excess volume of the same buffer. Chitinase activity was detected by agar plate assay and SDS-polyacrylaminde gel electrophoresis and protein content was determined. In the agar plate assay crude extract (0.3 mg protein) and 30-70% ammonium sulfate precipitation (0.1 mg protein) gave clear zone on the agar plate after incubated for 48 hours (Figure 7). For SDS-PAGE, it was shown that 30-50% and 50-70% ammonium sulfate fraction having highest chitinase activity as shown in figure 8. Therefore, it was dicided to collect the precipitate in the range 30-70 % for further study of the chitinase.

3.2.2 DEAE- cellulose column chromatography

The DEAE-cellulose was prepared as described in section 2.5.2.1. Dialysed 30-70% ammonium sulfate precipitated fraction of pericarp was loaded to DEAE-cellulose column, washed with 0.02M phosphate buffer pH 7.4 until absorption at 280 nm of the eluted fraction is negligible. The next step of elution was done by gradient of 0-0.6M NaCl in phosphate buffer pH 7.4. After the gradient elution, the remaining proteins in the column was washed out with 1 M NaCl. Each fraction was detected for chitinase activity by colorimetic method and the fractions with chitinase activity were pooled for further study. Figure 9 showed chromatographic profile of DEAE-cellulose column chromatography. Fractions 107-117 eluted at 0.18-0.2 M NaCl were shown to contain chitinase activity.

The pooled fraction was subjected to non denaturing polyarylamide gel electrophoresis and stained for chitinase activity as

