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ROZEBOOM, HJ; BUDIANI, A; BEINTEMA, JJ

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# Crystallization of Hevamine, an Enzyme with Lysozyme/Chitinase Activity from *Hevea brasiliensis* Latex

**Henriëtte J. Rozeboom, Asmini Budiani†, Jaap J. Beintema and Bauke W. Dijkstra‡**

*BIOSON Research Institute*
*Rijksuniversiteit Groningen*
*Nijenborgh 16, 9747 AG Groningen, The Netherlands*

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Hevamine, an enzyme with both lysozyme and chitinase activity, was isolated and purified from *Hevea brasiliensis* (rubber tree) latex. The enzyme (molecular weight 29,000) is homologous to certain "pathogenesis-related" proteins from plants, but not to hen egg-white or phage T4 lysozyme. To investigate the atomic details of the substrate specificity and the cause for hevamine's low pH optimum (pH 4·0), we have crystallized two hevamine isozymes as a first step towards a high-resolution X-ray structure determination. Suitable crystals were obtained at room temperature from hanging drop experiments by vapor diffusion against 1·7 M to 3·4 M-NaCl (pH 5·0 to 9·0) for the major isozyme, and by vapor diffusion against 2·5 M to 4·3 M-NaCl (pH 5·0 to 8·0) for the minor one. Both isozymes give the same crystal morphology and space group. Their space group is $P2_12_12_1$ with cell dimensions $a = 82{\cdot}3$ Å, $b = 58{\cdot}1$ Å and $c = 52{\cdot}5$ Å (1 Å = 0·1 nm). The crystals diffract to at least 2·0 Å resolution.

Many plants infected with pathogens develop local or systemic resistance against subsequent infections (Ross, 1961*a*,*b*). The induction of this pathogen resistance was found to be correlated with the production of "pathogenesis-related" proteins (Van Loon & Van Kammen, 1970; Gianazzi *et al.*, 1970). However, subsequent investigations have revealed that at least some of these pathogenesis-related proteins can also be found in healthy plants (Fraser, 1981; Pierpoint, 1986), and are expressed constitutively (Gianazzi & Ahl, 1983). The functions of these proteins are largely unknown, although both chitinase (Legrand *et al.*, 1987) and β-1,3-glucanase activities (Kauffmann *et al.*, 1987) have been observed for some of the pathogenesis-related proteins from tobacco. Chitinase activity has also been shown to be present in other plants (Metraux *et al.*, 1989), often in conjunction with lysozyme activity (Bernasconi *et al.*, 1987). Chitinase is a glucanohydrolase directed against chitin (poly-[1,4-(*N*-acetyl-β-D-glucosamine)]), a major component of the cell wall of many fungi and of the exoskeleton of insects; lysozyme hydrolyzes the glycosidic bond between C-1 of *N*-acetyl-β-D-muramate (NAM) and C-4 of *N*-acetyl-β-D-glucosamine (NAG). Bacterial cell walls consist of poly-[β-1,4-(NAG-β-1,4-NAM)]. The function of chitinase/lysozyme in plants thus seems to be to provide the plant with a general, unspecific defense against attack by microbial pathogens and insects.

Fresh latex, obtained by tapping the rubber tree *Hevea brasiliensis* can be separated by centrifugation into three main fractions (Moir, 1959). These are a white upper layer rubber particles, an aqueous layer containing the cytoplasma from the cells of the latex vessels and a "bottom fraction" which consists of lutoids; lutoids are cell organelles with a low internal pH that may be considered as the equivalent of animal lysosomes (Pujarniscle, 1968). The major basic protein from the bottom fraction, hevamine (Archer, 1976; Tata *et al.*, 1983), appears to be homologous to a pathogenesis-related chitinase from cucumber (Metraux *et al.*, 1989) and a pathogenesis-related basic lysozyme from *Parthenocissus quinquifolia* (Bernasconi *et al.*, 1987). Two isozymes of similar molecular weights and amino acid compositions have been found in the latex:

† Present address: Bogor Research Institute for Estate Crops, Bogor, Indonesia.

‡ Author to whom all correspondence should be addressed.



