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Studies on plant gums. Proteases in neem (*Azadirachta indica*) gum

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Abstract. Proteolytic activity was detected in neem (*Azadirachta indica*) exudate gum when tested with casein and albumin as substrates. The enzyme activity was separated into two fractions by chromatography on TEAE-cellulose after EDTA treatment. Both the enzyme fractions were fairly stable to high temperatures and wide range of pH conditions. The pH optima were found to be around 6·5. Phenylmethyl sulphonylfluoride inhibited the activity of both the fractions. EDTA, *β*-mercaptoethanol, tosylamide phenylethylchloromethylketone, tosyllysine chloroimethylketone, *p*-chloromercuribenzoate and dithio*bis*-2-nitrobenzoie acid did not affect the activity of the two enzyme fractions. The two fractions had no hydrolytic action on a variety of synthetic substrates tested.

Keywords. Neem exudate gum; serine proteases; *Azadirachta indica*; proteolytic activity.

Introduction

Plant exudate gums are known to contain varying amounts of proteinaceous materials (Anderson and Henrie, 1971; Anderson *et al.,* 1972; Usha Lakshmi and Pattabiraman, 1967). Little information is available on the biological functions and activities of these proteins. It Has been suggested that the gum proteins may arise as contaminants when the exuding gums come into contact with the stem of the tree (Jones and Smith, 1949). An alternate suggestion (Jones and Smith, 1949) that the proteins might be integral components of the gum as enzymes involved in polysaccharide formation, has not been verified. Neem (*Azadirachta indica*) gum occupies a special position among plant gums in that, it contains about one-third of its weight as proteins (Anderson and Henrie, 1971), the highest concentration reported for any plant gum. Thus, neem gum is an excellent experimental material for the study of the biological activities of proteins in exudate gums. In this paper, we report the presence of proteolytic activity in neem gum. Preliminary data on the properties of the two protease fractions separated from the gum are also presented.

Materials and methods

Materials

Collection of neem gum samples, their purification and the preparation of the aqueous solutions were reported earlier (Ramakrishna Nayak and Pattabiraman,

1978). The gum samples were stored at — 5° C until use. L-Benzoyl DL-arginyl *p*-nitroanilide, ethyl N-acetyl L-tyrosinate, hippuryl L-arginine, hippuryl L-phenylalanine, L-leucyl *p*-nitroanilide, Azocoll, tosylamide 2-phenylethyl chloromethylketone, N-tosyl L-lysine chloromethylketone, DL-methionyl *β*-naphthylamide HCl, L-leucyl *β*-naphthylamide, L-alanyl *β*-naphthylamide, L-cysteinyl *β*-naphthylamide and L-prolyl *β*-naphthylamide HCl were obtained from Sigma Chemical Company, St. Louis, MO, USA. TEAE-cellulose was purchased from Bio-Rad Laboratories, Richmond, Calif., USA. *p*-Chloromercurbinzoate and 5,5'-dithio *bis*-2-nitrobenzoic acid were the products of Pierce Chemical Company, Rockford, Illinois USA. Phenylmethyl sulphonyl fluoride and vitamin free casein were procured from Calbiochem, San Diego, Calif., USA. All other reagents were of analytical grade.

Protease assay

The caseinolytic activity was measured by the procedure of Kunitz (1947) with the following modifications. The reaction mixture consisting of 20 mg of casein, 200 μmol of sodium phosphate buffer, pH 6·5 and the enzyme solution in a volume of 2·0 ml was incubated at 37° C for 2h. The reaction was stopped by the addition of 3·0 ml of 5% trichloroacetic acid. After standing for 2 h, the solution was centrifuged at 2,500 × g for 15 min. One ml of the clear supernatant solution was assayed for trichloroacetic acid soluble peptide fragments by Lowry's method (Lowry *et al.,* 1951). Suitable controls were run simultaneously. In some experiments 20 mg of bovine serum albumin was used instead of casein. One unit of enzyme activity is defined as the amount that will liberate one mg equivalent of peptide fragments under the assay conditions. Bovine serum albumin was used as a standard in the determination of proteins and soluble peptides.

Assay with synthetic substrates

The esterolytic activity using ethyl N-acetyl tyrosinate was measured as described by Sudhakar Prabhu and Pattabiraman (1977). The amidolytic activity using benzoyl arginyl *p*-nitroanilide was measured as described by Sumathi and Pattabiraman (1977). Hydrolytic activities on different naphthylamide derivatives were estimated as described by Appel (1974). Hydrolytic activities on hippuryl arginine and hippuryl phenylalanine were measured as described by Snoke and Neurath (1949) using the colorimetric method of Moore and Stein (1948) for measuring the released amino acids. Hydrolysis of leucyl *p*-nitroanilide was monitored according to Tuppy (1962). Hydrolysis of Azocoll was estimated by measuring the increase in absorbance at 580 nm in the assay filtrate after treatment with the gum solutions (Ensign and Wolfe, 1965). In all these cases, the pH of the assay system was 6·5 and the time of incubation was 2 h. Protein (400–800 μg) of fraction *A* and 400-800 μg protein of fraction *B* were used in these studies.

