

*Chapter 3: Biotechnological
applications: possible utility of the
proteases from *Synadenium grantii*.*

INTRODUCTION

Proteases have a wide range of application in industries manufacturing food, detergents and pharmaceuticals. Other areas where proteases are used include the leather, waste treatment and chemical industries. Proteases are also used in the recovery of protein from animal remains, particularly bones, thus enabling the effective utilization of the same. The rate of hydrolysis of muscle fibre and connective tissue proteins like collagen and elastin, by plant proteinases is greater than microbial proteases (1).

Proteases, along with amylases and lipases, also constitute essential active ingredients of detergent formulations. Stability in the alkaline range and compatibility with detergents are the desirable attributes of a protease used in detergency. Similarly, proteases also have medicinal properties e.g. alkaline collagenases are used in the preparation of slow-release drugs (2). Bromelain, the latex protease from *Ananas cosmosus* that exhibits anti-inflammatory and tumor inhibition properties is yet another protease with therapeutic potential (3).

This chapter explores the properties of the two isolated proteases 'a' and 'b' that could enable their utilization in research as well as in food and detergent industries. Thermal and pH stability are among the desirable characteristics of the proteases, as discussed earlier. They also exhibit a remarkable tolerance to many of the known protease inhibitors.

Proteinase k and pronase are widely used in molecular biology for the removal of proteins during the isolation of nucleic acids (4-7). The search for more economic substitutes for these enzymes persists. Nagarse, for instance, is reported to be equally effective as proteinase k in the removal of protein contaminants (8). Owing to certain properties of the *S. grantii* proteases such as thermostability, resistance to metal chelators and SDS, the possibility of utilizing them as proteinase k substitutes in the isolation of genomic DNA was explored.

Papain, one of the earliest known plant latex proteases, has meat-tenderizing properties, which are of immense commercial significance (4). Other latex proteases such as bromelain and ficin have also been widely used in meat tenderization, since the last 4-5 decades (1). Likewise, the meat-tenderizing properties of the *S. grantii* latex proteases were also investigated in the present study.

The feasibility of using the *S. grantii* proteases as an ingredient of detergents was also investigated. The response of the proteases to the action of different additives, which may occur as ingredients of various formulations, was also studied.

MATERIALS

All buffers and chemicals used were of reagent or analytical grade. Commercially available meat samples were procured. Proteinase k, CTAB, sorbitol, potassium acetate, SDS, β -mercaptoethanol, lyticase, ethidium bromide and the Lambda/Hind III marker were obtained from Sigma Chemicals Co. Ltd, St. Louis, USA. HPLC-grade isopropyl alcohol was used for nucleic acid precipitation. 100% Ethanol was prepared by the distillation of rectified spirit. Culture media constituents such as yeast extract and peptone were procured from Hi-Media labs. All culture media, chemicals and glassware used for the DNA isolation experiments were autoclaved prior to use. CTAB/NaCl was filter-sterilized before use.

METHODS

Genomic DNA isolation

From yeast

Genomic DNA was isolated from the 16-24 hour-old cells of *Saccharomyces cerevisiae* by a modification of the process described in (9) as follows: -

- Cells were harvested by centrifugation, from 50 ml of an 18-24 hour-old culture and washed with sterile distilled water once.
- Washed cells were suspended in 0.5 ml sorbitol solution containing 0.9M sorbitol, 0.1 M Tris.Cl, pH 8.0 and 0.1 M EDTA.
- 0.05 ml of lyticase (stock=2500U/ml) in sorbitol solution was added to the cell suspension along with 0.05 ml of 0.28 M β -mercaptoethanol and vortexed gently to mix. The mixture was incubated at 37°C with shaking at 50-75 rpm for 60 min. After 60 min, the suspension was examined microscopically for determining the extent of spheroplast formation.
- Protoplasts were recovered by centrifugation at 3500 rpm for 10 min.
- Supernatant was discarded and the pellet was resuspended in 0.5 ml of lysis buffer (50 mM Tris.Cl pH 8.0, containing 20 mM EDTA).

- Equal activity units of the *S.grantii* proteases and proteinase k were added and the reaction mixture incubated at 60 °C for 60 min. Controls devoid of enzyme treatment were also set up.
- 10% SDS (0.05 ml) was also added and mixed thoroughly to a final concentration of 0.5% (w/v) by repeated inversions and incubated at 65 °C for 20 min.
- 0.02 ml of potassium acetate (5.0 M) was added and mixed thoroughly, after which the tubes were incubated on ice for 30 min. The resulting semi-solid suspension was centrifuged at 15,000 x g for 10 min at 4 °C.
- Supernatant was treated with 0.6 volumes of isopropyl alcohol for the precipitation of nucleic acids.
- Nucleic acid pellet was dissolved in 0.1 ml TE buffer and treated with 0.05 ml of RNase A (10 mg/ml) at 37 °C for 60 min.
- Solution treated with a TE-equilibrated mixture of phenol: chloroform (1:1) to remove the RNase. DNA was precipitated from the resulting supernatant with 0.6 volumes of isopropyl alcohol, followed by washing with 70% ethanol to remove any remaining salts in the mixture.
- DNA pellet was dissolved in TE buffer.
- 4-5µg of DNA was electrophoresced on a 0.7% agarose gel and subjected to staining with ethidium bromide by the method given in (10) and the spectra of the DNA samples read, using a Cary UV spectrophotometer.

From bacteria

Genomic DNA was isolated from an overnight culture of *E.coli* cells by the method described in (11) with slight modifications as follows:-

- Liquid culture (5.0 ml) was inoculated with *E. coli* and grown till saturation (18-24 hours).
- 1.5 ml of culture was centrifuged in order to yield a compact pellet; supernatant was discarded.
- Pellet was resuspended in 0.5 ml of TE buffer. 0.03 ml of 10% SDS was added for lysis of cells, followed by the addition of 0.08 ml of suitably diluted proteases (with an equal number of proteinase K units added to the positive control). Negative control consisted of

0.08 ml sterile water, instead of enzyme. Reaction mixtures were incubated at 37°C for 60 min.

- To the resulting viscous solution, 0.1 ml of 5.0 M NaCl was added and mixed thoroughly, to enable the removal of the denatured protein and cell wall debris.
- 0.08 ml of CTAB/NaCl solution was added, mixed and incubated for 10 min at 65°C.
- 0.7-0.8 ml of chloroform was added, mixed and centrifuged at 15,000 x g for 10 min in order to facilitate the removal of CTAB-protein-polysaccharide complexes that formed a white interface.
- Supernatant fraction was treated with an equal volume of phenol: chloroform and centrifuged at 15,000 x g for 10 min. The resulting aqueous supernatant was treated with 0.6 volumes of isopropyl alcohol for precipitation of the nucleic acids.
- Nucleic acids were pelleted by centrifugation at 15,000 x g for 10 min, after which the pellet was washed with 70% ethyl alcohol and respun. Traces of alcohol were removed from the pellet *in vacuo*. The pellet was dissolved in 50 µl TE buffer.
- 2-3 µl of RNase (10 mg/ml in TE) were added to the suspended nucleic acid pellet and incubation performed at 37 °C for 60 min.
- DNA was repelleted using isopropyl alcohol. 70% alcohol wash and removal of excess alcohol *in vacuo* was performed as before and the DNA suspended in minimal volume of TE buffer.
- Purity of the DNA was determined by agarose-gel electrophoresis and stained as described for yeast.

Meat tenderization properties

A known quantity of meat tissue was homogenized, using a mortar and pestle. The tissue homogenate was made up to 50 ml with distilled water. The suspension was centrifuged in a Beckman JA-20 rotor in order to precipitate the insoluble pellet and remove much of the soluble fraction. The pellet was washed 3-4 times with water (to remove the adherent peptides) until the supernatant yielded a ninhydrin value of 0.01-0.05. The pellet was incubated, with the aqueous latex extract (concentrated by ultra-filtration) at 37 and 50 °C. The reaction mixture was continuously stirred on a magnetic stirrer. At specified time intervals of 0, 5, 10, 20, 30, 40, 60, 120 and 180 min, 0.5 ml aliquots of the mixture were withdrawn (in triplicate) and precipitated with 10% TCA. The proteolytic digestion was monitored by the measurement of the amino-nitrogen released by the ninhydrin assay method

(12). Controls consisted of the homogenate incubated without the enzyme and the enzyme extract alone incubated for the same time period under the same temperature conditions in order to measure the blank hydrolysis, if any. The amount of N-terminal nitrogen released was plotted against time. The activity of the *S. grantii* enzyme extract was compared with that of papain.