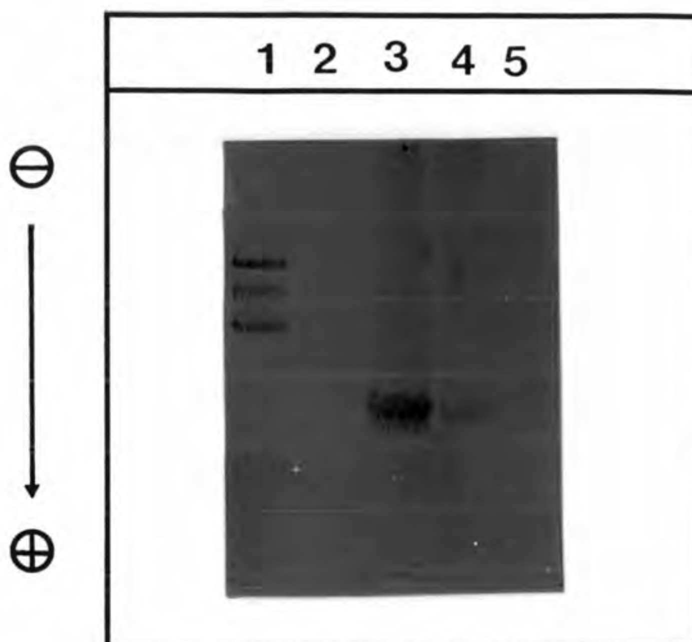

Figure 8 SDS-PAGE (12%) staining for chitinase of ammonium sulfate fractionation

Lane 1 commercial chitinase from *Serratia marcescens*

Lane 2 0-30% ammonium sulfate precipitation

Lane 3 30-50% ammonium sulfate precipitation

Lane 4 50-70% ammonium sulfate precipitation

Lane 5 70-100% ammonium sulfate precipitation

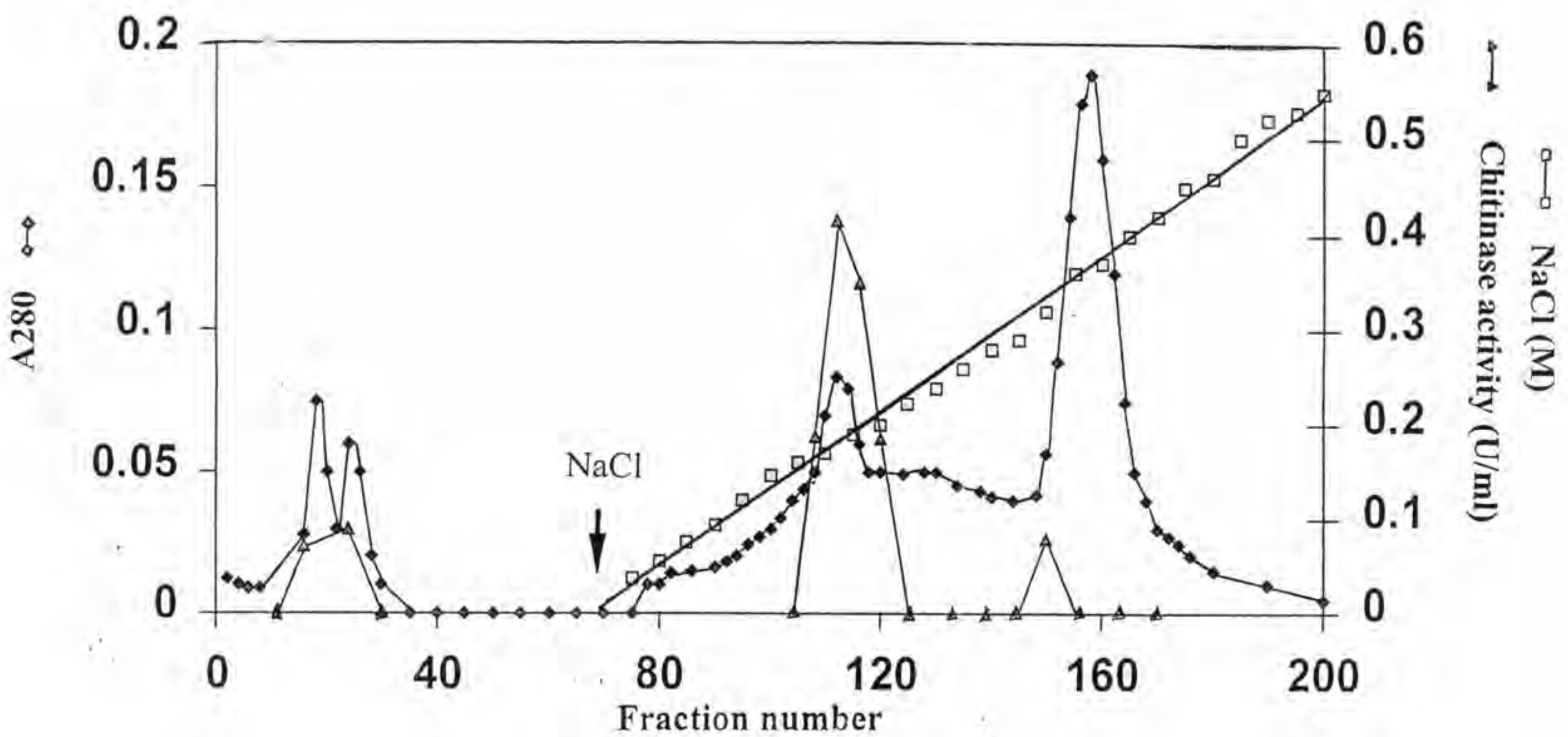

Figure 9 Chromatographic profile of chitinase from angled loofah fruit pericarp on DEAE-cellulose column.

30-70% ammonium sulfate percipitation was loaded on DEAE-cellulose column chromatography. The column size 2.4x16 cm, 5 ml/fraction at flow rate 30ml/hr.

described in section 2.3.3. Only the third peak (Figure 10 (b) lane 3) gave 2 band of chitinase activity (a and b) which corresponded to band of chitinase activity in crude and 30-70% precipitate (Figure 10 (b)).

In SDS polyacrylaminde gel electrophoresis for chitinase activity performed as described in section 2.3.3, the third peak (Figure 11, lane 3) gave one band of chitinase activity which corresponded to band of chitinase activity in crude and 30-70% ammonium sulfate precipitate (lanes 1, 2 respectively) as shown in figure 11.

Table 4 shows the yield and folds of purification of the chitinase from fruit pericarp of angled loofah. After, 30-70% ammonium sulfate precipitation, the chitinase activity recovered was 87.9 % with 6 folds of purity. Further purification with DEAE-cellose column gave activities of higher purity up to 18.9 folds and the yield of 6.7 %. Investigation of the purified samples in each step on SDS-PAGE (Figure 11) showed that the sample after DEAE-cellulose column was only slightly contaminated with other proteins.

3.3 Molecular weight determination of the chitinases

3.3.1 Molecular weight determination of pericarp chitinase by SDS-PAGE

The protein band of DEAE-cellulose fractions in ND-PAGE was cut to separate protein band a and b (Figure 10) and subjected to SDS-PAGE to determined molecular weight under reducing condition according to section 2.6. Figure 12, protein band a in ND-PAGE was

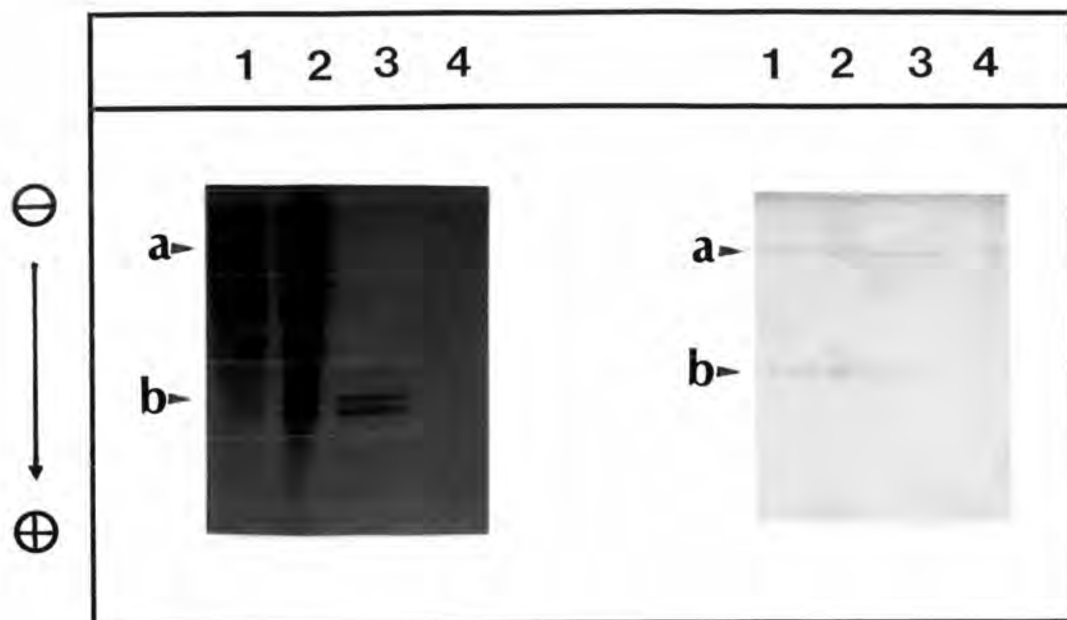

Figure 10 Native PAGE (12%) (a) staining for protein chitinase (b) staining of each peak from DEAE-cellulose column.