Protein was estimated by the method of Lowry *et al.* (1951) using bovine serum albumin as Standard.

Results

During the studies on pronase digestion of neem gum, it was observed that the concentration of ninhydrin positive materials in the control system (in the absence

of pronase) increased slightly on incubation of the gum solution for 16 h at 37°C at pH 7·0 (Ramakrishna Nayak and Pattabiraman, unpublished observations). This suggested autolysis, indicating the possible presence of proteases in the gum. Further studies showed that neem gum solutions hydrolysed casein. The activity was found to be linear over a narrow range of concentration of the gum as shown in figure 1.

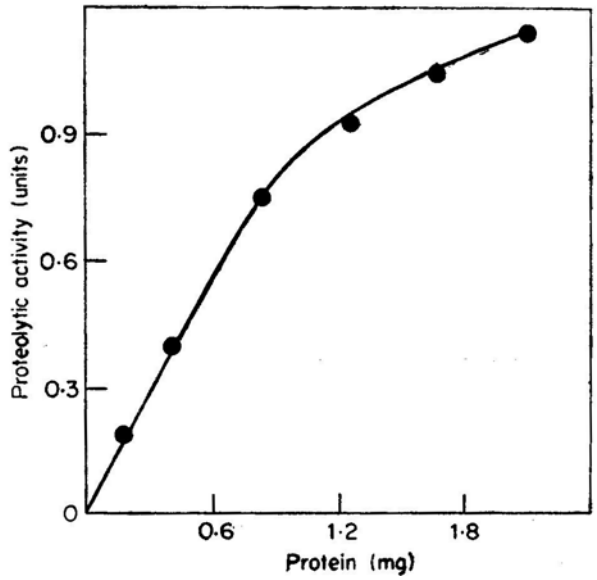

Figure 1. Effect of protein concentration in the gum on the hydrolysis of casein. The assay system was the same as described under 'materials and methods' except that varying amounts of the gum solution were used.

There was no loss in proteolytic activity when the gum solution was dialysed against water or EDTA solution. Earlier, it was observed that EDTA treatment of the gum followed by chromatography on TEAE-cellulose resulted in the separation of a carbohydrate-rich and two protein-rich fragments (Nayak *et al.,* 1979). When this technique was applied, the proteolytic activity separated into two fractions as shown in figure 2. Fraction A of proteolytic activity corresponded to the first protein peak which did not bind to the ion-exhanger and was eluted from the column with the equilibration buffer, 0·01 M sodium phosphate pH 7·0. The second peak of proteolytic activity (fraction B, figure 2) corresponded to the protein fraction eluted with 0·1 M sodium phosphate pH 7·0. The last fraction eluted with 0·1 M sodium phosphate pH 5·0, containing 1·0 M NaCl, was earlier shown to be rich in polysaccharides that was devoid of proteolytic activity (not shown in the figure). Total recovery of proteolytic activity after ion-exchange chromatography amounted to 60%. The relative ratio of caseinolytic activity in the fractions A and B were 1:2·8. Fraction A (tube No. 6, figure 2) and fraction B (tube No. 16, figure 2) were used for further studies.

The optimum pH for caseinolytic activity in the crude gum solution as well as in fractions A and B was around 6·5 as shown in figure 3. The enzyme activity was highly thermostable. No loss in activity was observed when fractions A, B