Wash performance in detergency

Swatches of size 8cm x 8 cm were cut from the artificially soiled fabric Wfk-Testgewebe GmbH, which contained the chocolate-milk stain. The initial reflectance of the swatches was read using a Gretag Macbeth Colour Eye 7000 A spectrophotometer at 460 nm. The wash solution consisted of distilled water containing the crude enzyme extract at different concentrations. Using a cloth: wash liquor ratio of 1: 25 (w/w), the swatches were placed in the pots of the launderometer (three replicate swatches per pot). After a period of soaking for 30 min in the washing solution, the swatches were washed for 15 min in the launderometer, at room temperature, maintaining a speed of 45 rpm, 9 balls per pot. The washed swatches were rinsed thrice with tap water, maintaining the same cloth: liquor ratio after which they were spin-dried. The final reflectance of the swatches was measured at 460 nm and the change in reflectance was plotted as a function of enzyme concentration. A control devoid of enzyme was also set up, under the same conditions.

INFLUENCE OF ADDITIVES/COMPOUNDS USED IN INDUSTRY ON THE PROTEOLYTIC ACTIVITY OF THE ENZYMES

Detergent additives

The effect of commonly used stabilizer compounds such as tri-sodium citrate, di-sodium tetraborate, diacids, diols and polyols used in liquid detergent formulations was tested at different concentrations against the proteases 'a' and 'b', with the objective of determining the stability of these proteases towards them. Similarly the effect of hydrogen peroxide, which is used as a component of powder formulations, was also studied.

Pharmaceutical additives

Diluents, binders, disintegrators and wetting agents are compounds used as pharmaceutical additives. Essentially excipients for compressed tablets, it is important that when enzymes are used as medicaments, they remain unaffected by these additives. The effect of these pharmaceutical additives were tested against the amino peptidase isolated from

Aspergillus japonica (13) in order to ascertain the feasibility of using the peptidase as an ingredient in pharmaceutical formulations. In our present study also, we have analyzed the response of the *S. grantii* proteases to some of these pharmaceutical additives.

Diluents

Diluents are finely powdered solids, which are mixed with medicaments in order to bring up the weight of tablets to be compressed up to a certain minimal level. They are essential additives in any tablet composition where the quantity of the medicament is small. Examples of commonly used diluents include sugars (e.g. dextrose, lactose, sucrose), starches, cellulose and inorganic compounds such as calcium carbonate, calcium lactate and tricalcium phosphate (14).

The crude enzyme powder (10 mg/ml) was incubated at 37 °C and 60 °C with 1% solution of various diluents used in the pharmaceutical industry as described in (13) for a period of 60 min. The residual activity was measured against azocasein. Positive controls consisted of the enzyme, devoid of diluent, while negative controls containing 1% solution of the diluent, but devoid of enzyme, were also set up.

Binders / disintegrants

Binders impart uniformity of size, hardness and compressibility to tablets. . Finely powdered materials are converted into granules by using binding agents e.g. gelatin, sugars, sorbitol, cellulose derivatives. Disintegrants are responsible for causing the compressed tablet to break, when the same is placed in aqueous media. Thus most of these compounds (e.g starches, alginic acid, kaolin, tragacanth) hydrate and swell up in contact with water, whereby pressure is exerted on the tablet, causing it to disintegrate, thus facilitating the release and uptake of the active medicament.

Different concentrations of compounds having binder properties were tested for their effect on the crude protease. Stock solutions of the compounds were prepared in water or a suitable solvent and tested at the final concentrations used in pharmaceutical formulations as given in (14). Crude enzyme powder (10mg/ml) was incubated with compounds at the various concentrations at 37 °C for 60 min, after which the residual activity against azocasein was measured. Controls devoid of enzyme/ binders were set up for comparison.

Wetting agents

'Wetting' is a term that denotes the spreading of a liquid over a solid surface (14). Among the wetting agents that were tested against the aminopeptidase in (13) were isopropyl alcohol, ethanol and acetone. In the present study also, the effect of these compounds were determined against the *S. grantii* proteases.

Aliquots of 5 μ l from a solution of the crude enzyme powder (20 mg/ml) were assayed against azocasein in a reaction volume of 0.5 ml that contained 0.4 ml of the buffered substrate and 95 μ l of each of the wetting agents at 30%, 60% and 90% concentration. The assay was performed as described earlier. Controls devoid of enzyme/wetting agent were also set up for reference.

Influence of other proteases

The *S. grantii* proteases were incubated with other proteases such as chymotrypsin, trypsin and papain under the conditions optimal for the activity of these proteases in order to ascertain whether the *S. grantii* proteases were acted upon by the other proteases. The combined caseinolytic activity of the *S. grantii* proteases with papain was also estimated.

RESULTS AND DISCUSSION

Protein removal during genomic DNA isolation

Genomic DNA is extracted from different cells for the purpose of constructing DNA libraries. DNA is associated with histone proteins in vivo forming the nucleosome. Isolation and purification of DNA necessitates the removal of all associated molecules including RNA (which is removed by treatment of the nucleic acid pellet with ribonuclease A) and proteins. Protein removal is usually achieved by treatment of the nucleic acid pellet with a non-specific protease like proteinase k, which is capable of cleaving native protein (15). As evident from the previous chapter, the *S. grantii* proteases were proved capable of hydrolyzing a wide range of substrates. Like proteinase k, the *S. grantii* proteases also displayed good activity at 60 °C and pH of 7.5-8.0 and were unaffected by EDTA or metal ions.

In the presence of 0.5% SDS, 70-75% of the initial activity was displayed by 'a' and 'b' following a pre-incubation of the proteases with different concentrations of SDS for 30 min prior to the assay. However at higher concentrations, a gradual decline in activity was observed and at 1% concentration of SDS, only 55% and 64% of the original activity were displayed by 'a' and 'b', respectively. The semi-purified enzyme fraction also exhibited a

similar response to SDS. Proteinase k, assayed against azocasein, under identical conditions, also exhibited only 62% of its initial activity in the presence of 1% (w/v) SDS (Fig 3.1).

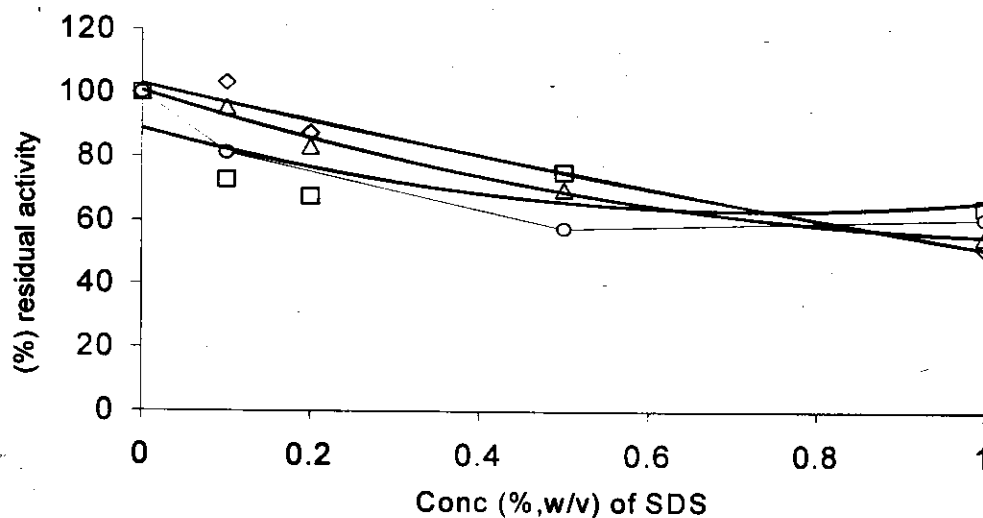


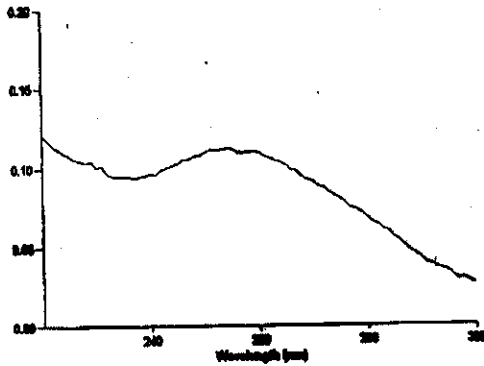
Fig 3.1: Effect of SDS on the *S. grantii* proteases

Proteases 'a' and 'b' (2.0 µg) were incubated at 37°C, pH 7.5 for 30 min in a total volume of 0.1 ml in SDS at final concentrations of 0.1-1.0% (w/v). Thereafter the residual activity of the proteases was examined against azocasein. Controls consisting of 0.1-1.0% (w/v) SDS devoid of enzyme were also set up under the same conditions and assayed against azocasein for estimating the absorbance increase resulting from any denaturation of substrate caused by SDS. The activity of the semi-purified protease (eluted from Sephacryl S-200) as well as that of proteinase k in the presence of 0.1-1.0% (w/v) SDS was also measured under the same conditions. Activity of semi-purified fraction (◇); a (□); b (Δ); proteinase k (○)

Since the *S. grantii* proteases retained 75% of their activity in the presence of 0.5% (w/v) SDS, their performance in the isolation of genomic DNA was investigated. The objective of these experiments was to determine whether the *S. grantii* proteases could be utilized as a substitute for proteinase k. Thus genomic DNA was isolated from yeast and bacterial cells (as described under Methods), using the purified protease fractions isolated from *S. grantii*. These DNA preparations were compared with those obtained by treatment with proteinase k.