Lane 1 crude extract of pericarp from angled loofah fruit

Lane 2 30-70% ammonium sulfate precipitation

Lane 3 peak III from DEAE-cellulose column

Lane 4 peak IV from DEAE-cellulose column

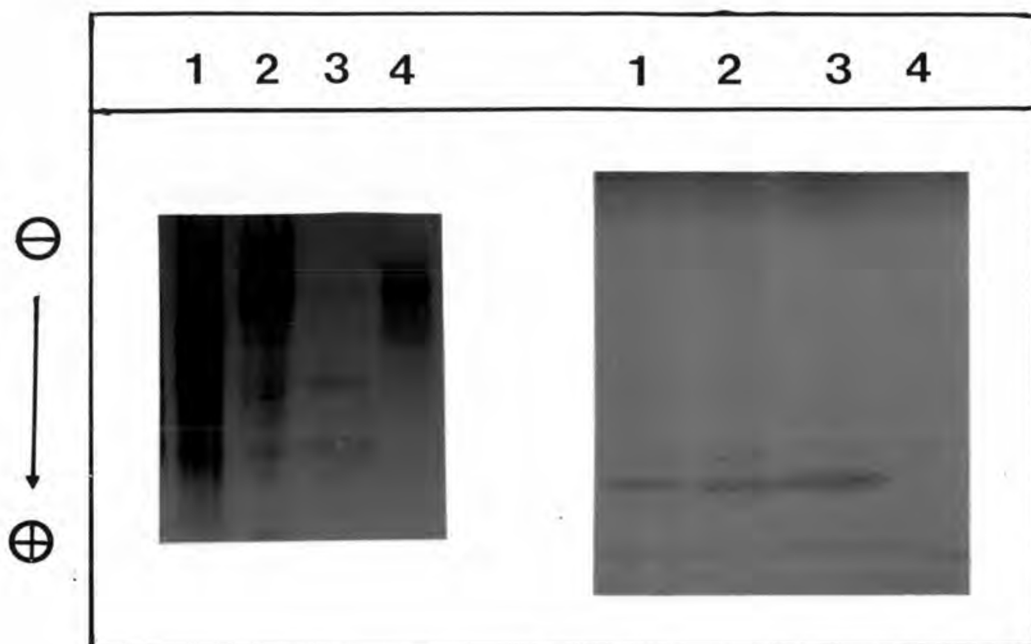

Figure 11 SDS-PAGE (12%) (a) staining for protein chitinase (b) staining of each peak from DEAE-cellulose column.

Lane 1 crude extract of pericarp from angled loofah fruit

Lane 2 30-70% ammonium sulfate precipitation

Lane 3 peak III from DEAE-cellulose column

Lane 4 peak IV from DEAE-cellulose column

Table 4 The purification of chitinase from pericarp of angled loofah fruit

Step	Total prot. (mg)	Total act. ($nmol\ min^{-1}$)	Specific act. ($nmol\ min^{-1}\ mg^{-1}$)	Recovery (%)	Purification (fold)
Crude	751.8	95.5	0.127	100	1.0
30-70% $(NH4)_2SO_4$	110.2	83.9	0.762	87.9	6.0
DEAE-cellulose	2.7	6.4	2.4	6.7	18.9

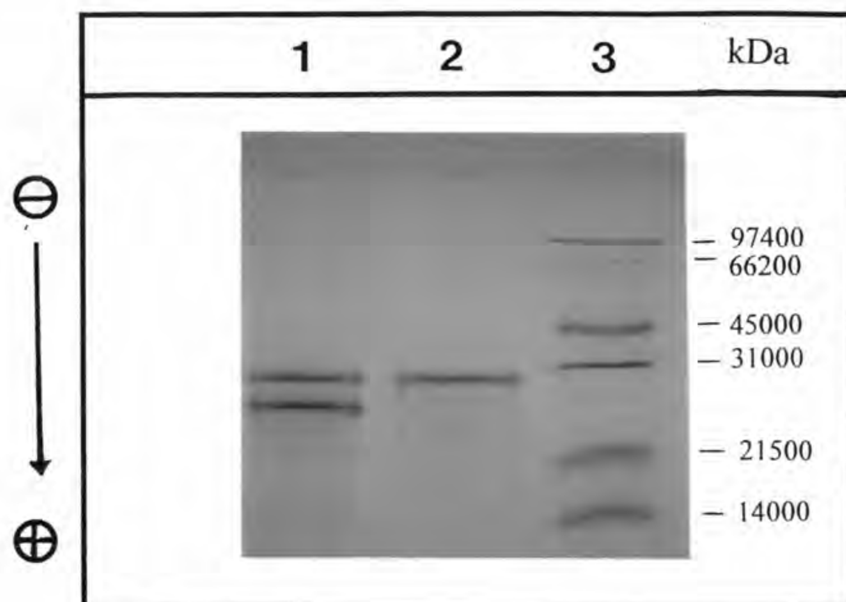

Figure 12 Molecular weight determination of chitinase from angled loofah fruit pericarp by SDS-PAGE (12%).

Lane 1 protein band a from native PAGE by gel slice

Lane 2 protein band b from native PAGE by gel slice

Lane 3 standard molecular weight

subjected to SDS-PAGE in lane 1 which showed two protein bands, their molecular weight were determined to be 25,000 and 29,000, respectively. Protein band b from ND-PAGE loaded in lane 2 which showed single protein band, its molecular weight was determined to be 29,000, from the calibration curve of standard proteins which include phosphorylase b, BSA, ovalbumin, carbonic anhydrase, trypsin inhibitor and lysozyme. The Rf of the proteins were plotted against log scale of its molecular weight (Figure 13). Only the protein band with molecular weight of 29,000 in both protein band a and b from ND-PAGE have a chitinase activity (data not show).

3.3.2 Sephadex G-100 column

Since the yield from DEAE-cellulose was very low, determination of native molecular weight of the chitinase on Sephadex G-100 was performed using 30-70 % ammonium sulfate precipitation instead.

