

COMMUNICATION V

Preliminary Studies of Acid Protease and its Inhibitor in Latex of *Hevea brasiliensis*

ABSTRAK

Asid protease (EC 3.4.23.-) khususnya terdapat pada fasa bawah lateks *Hevea brasiliensis* yang telah diultraempar. Enzim tersebut mempunyai aktiviti optima pada pH 3.5 dan direncat dengan kuat oleh perencat spesifik pepstatin. Kajian ini melaporkan bagi pertama kalinya kehadiran perencat protein untuk enzim tersebut pada fasa serum lateks *Hevea*.

ABSTRACT

Acid protease (EC 3.4.23-) is confined mainly to the bottom fraction of ultracentrifuged *Hevea brasiliensis* latex. The enzyme has an optimum activity at pH 3.5 and is strongly inhibited by the specific inhibitor, pepstatin. This study reports, for the first time, the presence of a protein inhibitor of the enzyme in the C serum of *Hevea* latex.

INTRODUCTION

Several serine proteases (EC 3.4.21.-) and alkaline proteases from the serum phase (C serum) and "bottom" (lutoids) fraction of *Hevea brasiliensis* latex have been isolated and described (Lynn & Clevette-Radford 1984, 1986; Low and Wiemken 1984). Acid or carboxyl protease is known to be present in the lutoid particles (Pujarniscle 1968) but to date has not been fully characterized. Other groups of proteases in *Hevea* have also not been reported. This communication describes some characteristics of acid protease isolated from the bottom fraction of *Hevea* latex.

MATERIALS AND METHODS

Cathepsin D and pepstatin were purchased from Sigma Chemical Company, St. Louis, USA. All other reagents were of A.R. quality. Haemoglobin substrate from fresh bovine blood, casein, gelatin and bovine serum albumin were prepared according to Barrett (1977).

Collection and ultracentrifugation of fresh latex (from *H. brasiliensis* clone RRIM 600) followed the methods described by Moir (1959). The bottom fraction and C serum were used as sources of the enzyme. The bottom fraction was

first resuspended in 0.1M formate buffer pH 4 containing 0.1% Triton X-100 (1 g fresh weight of B.F in 10 ml buffer) and disrupted gently in a Potter-Elvehjem homogenizer. The resulting suspension was centrifuged at 40,000 rpm (Beckman L5-65, Rotor 65) for 60 min to remove the particulate materials. The supernatant was then used as the enzyme source.

Assays for the proteolytic activity in the presence and absence of inhibitors were carried out as described by Barret (1977). Buffered enzyme (0.5 ml of bottom fraction suspension or C serum) was added into 0.25 ml 0.1M formate buffer pH 3.5 containing 0.25 ml 3% haemoglobin substrate at 37°C and incubated for 1 h. The reaction was terminated with 3% trichloroacetic acid (TCA) and then filtered. The amount of TCA soluble peptides was then determined using a modified Lowry's method. Activities were expressed as μmol tyrosine equivalents/h. Sodium formate/formic acid (0.1M, pH's 2.0-4.5) and sodium citrate/citric acid (0.1M, pH's, 3-6.2) buffers were used to investigate the effects of pHs on the proteolytic activity. Protein was determined according to the Lowry's method (Lowry *et al.* 1951), using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

The proteolytic activity against haemoglobin in the bottom fraction of *Hevea* latex showed an optimum pH of about 3.5 (Fig. 1). This highly acidic pH optimum and the strong inhibition by the specific protease inhibitor, pepstatin (Fig. 2), confirmed the presence of an acid or carboxyl (aspartyl) protease in the sample. Similar results were obtained with an authentic carboxyl protease, Cathepsin D (results not shown). Specific active site-directed irreversible inhibitors are generally used in classifying proteases into their respective groups (Fritz *et al.* 1974). Pepstatin, a pentapeptide obtained from the

filtrates of cultures of *Streptomyces* (Umezawa *et al.* 1970), inhibits competitively acid proteases in equimolar amounts and is thus used to differentiate this group of enzymes from other proteases. It can also be used to quantify the active forms of such enzymes by active site titration (Kunimoto *et al.* 1972). The amount of active enzyme estimated by this method in crude bottom fraction extract was about 50 μg per g of bottom fraction protein. The small amount of activity detected in C serum is probably due to leaching of the enzyme from labile luteoid particles present in the bottom fraction.

Members of acid protease include the gastric pepsin, chymosin, renin and gastricsin from a number of animal species, Cathepsin D and E in the lysosomes of many cells and from many microorganisms (Kay *et al.* 1981). Several acid proteases from higher plants have also been purified and studied in detail (Huffaker & Peterson 1974). However, the abundance of this type of protease in the bottom fraction of *Hevea* latex and the ease with which it can be isolated and purified provide a very convenient system for studying intracellular protein turnover in mature higher plant tissues. Another important reason is the possibility of harnessing the latex's own acid protease in removing contaminating proteins more effectively from rubber particles. In some industrial applications, the presence of proteins on rubber particles affect certain physical properties of the final products (Southorn 1980). Proteolytic enzymes from other sources have been used but with limited success (Archer 1983).