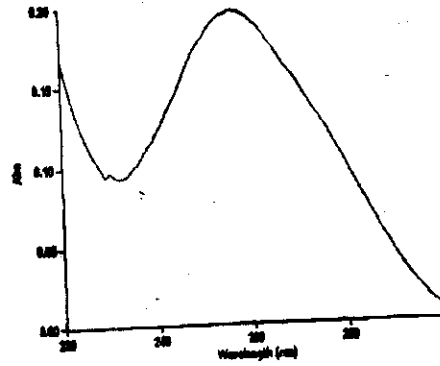
The *Saccharomyces cerevisiae* PMY strain was used for the extraction of DNA. The spectrum of the DNA samples extracted by each treatment was obtained using a Cary UV spectrophotometer (Fig 3.2).

i) Control (untreated)



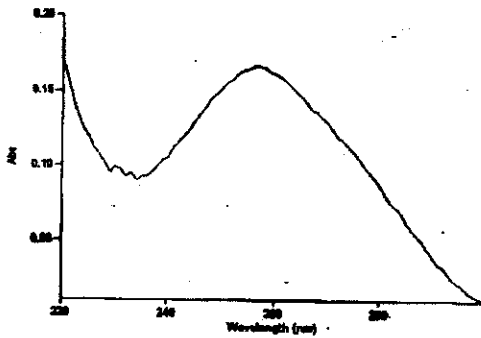
$A_{260/280}$ ratio: 1.48
DNA content = 0.2mg/ml

ii) Protease 'a'-treated



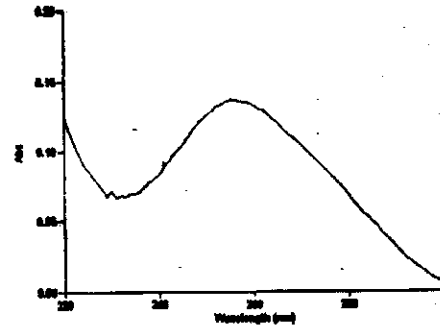
$A_{260/280}$ ratio: 1.96
DNA content = 0.15 mg/ml

iii) Proteinase-k treated



$A_{260/280}$ ratio: 2.01
DNA content = 0.16mg/ml

iv) Protease-b treated



$A_{260/280}$ ratio: 1.92
DNA content = 0.14 mg/ml

Fig 3.2: Extraction of DNA from *Saccharomyces cerevisiae*

DNA was isolated and purified from *Saccharomyces cerevisiae*. The spectra and the ratio of absorbance at 260 nm to 280 nm of each sample was measured and compared. For these analyses, 2 μ l of each DNA sample was diluted to 1000 μ l with sterile water and read against a blank consisting of 2 μ l TE buffer in water. The DNA concentration (mg/ml) was calculated as = 50 x A_{260} x dilution factor

It is evident from these figures that proteolytic treatment has a marked influence on improving the quality of DNA. Absence of proteinase treatment in the control was characterized by a reduction in the $A_{260/280}$ ratio, owing to protein contamination as seen in Fig 3.2 (i). Treatment with proteinase k yielded the best $A_{260/280}$ ratio, while the performance

of protease 'a' almost equalled that of proteinase k. These observations were also evident in the agarose-gel profile of the samples (Fig 3.3).

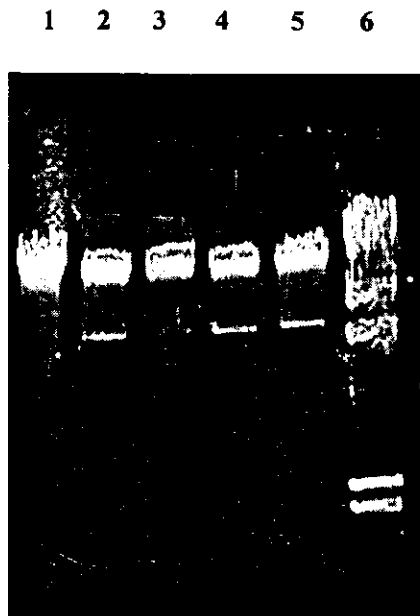
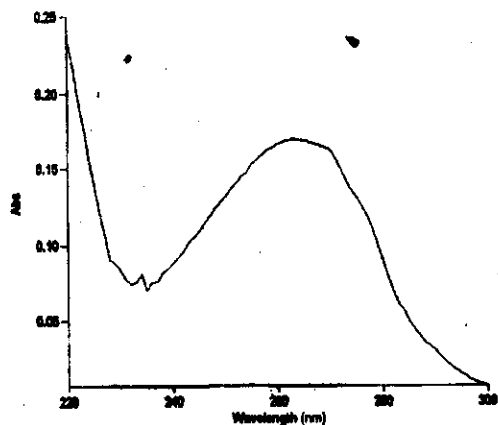


Fig 3.3: Agarose-gel electrophoresis of DNA extracted from *Saccharomyces cerevisiae* Lane 1 : Control (untreated) ; 2: Control (after phenol-chloroform treatment); 3: proteinase-k treated; 4 : protease 'a'-treated ; 5: protease 'b'-treated ; 6: Lambda/Hind III marker

The untreated control (lane 1) showed evidence of streaking, owing to the persistence of the protein contaminants as seen in the gel-profile. Treatment of the DNA with phenol-chloroform (lane 2) eliminated streaking as this process eliminates some of the protein. Treatment with the *S. grantii* proteases (especially protease 'a') yielded a DNA preparation that compared favourably with that produced upon treatment with proteinase k.

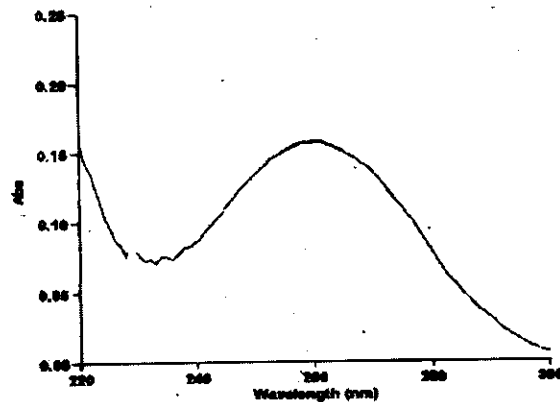
Genomic DNA was extracted from *E.coli* strain JM 109. The spectra of the DNA samples along with the calculation of $A_{260/280}$ ratio were performed as for yeast. As evident from the spectra, the yields of DNA were 1.2-1.8 times higher in the absence of proteolytic treatment; however the slight distortion in the spectrum owing to absorbance at 280 nm indicates presence of small amounts of protein. The DNA obtained by proteolytic treatment with proteases 'a' and 'b' was comparable to that obtained with proteinase k. Of the two proteases, the activity of protease 'b' against the DNA-associated proteins appeared to be marginally higher than that of 'a'. However, in the case of *E. coli*, proteolytic treatment was not mandatory, as evidenced from the higher $A_{260/280}$ ratio of the control sample (Fig 3.4).

a) Control-untreated



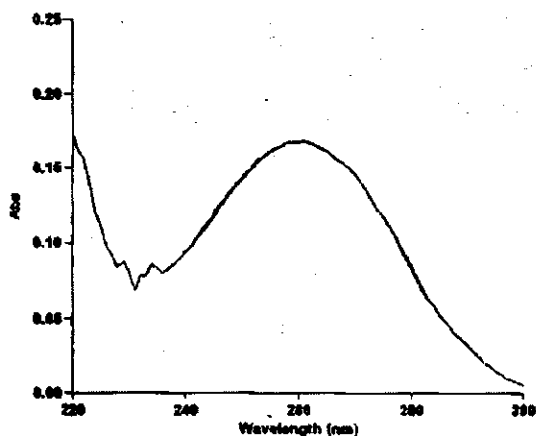
$A_{260/280}$ ratio: 2.01;
DNA content = 0.43mg/ml

c) Protease 'a'-treated



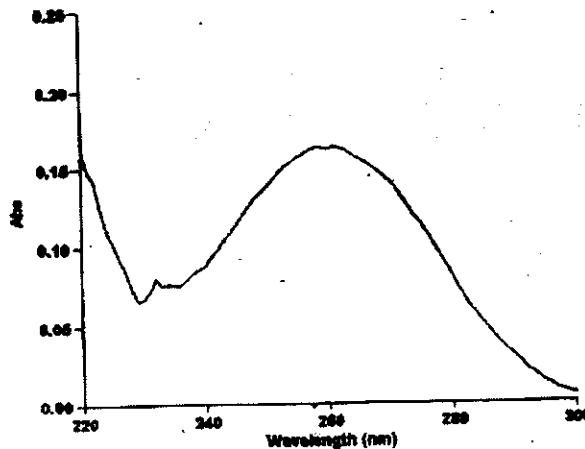
$A_{260/280}$ ratio: 1.85;
DNA content = 0.25 mg/ml

b) Proteinase k-treated



$A_{260/280}$ ratio: 1.96
DNA content = 0.35 mg/ml

d) Protease 'b'-treated



$A_{260/280}$ ratio = 1.91
DNA content = 0.32 mg/ml

Fig 3.4: Extraction of DNA from *E. coli*

DNA was isolated and purified from *E. coli*. The spectra and the ratio of absorbance at 260 nm to 280 nm of each sample was measured and compared. For these analyses, 5 μ l of each DNA sample was diluted to 1000 μ l with sterile water and read against a blank consisting of 5 μ l TE buffer in water.