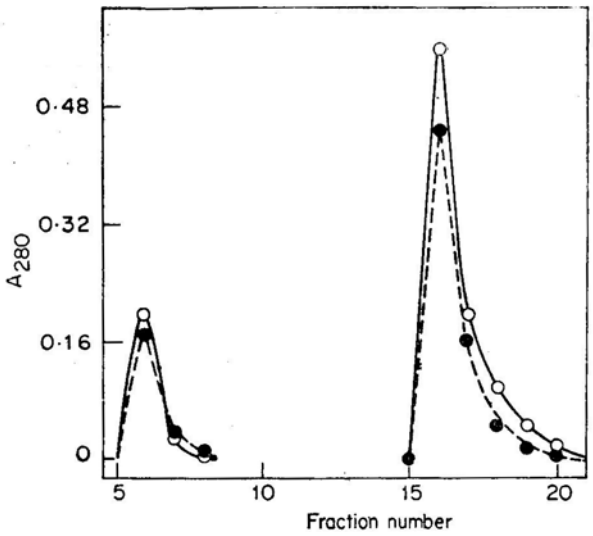

Figure 2. Fractionation of EDTA-treated gum on TEAE-cellulose:
EDTA-treated gum solution (gum solution dialysed against 50 vol of 0·01 M EDTA for 6 h twice, followed by dialysis against 50 vol of water for 8 h twice) containing 60 mg of carbohydrate and 33 mg protein was applied to a TEAE-cellulose column (1·8 × 20 cm, bed vol 50 ml) equilibrated with 0·01 M sodium phosphate buffer pH 7·0. The column was eluted with 100 ml of equilibration buffer followed by 100 ml of 0·1 M sodium phosphate buffer pH 7. Ten ml fractions were collected at a flow rate of 40ml/h. Absorbance of the different fractions were recorded at 280 nm and the fractions were assayed for caseinolytic activity as described under ' materials and methods'.O—O Absorbance at 280nm., ●– – –● Proteolytic activity.

or the crude gum solution was heated for 60 mm at 70° C or 80° G. However, nearly 50% of the proteolytic activity of fractions A and B were lost on heating at 90° C (figure 4). Heat treatment at 100° C for 5 min resulted in the complete loss of enzyme activity in both the fractions. The proteolytic activity in fractions A and B were also found to be stable to a wide range of pH conditions as shown in figure 5. There was appreciable loss in proteolytic activity only when the fractions were exposed to highly acidic conditions. Gum samples, stored at room temperature for a year did not show any appreciable loss of proteolytic activity.

p-Chloromercuribenzoate (0·lmM) or dithionitrobenzoic acid (0·1 mM) did not inhibit the proteolytic activities of fractions A and B upon preincubation for 30 min; thus fractions *A* and *B* are not sulfhydryl proteases. EDTA (0·1 mM–1 mM) did not reduce the caseinolytic activities of both the fractions suggesting that the enzymes are not metalloproteases.

Both the fractions did not display any amidolytic activity against benzoyl arginine *p*-nitroanilide or esterolytic activity against ethyl N-acetyl tyrosinate when incubated for 2 h. These data tend to suggest that proteolytic activities in the gum fractions are not probably due to trypsin (or related enzymes) or chymotrypsin type of activities.

Fractions A and B also did not exhibit any esterolytic or amidolytic activities with hippuryl arginine, hippuryl phenylalanine, leucyl *p*-nitroanilide, leucyl naphthylamide, methionyl naphthylamide, alanyl naphthylamide, cysteinyl naphthylamide

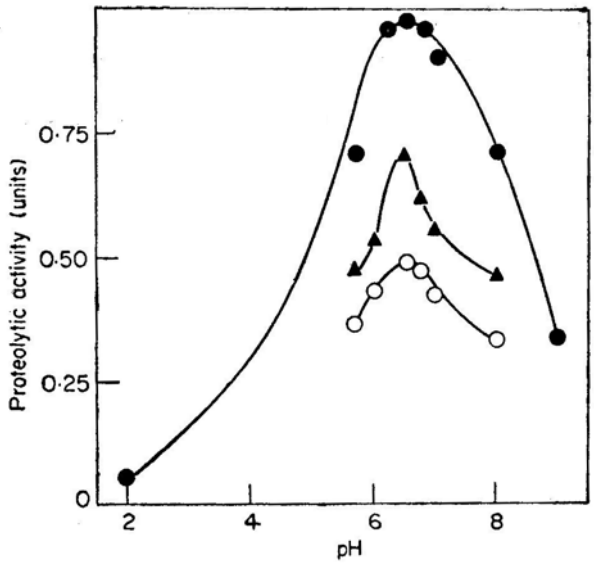

Figure 3. Effect of pH on enzyme activity.
Assay conditions were the same as described under 'materials and methods' except that 200 μmol of buffers of different pH were used in the assay system. The buffers used were, pH 2·0—KCl-HCl, pH 5·7—Citric acid-sodium citrate, pH 6·25, 6·6, 6·7, 7·0 and 8·0—sodium phosphate, pH 9·0—Tris-HCl. ●—● Crude gum O—O Fraction A. ▲—▲ Fraction B.