Sephadex G-100 was prepared as described in section 2.5.2.2 and the sample was loaded to Sephadex G-100 column, eluted with 0.02 M sodium phosphate buffer (pH 7.4) until absorption at 280 nm of the eluted fraction is negligible the elution profile was shown in Figure 14. Chitinase activity of the eluted fractions was determined on polyacrylamide gel electrophoresis. Only fractions from peak I gave positive chitinase stain on the gel (Figure 15). However, the protein loaded was too minute, it cannot be detected with protein staining. Molecular weight calibration curve was constructed from running a series of molecular weight standards which include acid phosphatase,

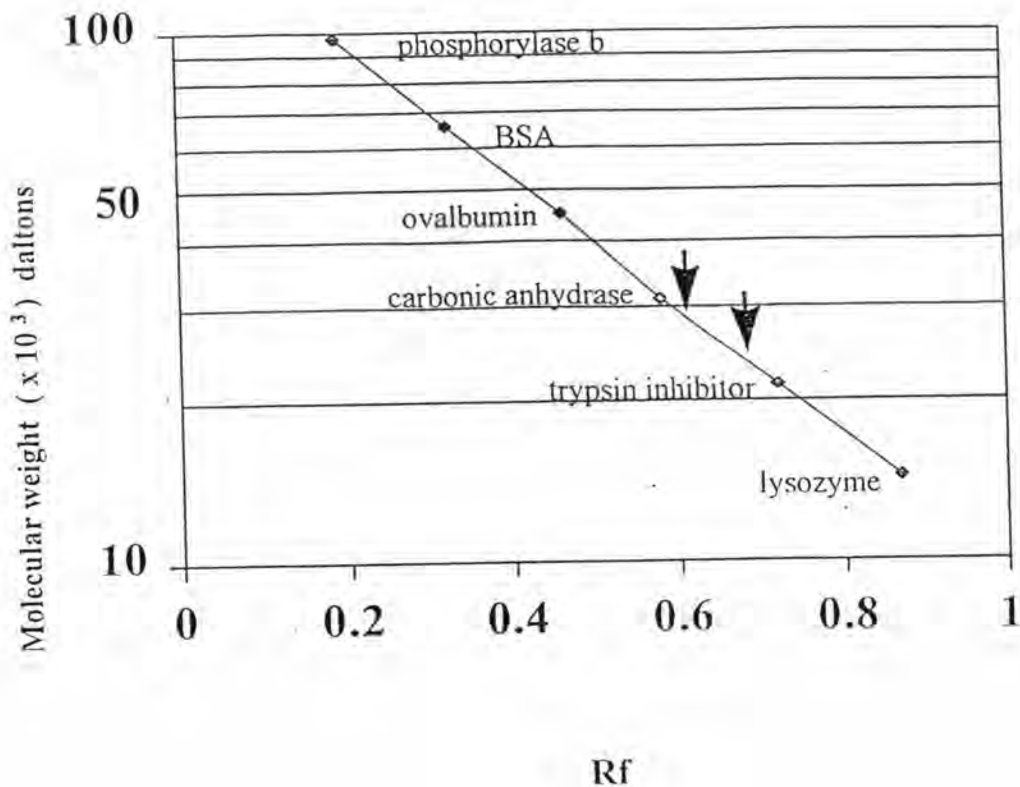

Figure 13 Molecular weight calibration curve of standard protein separated on 12 % SDS-PAGE.

Molecular weight of standard markers (as shown in the figure) were plotted on semilog scale against their relative mobility on 12% SDS-PAGE. The arrow indicated chitinase from pericarp of angled loofah fruit.

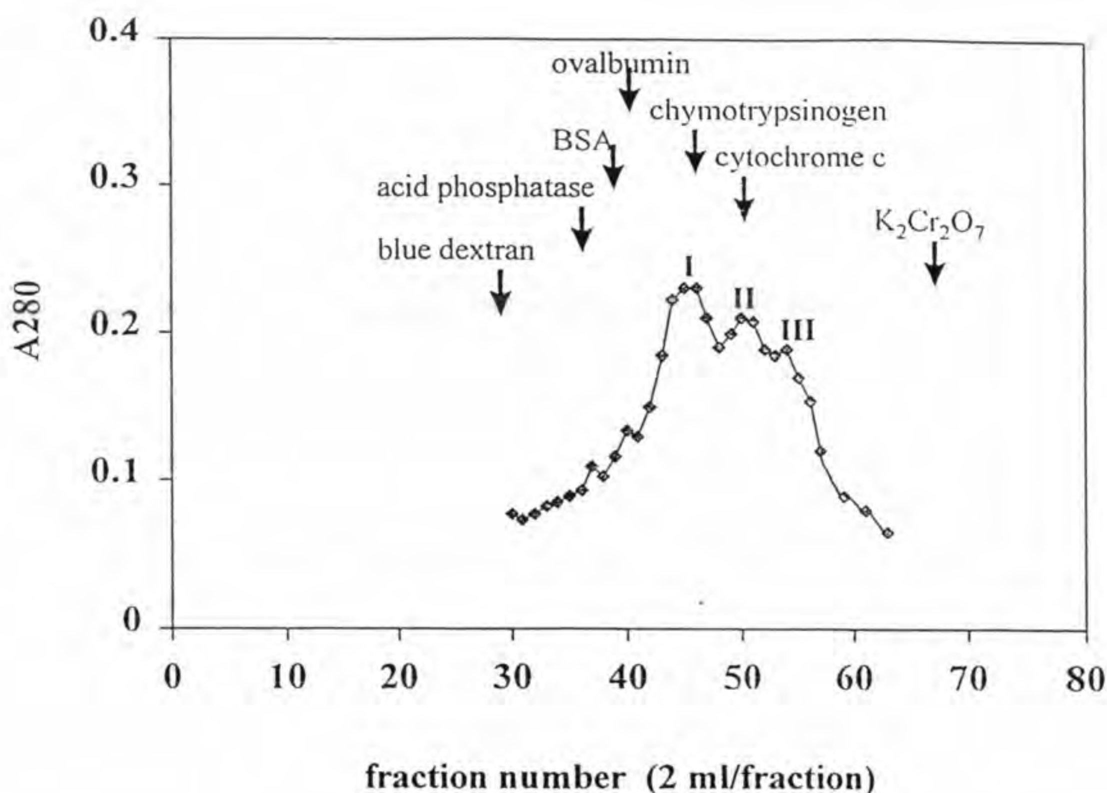

Figure 14 Elution profile of partial purified pericarp chitinase on Sephadex G-100 column.