The DNA concentration (mg/ml) was calculated as = 50 x A_{260} x dilution factor

The DNA samples were electrophoresced on a 0.7% agarose gel. (Fig 3.5).

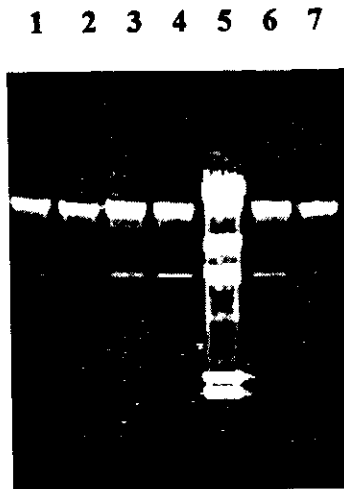


Fig 3.5: Agarose-gel electrophoresis of the DNA samples

Lanes 1: Control; 2: Proteinase k-treated; 3& 6: 'a'-treated; 4&7:'b'-treated; 5: Lambda/Hind III marker.

The gel profiles of the samples also did not seem to be much different from one another. This indicated that the protein contaminants present in the untreated control probably did not cause much interference in the gel-profile, though they produced a minor distortion, when viewed in the spectrum profile.

Meat-tenderization

Plant proteases such as papain have found extensive application in the meat processing industry as mentioned earlier. It was therefore thought worthwhile to investigate the usefulness of the *S. grantii* proteases for this purpose. The performance of papain was also monitored under the same conditions, in order to facilitate comparison with the *S. grantii* proteases.

a) Chicken meat

Time-course assays revealed that the incubation of chicken meat with the latex extract (1/10, w/v) at room temperature resulted in proteolysis of the meat. Proteolytic digestion was linear for the first 60 min of incubation. Between 60-180 min a two-fold increase was observed in the activity of the *S. grantii* latex. Similar observations were made in the case of crude papain also, which yielded a total activity of 92.8 U/mg of protein (where 1U= amount of enzyme required for proteolysis leading to the release of 1 μ M of leucine per min) at the end of 60 min. The *S. grantii* latex possessed 85% of the activity of papain as measured at the end of 180 min (Fig 3.6).

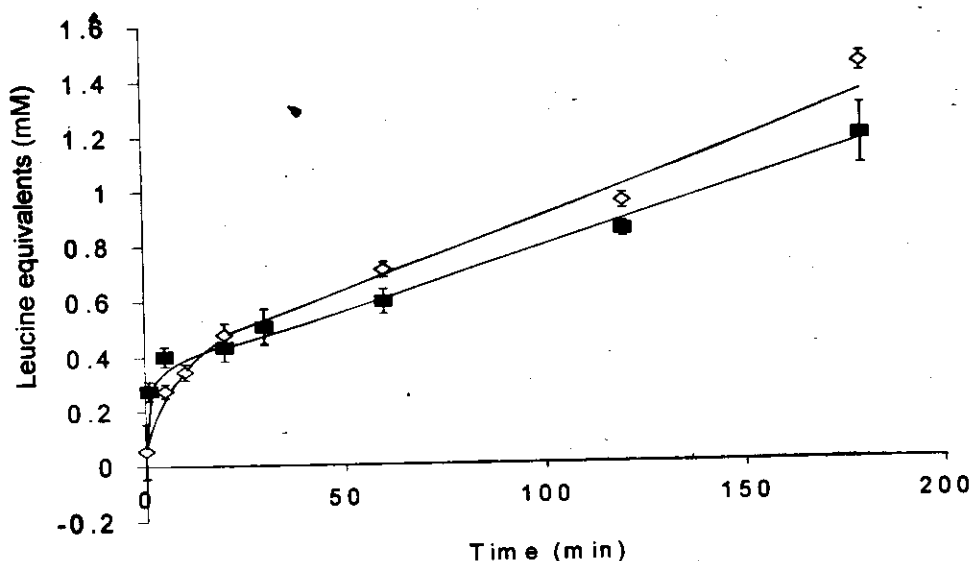


Fig 3.6: Time-course of the activity of the latex-extract compared with that of papain at 37 °C

Raw chicken (5.0 gm) was macerated with small amounts of distilled water and made up to a 50ml. The insoluble meat pellet was washed 3-4 times and incubated in 50 ml of the aqueous latex extract of *S. grantii* that contained a total caseinolytic activity of 10units/min. Incubation was performed for 180 min at a temperature of 37°C on a magnetic stirrer and the ninhydrin values of the reaction mixture estimated at regular time intervals. Digestion was also performed with crude papain, having the same number of units of caseinolytic activity. Controls devoid of enzyme were also set up. All readings represent the average of triplicates. Activity of *S. grantii*; ◇ Activity of papain: ■

At 50 °C, the activity of the *S. grantii* latex and papain showed a relatively gradual increase. Despite the fact that there is an increase in the caseinolytic activity of both enzymes between 37-60 °C, there was no considerable increase in the resultant activity of either enzyme at 50 °C, when compared to the activity at 37 °C. Papain exhibited an initial burst phase followed by a slow phase, with an activity of 73.3U/mg/min at the end of 180 min, whereas the activity of *S. grantii* latex was linear throughout and attained a value of 53.3 U/mg/min at the end of 180 min. The corresponding increase in spontaneous proteolysis with increase in temperature could be one of the factors responsible for the same. Moreover, since, distilled water was used in place of the buffer; the pH was only around 6.5, which is less than the optimal pH (7.0-7.5) for both the enzymes (Fig 3.7).

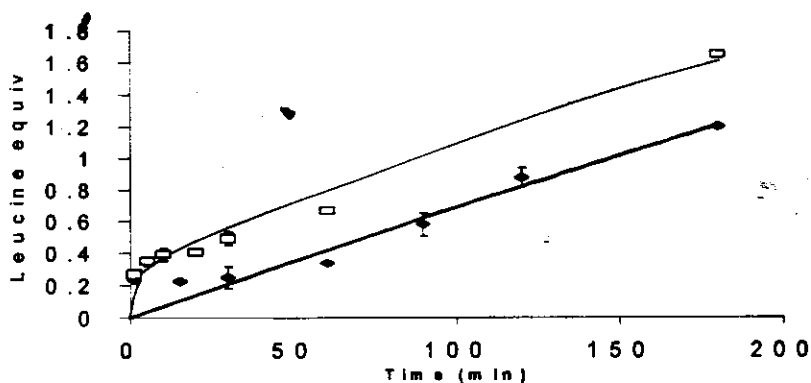


Fig 3.7: Time-course of the activity of the latex-extract against chicken meat compared with that of papain at 50 °C

Raw chicken (5.0 gm) was macerated with small amounts of distilled water and made up to 50ml. The insoluble meat pellet was washed 3-4 times and incubated in 50 ml of the aqueous latex extract of *S. grantii* (total caseinolytic activity of 10units/min). Incubation was performed for 180 min at a temperature of 50 °C on a magnetic stirrer. Protein hydrolysis was measured as given in Fig 3.4. Digestion was also performed with crude papain, having the same number of units of caseinolytic activity. Controls devoid of enzyme were also set up. All readings represent the average of triplicates. Activity of *S. grantii*: ◆; Activity of papain : □

Fish remains

The aqueous latex extract was also found capable of hydrolyzing proteins contained in fish wastes at 37 °C (Fig 3.8).