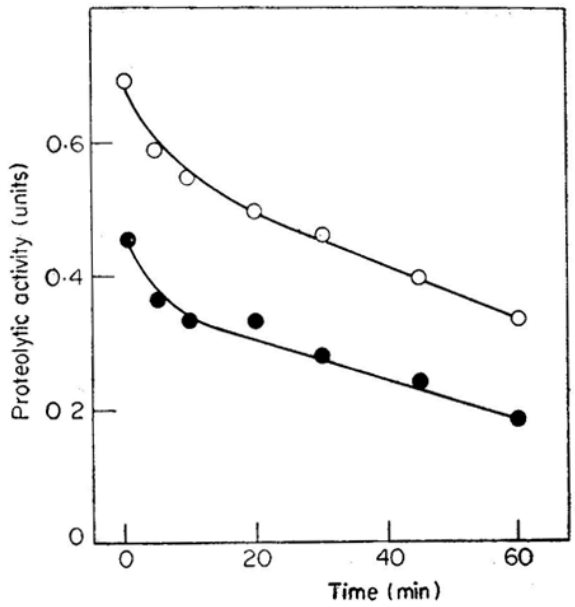

Figure 4. Effect of heat treatment at 90° C on enzyme activity:
Protein (400 μg) from fraction A and 400 μg of protein from fraction B were heat treated at 90° C for different intervals of time. The beat treated fractions were assayed for proteolytic. activity as described under 'materials and methods', ●——● Fraction A O——O Fraction B.

and prolyl naphthylamide. No hydrolytic activity was observed when Azocoll (collagen complexed with azo dye) was tested as a substrate. Even though the crude gum solution, fraction A and fraction B can hydrolyse albumin, the relative activities with respect to casein as substrate was low and was 50%, 60% and 33%, respectively.

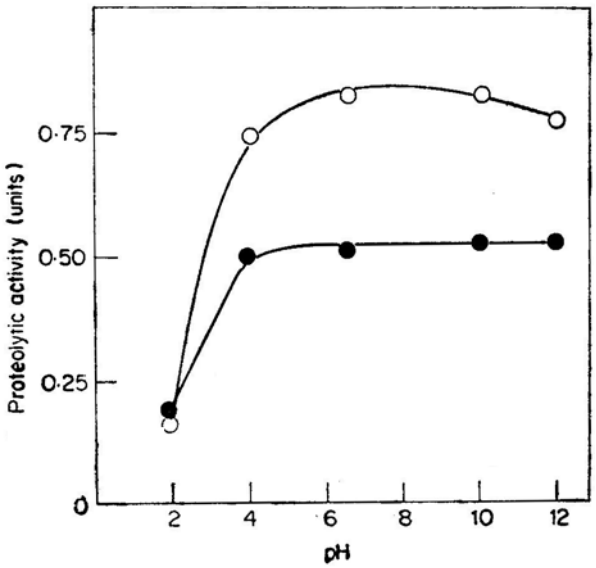

Figure 5. Effect of pH on the stability of the enzyme activity.
Protein (400 μg) from fraction A and 400 μg of protein from fraction B were incubated with 100 μmol of buffers at different pH values in a volume of 1 ml for 17 heat room temperature. After dialysing the samples against 100 vol of 0·2 M sodium phosphate buffer pH 6·5 for 9 h twice, the proteolytic activity was measured as described under 'materials and methods'. ●—● Fraction A. O—O Fraction B.

Both fractions A and B were susceptible to the action of phenylmethyl sulphonyl-fluoride as shown in table 1. The data suggest that the two fractions are serine

Table 1. Effect of phenylmethyl sulphonyl fluoride (PMSF) on fractions A and B.

Period of preincubation (min)	Caseinolytic activity (%)	
	Fraction A	Fraction B
Control (minus PMSF)	100	100
0	90	83
60	31	21
120	0	0

The enzyme solutions were preincubated with PMSF (0·1mM) for different time intervals indicated in the table in 200 μmol of sodium phosphate buffer and the reaction was initiated by the addtion of casein solution.

proteases. However, treatment with tosylamide phenylethylchloromethylketone (50μM) or tosyllysine chloromethylketone (50μM) for2h did not abolish the caseinolytic activities of fractions A and B, again indicating that the enzymes are different from trypsin or chymotrypsin.

The proteolytic activity in neem gum does not appear to be due to microbial contamination. The fresh neem gum samples used in these studies neither displayed any detectable bacterial count nor there was any mycelial fragments. In addition, an ultrafiltrate of the gum solution (filtered through membrane filter, 0·45μ) had the same amount of proteolytic activity as the original gum solution.

Discussion

These investigations establish for the first time the presence of proteolytic activity in neem exudate gum, a typical plant exudate gum. The enzyme activity was separated into two fractions by chromatography on TEAE-cellulose corresponding to the two protein components in neem gum. However, this cannot be taken as evidence to indicate that the two protein fractions in the gum represent two distinct proteases. Both the fractions were similar in many respects as far as the catalytic activity was concerned. In all probability they are isoenzymes. Alternatively, they could be artifacts due to the interaction of a single enzyme with other proteins in the gum.

The proteolytic activities in neem gum appear to be highly heat stable and also stable on exposure to a wide variation in pH conditions. The proteases appear to be neutral serine proteases, with extremely limited substrate specificities. Further studies are needed to establish the type of bonds cleaved by these proteases in protein substrates and to understand their biological role.

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