Partial purified chitinase (30-70 % ammonium sulfate percipitation) from angled loofah fruit (2 ml of 5 mg) was loaded on Sephadex G-100 column equilibrated and eluted as described in section 2.7.2. This column was calibrated with blue dextran, $K_2Cr_2O_7$ and the following standard proteins : acid phosphatase (95,000), BSA (68,000), ovalbumin (43,000), chymotrypsinogen (23,240) and cytochrome c (12,380).

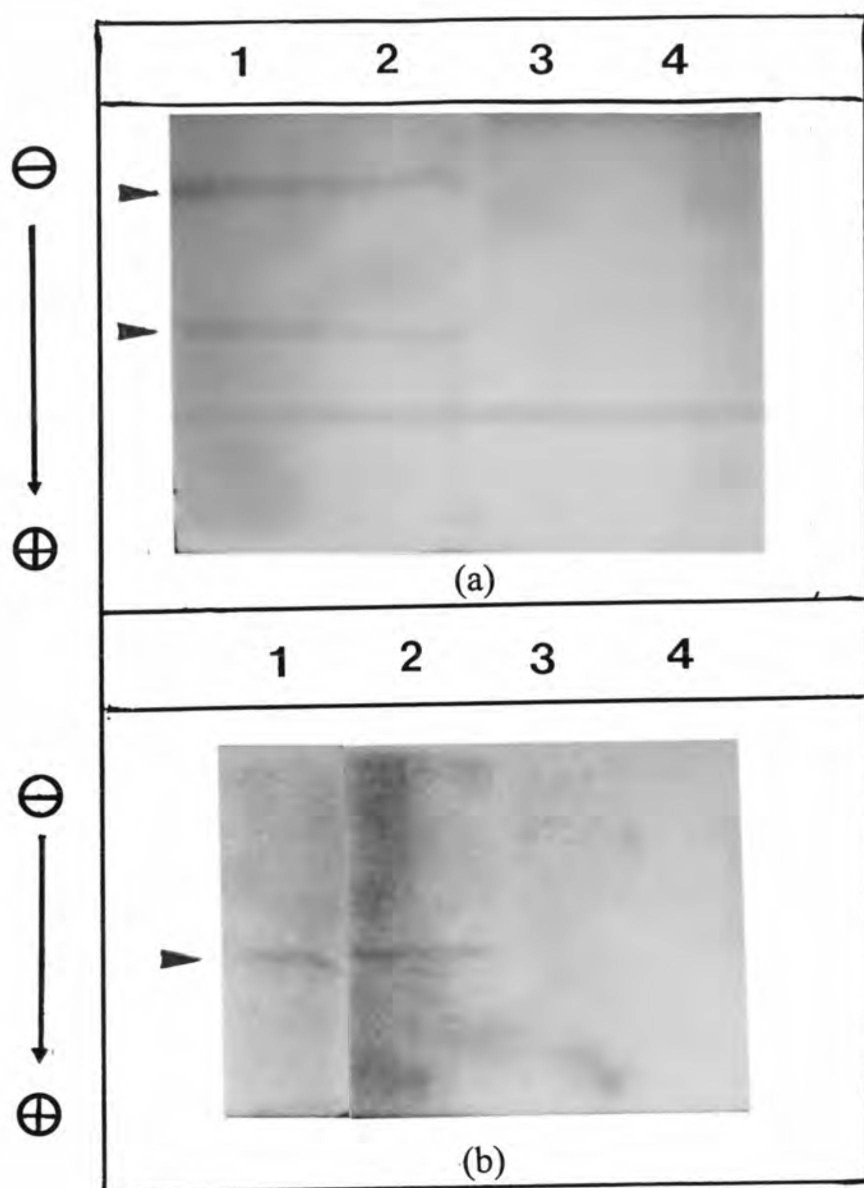

Figure 15 (a) Native-PAGE (12%), (b) SDS-PAGE (12%) staining for chitinase activity of the eluted fractions from Sepahdex G-100 column.