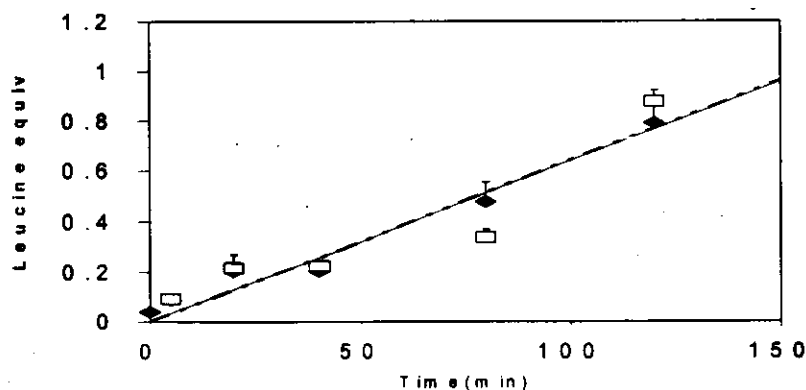


Fig 3.8: Effect of the latex extract on fish wastes (incubated at room temperature)

Fish remains (5.0 gm) were ground with distilled water and made upto 50ml. The insoluble pellet was washed as described for chicken homogenate and incubated in 50 ml of the aqueous extract of the latex containing total caseinolytic activity of 10units/min. Incubation was performed for 120 min at room temperature on a magnetic stirrer. The activity of an aqueous solution of crude papain diluted in order to contain equal units of caseinolytic activity was also monitored under the same conditions. Ninhydrin values were estimated at regular time intervals as given earlier. Controls devoid of enzyme were also set up. Activity of *S. grantii* latex: ◆; papain: □

Macerated fish remains comprising the skin, parts of the flesh and bones were incubated with the aqueous latex extract (1/10, w/v) at room temperature, with continuous stirring as described under Methods. Aliquots of 0.5 ml were withdrawn in triplicate and assayed by the ninhydrin method. The proteolysis of fish remains showed a linear increase against time during the entire 120 min of the assay with a total enzyme activity of 52.6 U/mg/min for the *S. grantii* latex. Similar observations were made in the case of crude papain (used for comparison), which exhibited an activity of 58.0U/mg/min

b) Mutton

The *S. grantii* latex also cleaved linkages in mutton homogenate, exhibiting only 56% of the activity of papain (Fig 3.9).

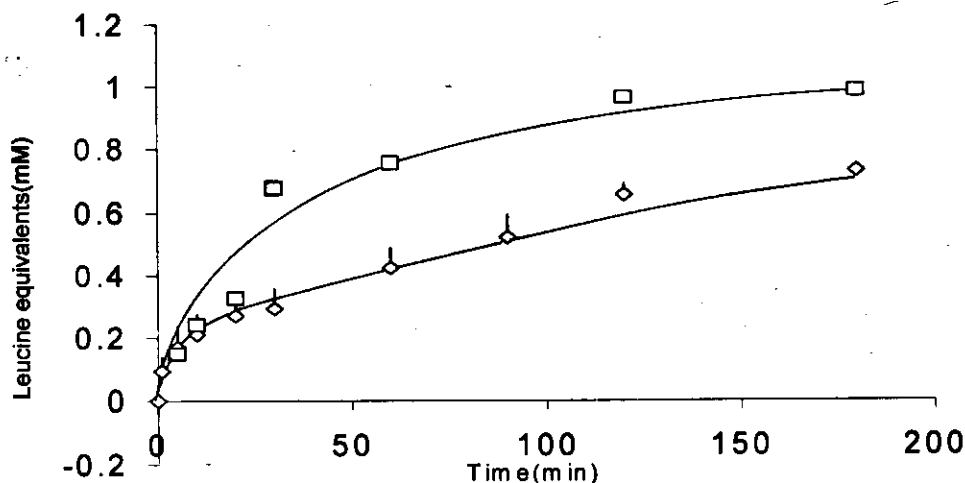


Fig 3.9: Effect of latex extract of *S. grantii* against mutton tissue (compared with papain)
Mutton (5.0 gm) was macerated with small amounts of distilled water and made upto 50ml. The insoluble meat pellet obtained by centrifugation was washed 3-4 times and incubated in 50 ml of the aqueous extract of the latex containing caseinolytic activity of 10U/min. Incubation was performed for 180 min at 50°C on a magnetic stirrer. The activity of an aqueous solution of crude papain diluted in order to contain equal units of caseinolytic activity was also monitored under the same conditions. Controls devoid of enzyme were also set up. Activity of *S. grantii* latex: ◇; papain: □

The initial burst of activity with papain was much higher than that of the *S. grantii* proteases. The second phase of activity seen from 30 to 180 min was also of a similar order

Enzymes in detergency

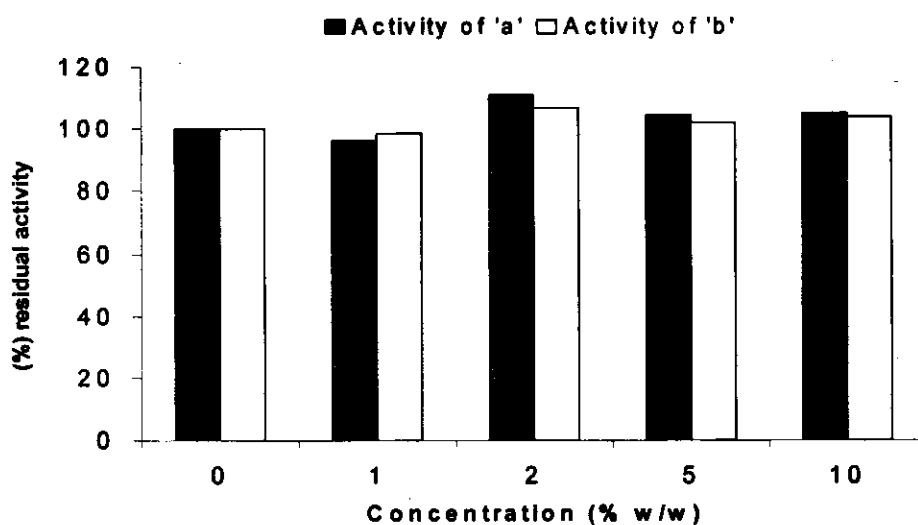
Proteases have been widely used in detergency. Properties such as stability towards alkaline pH, high temperatures and denaturing compounds are advantageous for detergent

proteases. Presence of detergents and detergent additives causing partial unfolding has been among the major reasons for the instability of various proteolytic enzymes such as papain and trypsin whose usage in detergent formulations was attempted with little success (16). As discussed in the previous chapter, the *S. grantii* proteases are capable of hydrolyzing a number of proteins, of food as well as blood origin. Therefore the possibility of their use in detergency for the removal of protein-based stains was explored. Prior to studying their wash performance, their activity under the influence of some of the stabilizer compounds used in detergent formulations (17) listed in Table 3.1 was studied (Fig 3.10).

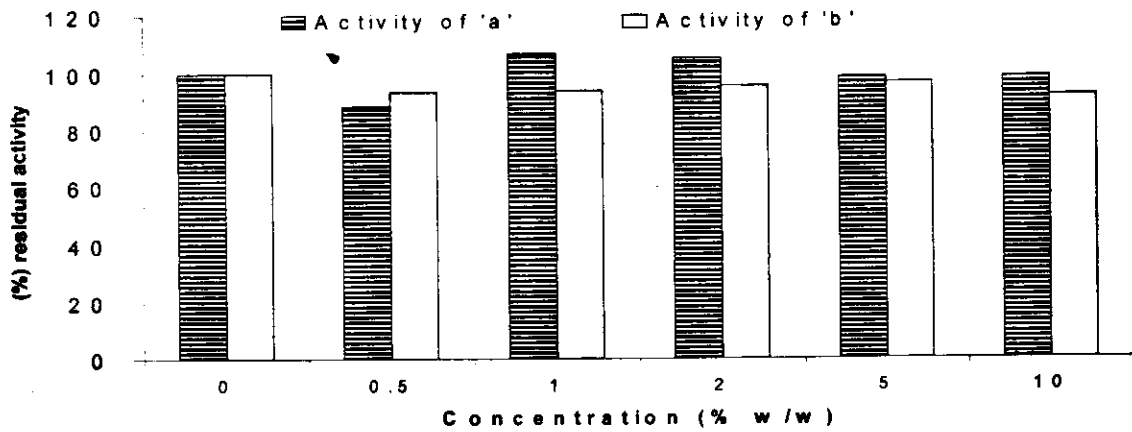
Table 3.1

Stabilizer	Concentration (% w/w)
Calcium ions	0.5-2.0
Citrates of sodium	1.0-5.0
Borates (sodium tetraborate)	2.0-5.0
Malonic acid/organic diacids	0.5-2.0
Glycerine/polyols	2.0-5.0
Propylene glycol/diols	5.0-20.0