The effect of different substrates on the proteolytic activity of acid protease at pH 3.5 is summarized in Table 1. The enzyme showed a higher affinity for freshly prepared haemoglobin compared to other substrates used in the assay. It has several similarities with Cathepsin D (Barrett, 1977), an acid protease found in animal tissues. Both enzymes are lysosomal, and showed similar pH optimum and substrate specificity. Cathepsin D, however, has been recognized to be involved in intracellular protein degradation in animal tissue. The biological role of the *Hevea* enzyme is yet to be defined but probably has analogous functions in plant.

Using haemoglobin as a substrate for the *Hevea* enzyme, however, will not measure its true *in vivo* activity. To investigate its true biological

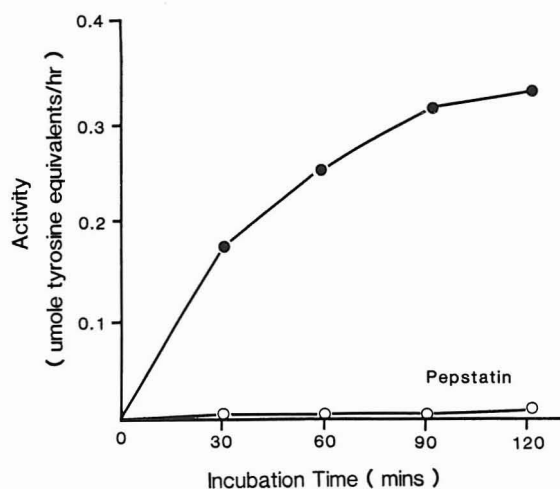


Fig. 1: Proteolytic activities in the bottom fraction and C serum of *Hevea brasiliensis* latex.

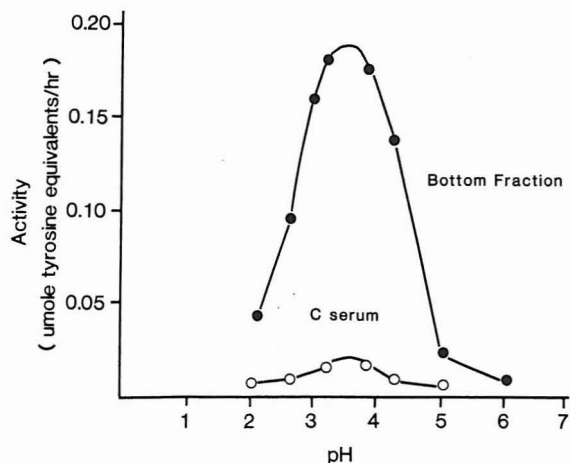


Fig. 2: Inhibition of proteolytic activity in the bottom fraction of *Hevea* latex by pepstatin.

TABLE 1

The effect of different protein substrates on the proteolytic activity of acid protease from *Hevea* bottom fraction

Substrate	Activity as % of fresh haemoglobin
Haemoglobin (freshly prepared)	100
Bovine serum albumin	12.5
Casein	32
C serum (concentrated to 3% w/v)	3
Haemoglobin + C serum (fresh, 150 ul)	77
Haemoglobin + pepstatin (1 ug)	0

The concentration of each substrate was adjusted to 3% w/v.

function, its natural protein substrate(s) should be identified and used. Concentrated C serum (3% w/v protein) was, therefore, examined as a substrate for the enzyme but was found to be inhibitory (Table 1). This suggests that an inhibitor may be present. The degree of inhibition is dependent upon the concentration of C serum (Fig. 3). The inhibitor is non dialysable and is destroyed by boiling. Salleh (1982) had shown that it is a protein of molecular weight of about 18,000D. While the presence of an alkaline

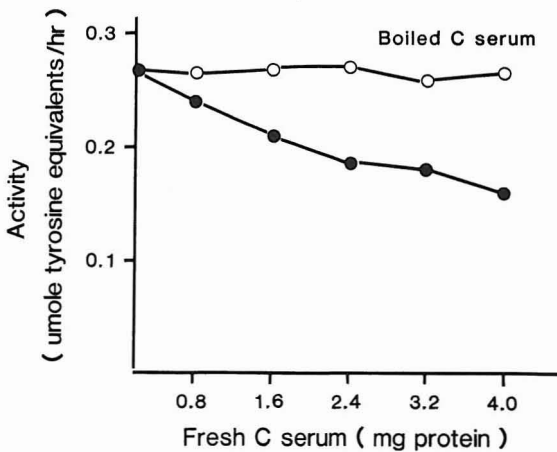


Fig. 3: Proteolytic activity of *Hevea* bottom fraction in the presence of C serum.

protease inhibitor in *Hevea* latex has been reported (Archer 1983), an acid protease inhibitor has not been described. A report on the isolation and characterization of this protein inhibitor is currently being prepared (Ampon & Salleh 1989).

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