Lane 1 30-70% ammonium sulfate precipitation

Lane 2 Eluted fraction peak I

Lane 3 Eluted fraction peak II

Lane 4 Eluted fraction peak III

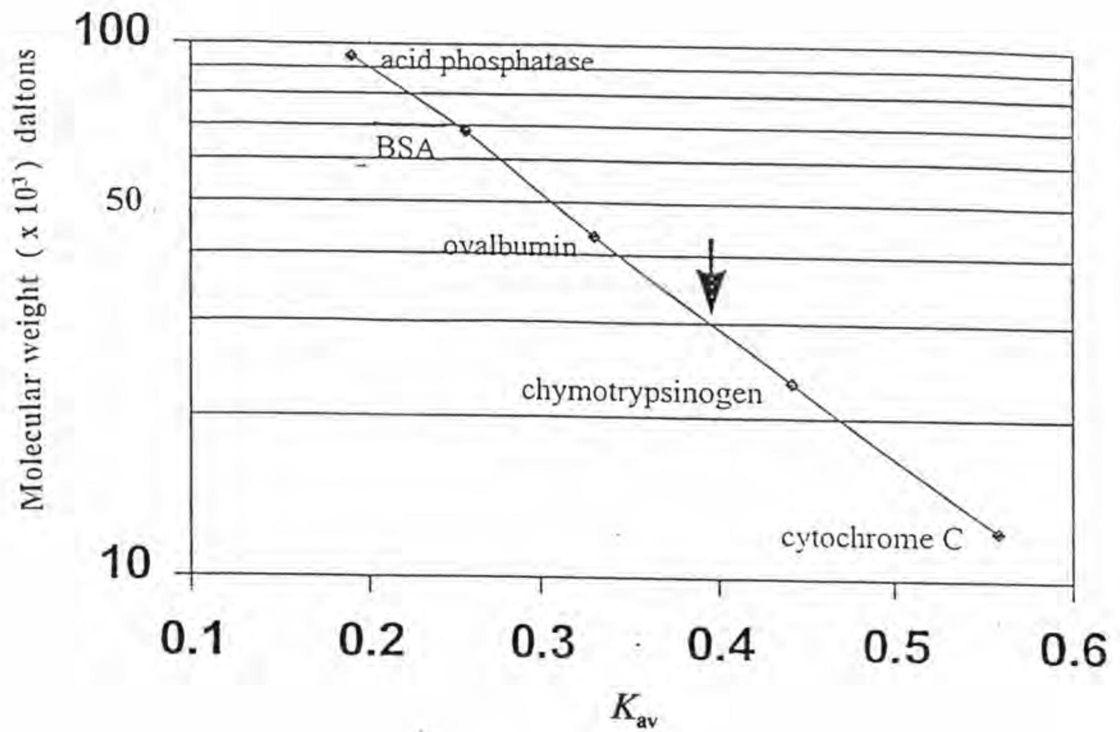

Figure 16 Molecular weight calibration curve for determination of molecular weight by gel filtration on Sephadex G-100.

The K_{av} of each standard protein was calculated from Figure 15 and were plotted against its molecular weight. The K_{av} of partial purified pericarp chitinase was indicated by the arrow.

BSA, ovalbumin, chymotrypsinogen and cytochrome C. The K_{av} of the protein were plotted againt log scale of its molecular weight (Figure 16). The molecular weight of the chitinase in peak was then determined from its K_{av}, which turned out to be 30,000.

3.4 Some biochemical properties of the chitinase

Experiments were carried out to study some of the biochemical properties of the partial-purified chitinase.

3.4.1 Determination of isoelectric point

Partial-purified pericarp chitinase was analysed for its pI by isoelectric focusing on polyacrylamide gel (IEF) as described in section 2.8 and the result shown in figure 16. The range of ampholine used was 4-7 and the protein standards used were mixture of proteins with different pI's : Phycocyanin (4.65), β-lactalbumin B (5.10), bovine carbonic anhydrase (6.00), human carbonic anhydrase (6.50), Equine myoglobin (7.00), human hemoglobin A (7.10), human hemoglobin C (7.50), lentin lectin (7.80, 8.00, 8.20) and cytochrome c (9.60). Figure 18 showed the calibration curve of standard pI. As narrow range ampholines was used, the standards with pI's in the more basic regions were compressed into thick bands and cannot be accurately identified. Therefore, only the pI standards in the range 4-7 were used to plot the standard curve. The pI of the fraction from protein band b which have chitinase activity was determined to be 5.4 from the standard curve

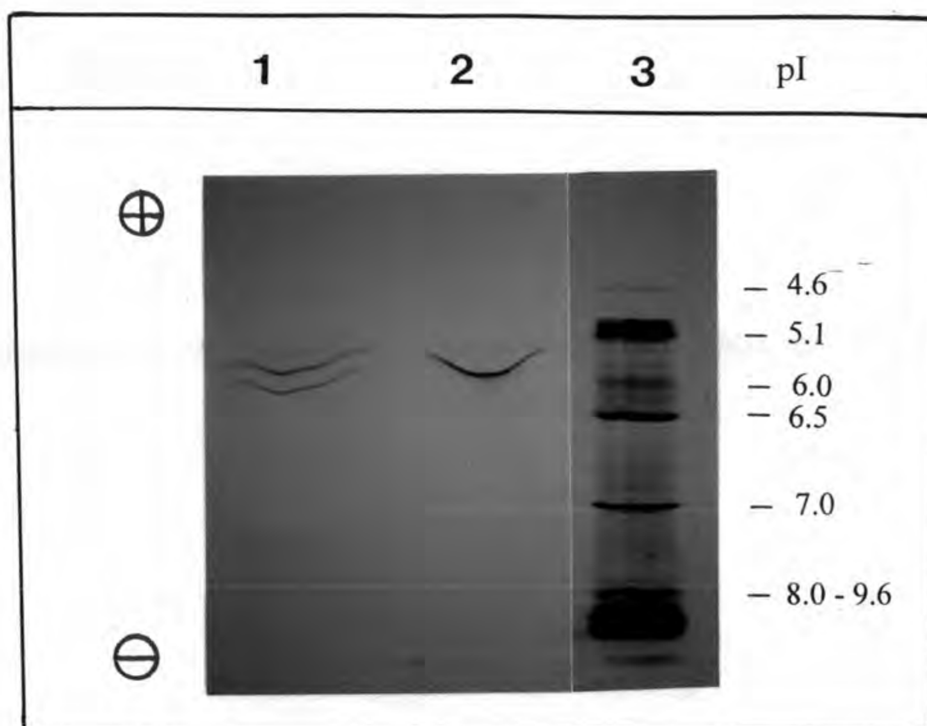

Figure 17 Isoelectric focusing polyacrylamide gel electrophoresis of partial purified pericarp chitinase. Standard pI markers used were described in section 2.8.