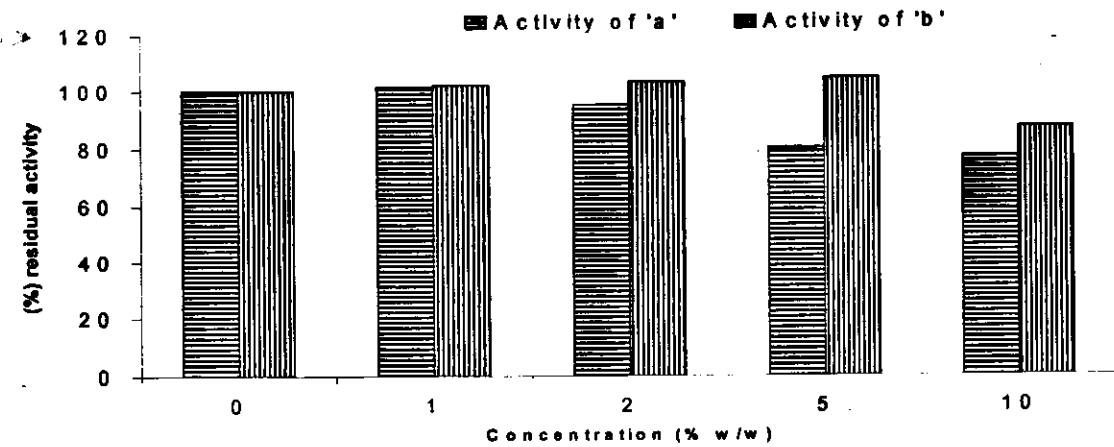
i) Sodium citrate



ii) Malonic acid



iii) Sodium tetraborate



iv) Propylene glycol

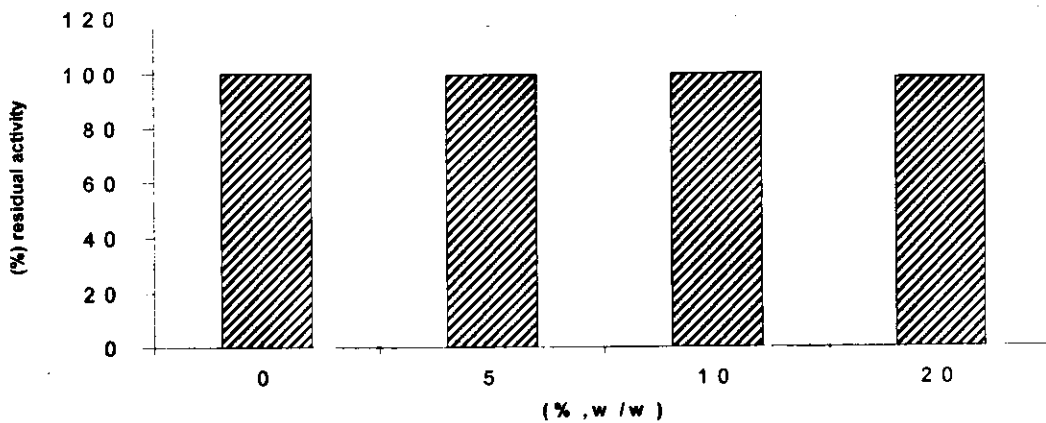


Fig 3.10: Effect of detergent additives/stabilizers on the *S. grantii* proteases

Proteases 'a' and 'b' were incubated in the presence of different concentrations of the detergent stabilizers (according to the % w/w concentrations indicated in Table 3.1) maintaining a constant pH of 8.5. Incubation was performed for 30 min at room temperature after which the residual activity of the proteases was measured against azocasein as previously described

Both the *S. grantii* proteases were unaffected by the presence of sodium citrate and malonate, at concentrations within the specified range indicated in Table 3.1. There was no loss of activity even when the enzymes were incubated at higher concentrations such as 10% (w/w) of these compounds. However, protease 'a' was found to undergo a 20% decline in activity in the presence of 5% (w/w) sodium tetraborate. Protease 'b' remained unaffected at this concentration of the compound though a 12% decline in activity was seen at 10% (w/w).

The effect of hydrogen peroxide on the proteases was also studied. As indicated in (16), storage stability of enzymes in the presence of peroxygen-bleaching systems is a major cause of concern. Both the *S. grantii* proteases were inactivated by hydrogen peroxide at concentrations of 0.1-1.0M (Fig 3.11).

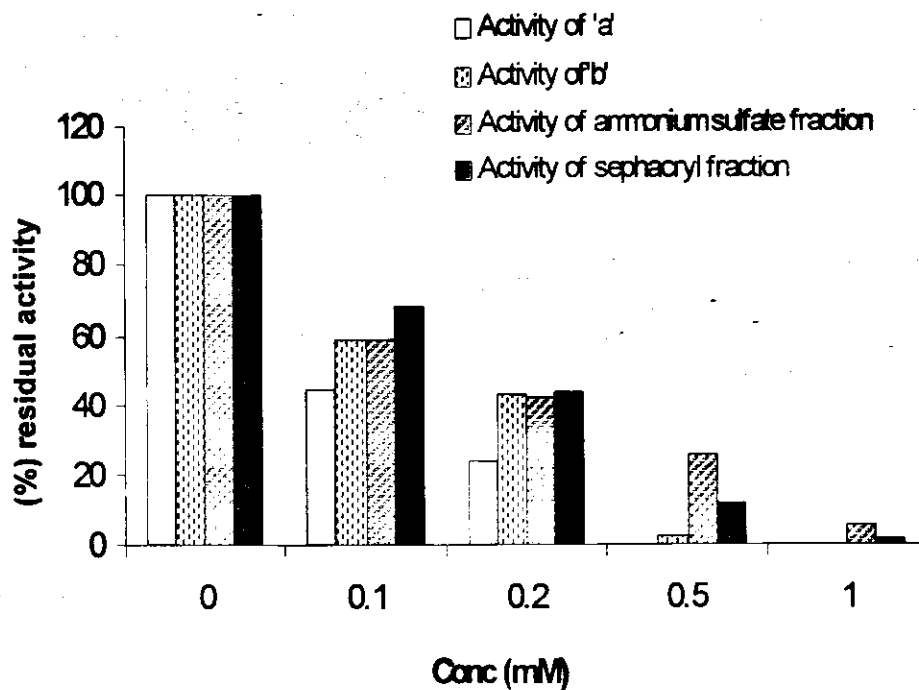


Fig 3.11: Effect of hydrogen peroxide on the *S. grantii* proteases
 Proteases 'a' and 'b' were incubated in the presence of 0.1-1.0 M of hydrogen-peroxide. Incubation was performed for 30 min at room temperature after which the residual activity of the proteases was measured against azocasein as previously described

Similar observations were made with regard to the semi-purified (Sephacryl) fraction as well as the ammonium sulfate fraction.

Wash performance in detergency

The crude enzyme powder was dissolved in water at different final concentrations ranging from 0-0.5 gm/l (ranging from an activity of 7.0-70 units/mg against azocasein, under the assay conditions) in the wash liquor. The washing process was performed as given under Methods. However, the performance of the enzyme when used alone seemed to have no special impact on the washing process as evidenced by the difference between the initial (pre-wash) and final (post-wash) reflectance values of the swatches at 460 nm. The presence of soda also did not seem to exert any influence on the activity of the enzyme (Fig 3.12).

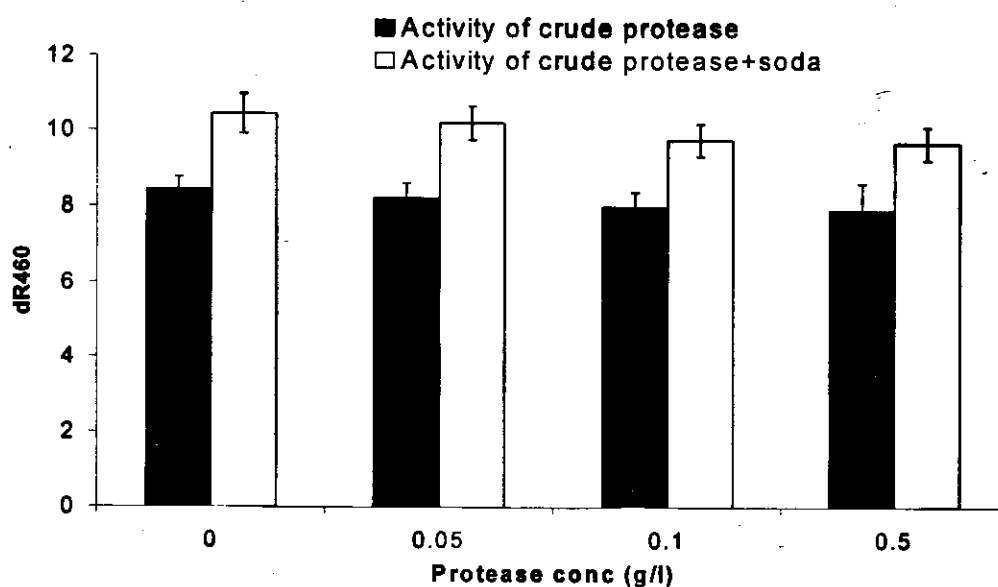


Fig 3.12: Wash performance of the crude lyophilized powder

The crude enzyme was added to the wash liquor at different final concentrations, ranging from 0-0.5 gm/l in the presence and absence of soda. The difference between the initial and final reflectance values was measured at an average of 3 replicates per pot and the same plotted as a function of the protease concentration.

The washing process was repeated, using the enzyme (0.25 gm/l) as a supplement to linear alkyl sulphonate (LAS) and a surfactant formulation developed by the Unilever research laboratories, Ltd. Only a marginal increase in activity (2 units increase in reflectance) was observed, when the enzyme was used in combination with LAS. There was no significant increase reported in the case of the surfactant either (Fig 3.13).

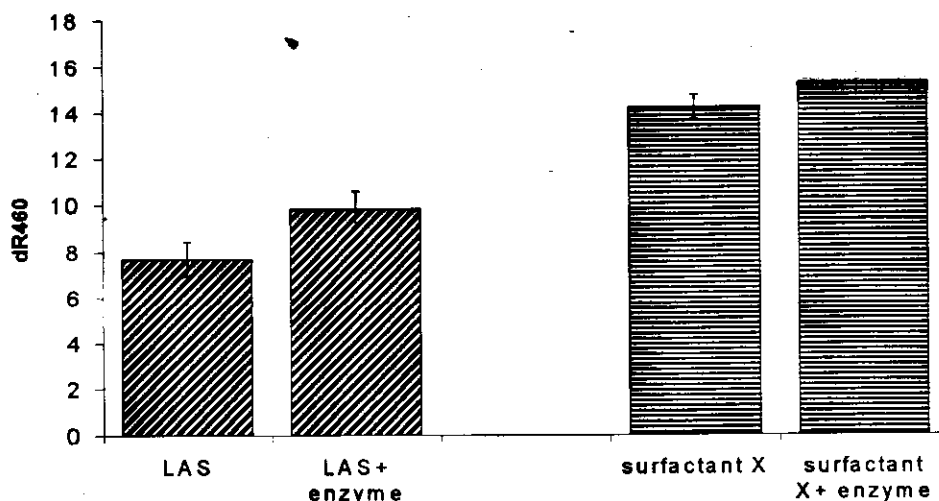


Fig 3.13: Wash performance of protease as supplement to detergent

The crude enzyme powder (0.25 mg/ml) was added to 0.5% solution of LAS and surfactant 'X' and the washing process performed as earlier at room temperature. The difference in reflectance values was plotted as an average of 3 replicates.

Effect of various proteases against the *S. grantii* proteases

The effect of trypsin, chymotrypsin and papain was determined against the *S. grantii* proteases, in order to determine their compatibility with one another, if used in a multi-enzyme preparation. Following incubation of the proteases 'a' and 'b' (2 µg) with 2 µg of trypsin, chymotrypsin and papain at 37 °C, pH 7.5, in a volume of 0.1 ml, the reaction was terminated by adding TLCK /TPCK to a final concentration of 5 mM in the reaction mixture. The residual activity (of the *S. grantii* proteases, which were unaffected by the inhibitors) was measured against azocasein as indicated in Table 3.2.

Table 3.2: Effect of other proteases against the *S. grantii* proteases

Protease	'a'	'b'
Control*	100	100
papain	95	101
chymotrypsin	100	96
trypsin	99	98

* Containing only the *S. grantii* proteases

The caseinolytic activity of the *S. grantii* latex extract in conjunction with papain was also determined as indicated in Fig 3.14.

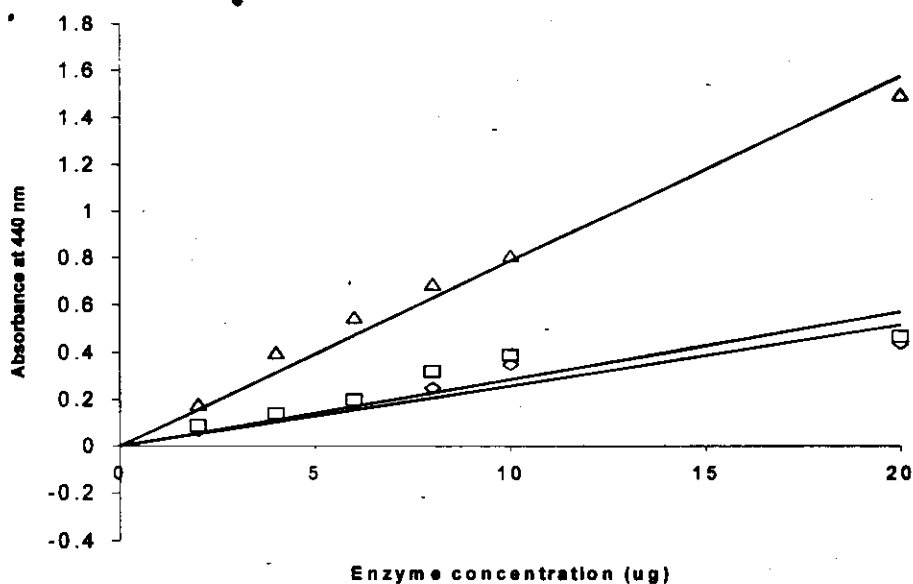


Fig 3.14: Effect of papain on the activity of the *S. grantii* latex extract

Different concentrations of the *S. grantii* latex extract and papain were assayed for their caseinolytic activity in the range of 2-20 μg at pH 7.5, 37 °C for 30 min. Thereafter, both proteases were combined and the activity of the protein mixture monitored within the same range of protein concentration. \diamond : *S. grantii*; \square : papain ; Δ :mixture of both *S. grantii* & papain.

As indicated in Fig 3.14, the mixture of the *S. grantii* latex extract and papain, yielded higher caseinolytic activity than when used individually. The activity of the protease mixture was 2-fold greater than those of the individual proteases within the concentration range of 2-10 μg and showed a linear increase upto a concentration of 20 μg .

Hence papain and the *S. grantii* proteases are capable of augmenting the action of one another. While papain served as the positive control in the meat tenderization experiments, the results of the aforesaid experiment indicates that the *S. grantii* proteases are compatible with papain and could be used in multienzyme preparations.

Effects of additives used in the pharmaceutical industry

Proteases find increasing application in therapy. Hence their importance in the pharmaceutical industry is an area with much scope for research. Ananain and related proteases from *Ananas cosmosus* are used in the debridement of burns (18-19). Likewise

curcain from *Jatropha curcas* has been shown to possess wound-healing properties (20). Use of enzymes in the pharmaceutical industry often necessitates their ability to withstand the action of different additives such as diluents, binders/disintegrators and wetting agents.

The effect of pharmaceutical additives was studied against the aminopeptidase from *Aspergillus japonica* (13), with a view to assess the stability of the protease in the presence of these compounds. This aminopeptidase was investigated for its ability to function in association with digestive proteases such as trypsin and chymotrypsin as a constituent of digestive mixtures. Given the desirable traits of the *S. grantii* proteases, similar studies were performed on them as well. As given under Methods, the semi-purified fraction was incubated with the different diluents at 37 °C as well as at 60 °C (optimal temperature of the proteases). The proteases were unaffected by the diluents at 37 °C. At 60 °C, a slight escalation of proteolytic activity (about 5-10%) in the presence of the diluents was reported though this increase is not very significant (Fig 3.15).

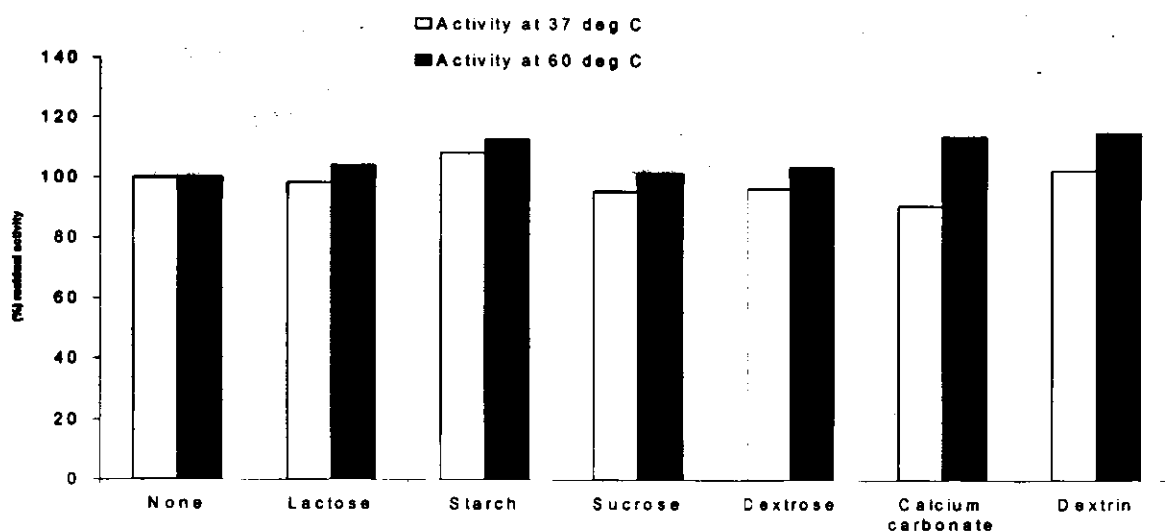


Fig 3.15: Activity of the semi-purified proteolytic fractions in the presence of pharmaceutical diluents.