Lane 1 protein band (a) from native PAGE by gel slice

Lane 2 protein band (b) from native PAGE by gel slice

Lane 3 standard pI markers

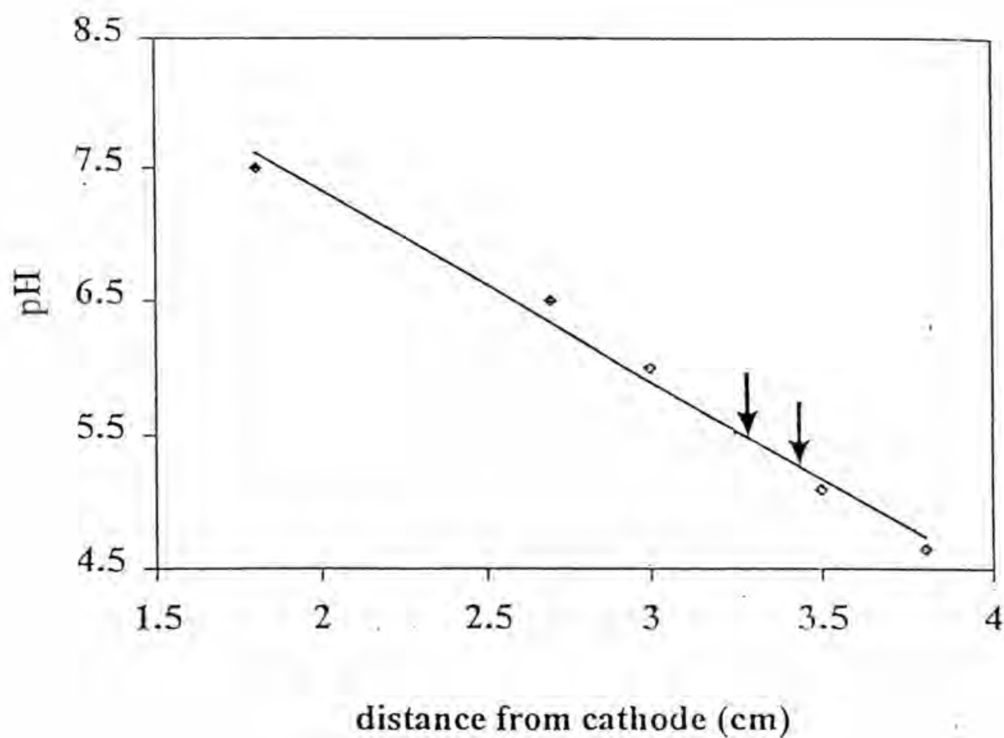

Figure 18 Calibration curve of standard pI markers for determination of isoelectric pH of pericarp chitinse.

The migration distance from cathode of the pI standard markers (as described in Figure 17) were measured and plotted against pI's. Migration position of the partial purified chitinase were indicated by arrow.

(Figure 17 lane 2). while protein band a showed two protein bands with the pI of 5.4 and 5.5, respectively (lane 1).

3.4.2 Effect of pH on chitinase activity

Experiment was performed to investigate the optimum pH for the activity of the partial purified chitinase. The enzyme (24.4 μg) was incubated at 37°C at various pHs : 1M sodium citrate buffer pH 3.0 and 3.5, 1M sodium acetate buffer pH 3.5, 4.0, 4.5 and 5.0, 1M sodium phosphate buffer pH 5.0, 6.0 and 7.0 and 1M Tris-HCl pH 7.0, 8.0 and 9.0. Figure 19 shows that the enzyme exhibited maximal activity at pH 3.5. Therefore, the other enzyme assay were run at pH 3.5.

3.4.3 Effect of temperature on chitinase activity

Experiments on temperature effect on the activity of the partial purified chitinase was performed in 1M sodium citrate buffer pH 3.5. The enzyme (24.4 μg) was incubated with appropriate reaction mixture at various temperatures : 25, 30, 35, 37, 40, 50 and 60°C. The enzyme showed maximal activity at 37°C (Figure 20). Therefore, the suitable condition for chitinase assay should be at pH 3.5 and 37°C.

3.4.4 The subtrate specificity test

For the substrate specificity test, the enzyme was assay in 1M sodium citrate buffer pH 3.5 at 37°C, and the reaction was started by

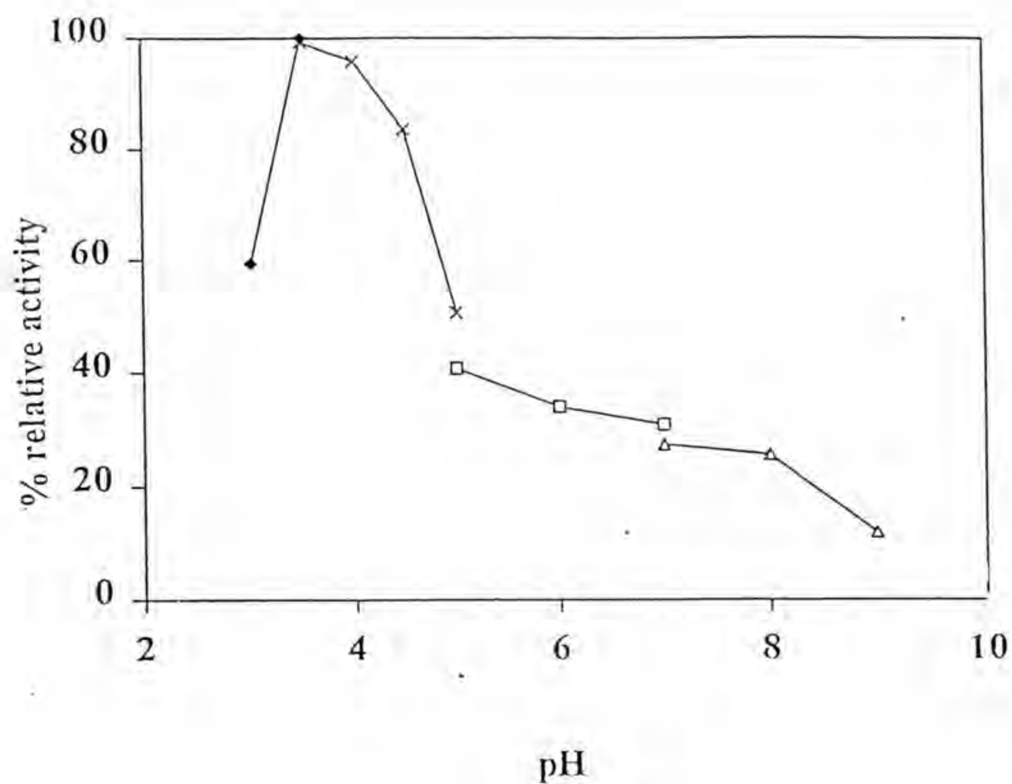

Figure 19 Effect of pH on pericarp chitinase activity.