The semi-purified protease preparation (5mg) was incubated with an equal weight of the various diluents at temperatures of 37 °C and 60 °C for a period of 60 min. At the end of the incubation period, the enzyme samples were diluted in 10 mM Tris .Cl ,pH 8.0 and residual activity assayed against azocasein. Activity without diluent was taken as 100%.

Likewise the activity of the proteases was unaffected by all the binder compounds, tested, with the exception of SDS as shown in Table 3.3.

While the proteases exhibited very little loss of activity at concentrations less than 1% SDS, thus enabling their use in DNA isolation, concentrations greater than 1% proved detrimental to their proteolytic activity, as indicated in Table 3.3 (i). As in the case of the aminopeptidase from *Aspergillus japonica* (13), ANOVA analysis was performed in the present study also, in order to determine the extent to which binder compounds influenced proteolytic activity.

Table 3.3: i) Effect of binders/disintegrators on the proteolytic activity

Binder/disintegrator	Residual activity at each concentration (%w/w)		
	1%	3%	5%
PEG	81.6	89.4	92.8
Gelatin	103.3	131.1	107.6
Starch	96.2	77.4	97
Sorbitol	127	108.6	107
Sodium dodecyl sulphate	55	39	17

Comparison between the critical and the calculated F-values indicate that statistically significant differences were seen among the different binders/ disintegrators at a probability of 5%. A statistically significant variation among the different concentrations was seen only in the case of SDS as shown in Table 3.3 (ii).

Table 3.3: ii) Analysis of variance

Source of Variation	SS	df	MS	F	P-value	F crit
Binder	11898.2	4	2974.5	15.3	0.0008	3.8
Concentration (%)	175.3	2	87.6	0.45	0.65	4.4
Error	1559.6	8	194.9			
Total	13633.1	14				

SS: sum of squares or variation; df:degrees of freedom; MS= SS/df or variance ; F =MS within groups/between groups

Since, different concentrations did not display any significant variations in the majority of binder compounds; the P-value was insignificant.

However, it was observed that the wetting agents such as acetone, isopropanol and ethyl alcohol inhibited the proteolytic activity, with increasing (% v/v) concentrations of the solvents as indicated in Table 3.4 (i).

Table 3.4: (i) Effect of wetting agents on the proteolytic activity

Wetting agent	Residual activity at each concentration (% v/v)		
	30 %	60 %	90 %
Acetone	90.5	69.3	73.3
Ethyl alcohol	79.8	67.1	39.5
isopropanol	49.1	25.7	24.2

Maximal decline in proteolytic activity of the *S. grantii* proteases was observed in the presence of isopropanol and ethyl alcohol.

Table 3.4 (ii) Analysis of variance

Source of Variation	SS	df	MS	F	P-value	F crit
Wetting agent	3092.1	2	1546.04	20.4	0.008	17.9
Concentration	1189.8	2	594.91	7.8	0.041	6.9
Error	303.04	4	75.7			
Total	4584.94	8				

SS: sum of squares or variation; df: degrees of freedom; MS= SS/df or variance;
F =MS within groups/between groups

As evident from Table 3.4 (ii), statistically significant differences were found among the wetting agents at 5% probability and also among the concentrations of the wetting agents at 5% probability, as per the comparison of the F_{obs} value with the specified F_{crit} value.

DISCUSSION

Preliminary studies indicate that the *S. grantii* proteases appear to have a reasonably good potential for biotechnological application. In the food industry, they could be used in

meat processing and in the production of protein hydrolysates, which constitute a rich source of amino acids.

Various proteases of microbial origin possess meat-hydrolysing properties (21-22), while among plants, papain and bromelain are the well-characterized proteases find application in meat-tenderization as already discussed. In the present study, the *S. grantii* latex proteases have been found to display good activity, which is 75-85% of that exhibited by papain against chicken meat. Among recently reported plant proteases that possess the property of meat-hydrolysis is the ginger extract (*Zinziber officinale*), which is reported to display extensive proteolysis of spent hen meat, when the latter was marinated, using different concentrations of the ginger extract (23).

Proteases are also used as a constituent of multi-enzyme preparations for hydrolyzing meat. e.g. *Streptomyces griseus* protease along with the *Candida cylindracea* lipase yielded substantial hydrolysis of bovine liver, cod fillet and chicken egg tissues (24). There is every likelihood that the stable proteolytic enzymes from *S. grantii* could also be used either singly, or in conjunction with other enzymes, especially considering the fact that the proteases are compatible with other proteases like papain, trypsin, chymotrypsin and also various compounds used as additives. The combination of papain and *S. grantii* proteases has been shown to possess 2-3 fold greater activity than when used separately.

Protein hydrolysates form an important food ingredient in a number of countries. Meat hydrolysates from pepitona (*Arca zebra*) have rich nutritional properties. Papain and bromelain were found to hydrolyze the insoluble pepitona to yield soluble peptides at concentrations of 0.3g and 0.2g enzyme/100gm of pepitona, respectively, at a temperature of 40 °C and pH of 7.0, for papain (25). Since the *S. grantii* proteases exhibit good activity under these conditions, they could also be used in studies of this nature.

Recovery of protein from fish wastes constitutes an important area in which enzymes are used, in order to facilitate the twin objectives of waste recycling as well as protein utilization. Like vegetable and milk proteins, fish hydrolysates are also nutritionally-rich ingredients (26). The present study shows that the activity of the *S. grantii* proteases is comparable to papain with respect to fish wastes. Hence, the *S. grantii* proteases could be used in the effective treatment of fish wastes.

In human diet, higher uptake of amino acids from protein hydrolysates such as di/tripeptides is observed when compared to the free amino acids. Clinical studies on human

subjects have revealed that casein and lactalbumin hydrolysates enhanced the absorption of water and electrolytes in the jejunum (27). Since the *S. grantii* proteases possess good caseinolytic activity, apart from activity against several other proteins as discussed in earlier chapters, they could be used for the generation of protein hydrolysates that are of nutritional as well as medical significance.

Ervatamins a and b isolated from the latex of *Ervatamia coronaria* that are also resistant to SBTI have been described as having potential for food industry application (28). The *S. grantii* proteases are also resistant to many of the naturally occurring protease inhibitors such as the ovomucoid and soyabean trypsin inhibitors and thus could also find similar application. The recovery of complete activity from the proteases following incubation with diluents and binders makes them capable of being used as a pharmaceutical ingredient in conjunction with these compounds as well, like the aminopeptidase from *Aspergillus japonica* (13).

Both the *S. grantii* proteases exhibited the ability to function in the presence of EDTA and 0.5% SDS (pH 8.0). At 1% concentration of SDS, however, only 50-60% of the proteolytic activity remained. Interestingly, a similar observation was made with proteinase k, when it was incubated for 30 min at this concentration of SDS and assayed against azocasein (when compared with a control devoid of enzyme, but containing 1% SDS). This is surprising, because SDS is reported to activate proteinase k (15). However it has also been stated that this apparent activation maybe the result of an alteration of the protein substrate (as observed in the case of bovine serum albumin), thus making it more prone to proteolytic attack. When assayed against Z-(Ala)₂-Nan, only 5% of the initial activity of proteinase k remained at 1% SDS concentration (29). This could explain why, in the present study also, the activity of proteinase k showed a gradual decline, as measured in the presence of controls consisting of buffered substrate incubated with SDS.

The proteases showed the ability to hydrolyze and eliminate protein contaminants during genomic DNA isolation. Their performance is comparable with that of proteinase k, which is widely used, on account of its broad substrate specificity. Despite the fact that they have narrower substrate specificity, when compared to proteinase k, the *S. grantii* proteases yielded a DNA preparation, from *Saccharomyces cerevisiae*, which was free from protein contaminants. Nagarse which is cost-effective alternative to proteinase k is another protease reported to yield DNA of high purity from bacterial cells and leukocytes (8). Similarly the *S.*

grantii proteases could also serve as good substitutes for proteinase k as evidenced from the present studies.

Present observations do not indicate potential for application of the *S. grantii* proteases in detergency, despite the fact that they are unaffected by the detergent stabilizers. Owing to their inactivation by hydrogen peroxide, they however appear unsuitable for use in bleaches, unless subjected to encapsulation. Present evidence indicates a poor wash performance, despite the fact that they exhibited good proteolysis of casein, in vitro. The reason for this remains unclear. However, it should be noted that papain, which was originally considered unsuitable for detergency, was chemically modified, using succinic anhydride, thereby yielding a molecule, which exhibited a good wash performance. The chemically modified papain was also similar to the native papain in its activity and stability towards detergents (30). Similarly new approaches for enabling the use of the trypsin family of proteases in detergency are also being attempted (16). Against the background of these developments, newer approaches involving chemical modification of the *S. grantii* proteases could also be attempted, which could enable a fuller utilization of their potential.

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