- • sodium citrate buffer
- × sodium acetate buffer
- □ sodium phosphate buffer
- △ Tris-HCl buffer

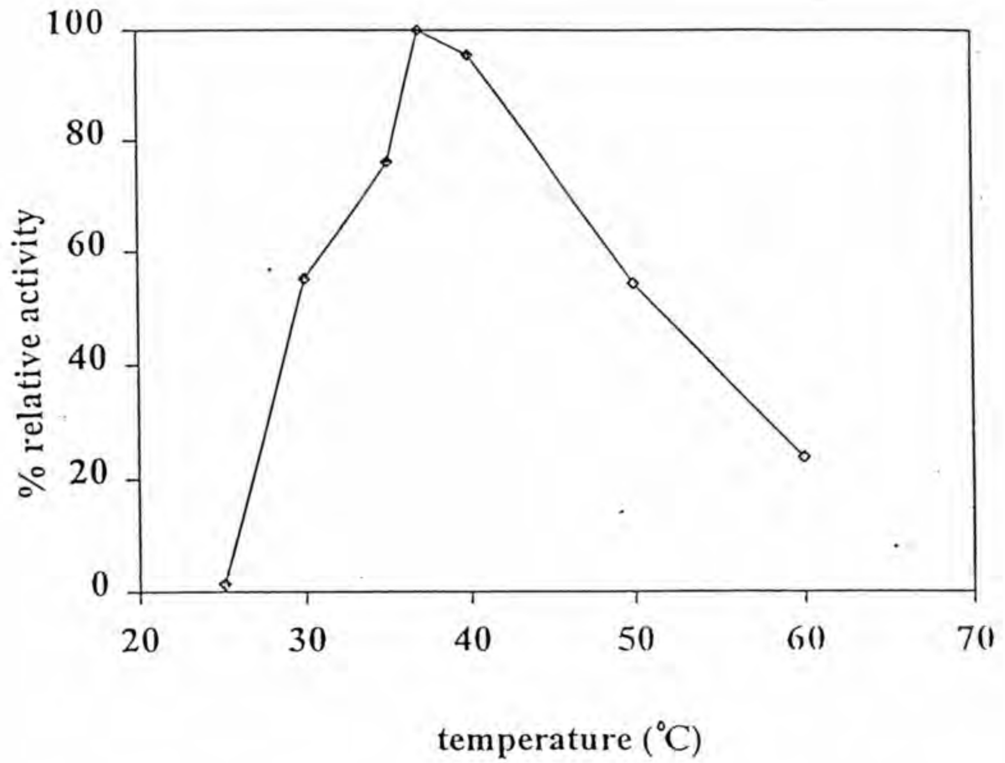

Figure 20 Effect of temperature on pericarp chitinase activity.

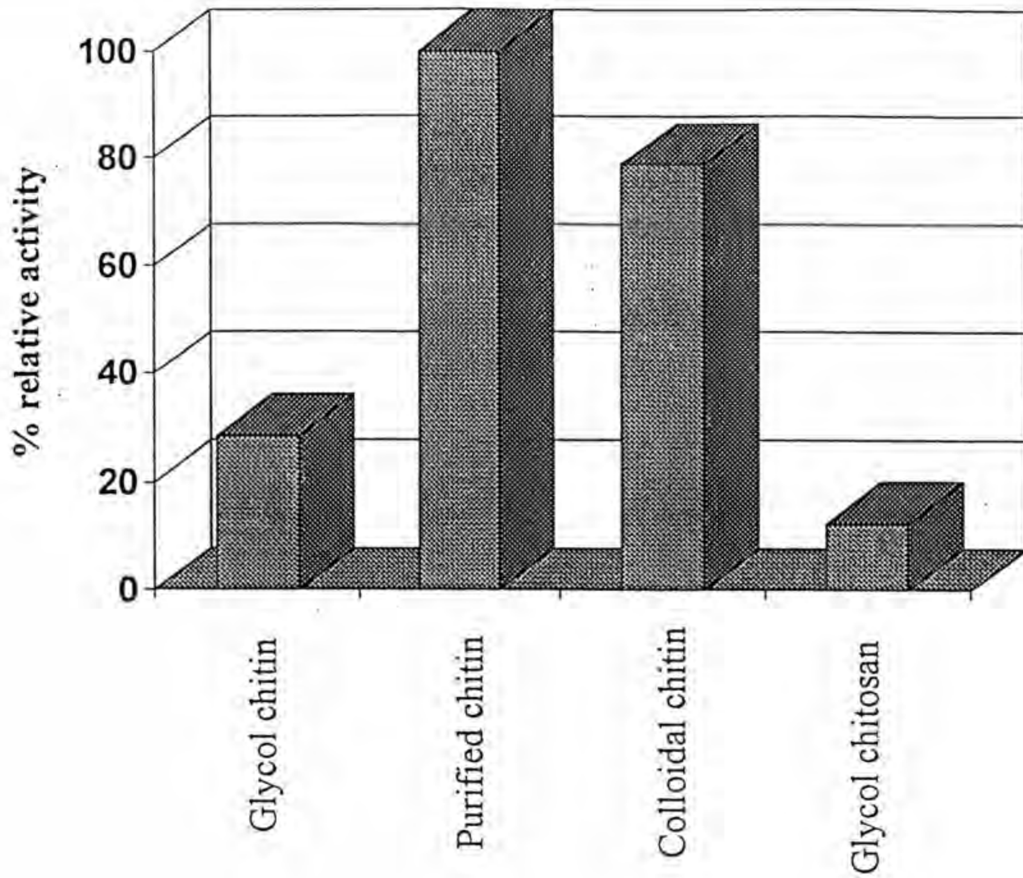

Figure 21 Substrate specificity test

Partial purified pericarp chitinase from anled loofah fruit was tested for substrate specificity with chitin analog : glycol chitin, purified chitin, colloidal chitin and glycol chitosan.

adding different substrate analogs. Three substrates analogs (purified chitin, colloidal chitin, glycol chitin and glycol chitosan) were tested. Figure 21 shows the % relative activity of the enzyme in the presence of each analogs.