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hevamine A, the most abundant isozyme and a minor fraction, hevamine B. Hevamine B differs from hevamine A probably only in the replacement of leucine by an arginine in the C-terminal part of the molecule (P. A. Jekel & J. J. Beintema, unpublished results). Hevamine has both lysozyme and chitinase activity (Tata, 1980; Tata *et al.*, 1983). The lysozyme activity of hevamine has been investigated in most detail (Tata *et al.*, 1983). The pH optimum is 4·0, rather different from the pH optima of hen egg-white and phage T4 lysozyme, which have their pH optima in the range 5·9 to 6·3. The reported molecular weight of 26,000 is also significantly different from that of hen egg white lysozyme ($M_r$ = 14,000) and T4 lysozyme ($M_r$ = 17,000). The sequence of the first 21 N-terminal amino acid residues of hevamine has been published (Tata *et al.*, 1983). No sequence homology with hen egg-white or T4 lysozyme could be found. Recent results on the further elucidation of the primary structure of hevamine (P. A. Jekel & J. J. Beintema, unpublished results) corroborate this observation. Instead, they indicate about 95% sequence identity with the N-terminal amino acid sequence of a lysozyme from *Parthenocissus quinquifolia* (Bernasconi *et al.*, 1987), which also has a low pH optimum, and about 60% sequence identity with an extracellular cucumber chitinase sequence (Metraux *et al.*, 1989).

On account of its molecular weight, combined lysozyme/chitinase activity and amino acid sequence it is clear that hevamine is a member of a new class of lysozymes, which does not seem to be related to the hen egg-white or T4 lysozymes. To investigate this new class of lysozymes/chitinases in more detail we set out to determine the three-dimensional structure of this enzyme by X-ray crystallography. A three-dimensional structure will provide us with the architecture of the active site, and might give us an explanation for the enzyme's substrate specificity and low pH optimum. Also any evolutionary relationships with T4 and hen egg-white lysozyme might become apparent on the level of the three-dimensional structure (Matthews *et al.*, 1981). In the future, hevamine mutants might be envisaged for possible application in the genetic engineering of disease-resistant plants as bacteriocides/fungicides. This paper describes the production of crystals of both isozymes of hevamine, which are suitable for a high-resolution structure determination by X-ray analysis.

Hevamine was isolated and purified from freeze dried *Hevea brasiliensis* latex bottom fraction, which we obtained as a gift from Dr A. Soedarsan, Bogor Research Institute for Estate Crops, Bogor, Indonesia. A total of 94 g of this material were homogenized in 900 ml of water, containing 0·05% (w/v) sodium dithionite to inhibit polyphenoloxidases. After centrifugation, the solution was saturated to 65% with ammonium sulfate. The precipitate was dissolved in buffer (0·04 M-borate buffer with 0·05% dithionite, pH 8·9) and dialyzed overnight against this buffer. Purification of hevamine was accomplished by carboxymethyl-cellulose column chromatography as described by Archer (1976). Two peaks with lysozyme activity were obtained, corresponding with hevamine A and B. These were desalted by a gel filtration in 0·2 M-acetic acid and freeze drying. Totals of 166 mg of hevamine A and 43 mg hevamine B were obtained. The enzyme preparations showed only one band on polyacrylamide/sodium dodecylsulfate gel electrophoresis with a molecular mass of 29,000. This is a higher value than reported by Tata *et al.* (1983), but is in better agreement with amino acid sequence studies and the molecular masses of homologous proteins (Bernasconi *et al.*, 1987; Metraux *et al.*, 1989).

For crystallization experiments the freeze dried hevamine preparations were dissolved to a concentration of 10 mg/ml in 20 mM-BES buffer (*N*,*N*-bis[2-hydroxyethyl-]2-aminoethane sulfonic acid), pH 7·0, and dialyzed against this buffer for 24 hours at 4 °C. Crystallization conditions were screened using the hanging drop method of vapor diffusion (McPherson, 1982). Protein solution (3 $\mu$l) and 3 $\mu$l of precipitant solution were mixed and suspended over a 1 ml reservoir of precipitant solution. Several precipitating agents (sodium chloride, ammonium sulfate, 2-methyl,2,4-pentanediol and polyethyleneglycol 6000) were tested at various pH values ranging from 5·0 to 9·0. Crystals of hevamine A grew within two weeks at room temperature both with ammonium sulfate (35 to 65% saturated, pH 5·0 to 9·0) and with sodium chloride (1·7 M to 3·4 M, pH 5·0 to 9·0) as precipitants. However, the crystals grown from ammonium sulfate are intergrown, and not suitable for a structure determination, but the crystals obtained with NaCl as a precipitant are single rod-shaped crystals with dimensions of up to 0·2 mm × 0·2 mm × 0·5 mm. Routinely, crystals grown at different conditions are transferred to a standard mother liquor (3·4 M-NaCl, pH 7·0) before diffraction experiments. The crystals belong to the orthorhombic space group $P2_12_12_1$ with unit cell dimensions $a$ = 82·3 Å, $b$ = 58·1 Å and $c$ = 52·5 Å (1 Å = 0·1 nm) and diffract to at least 2·0 Å resolution. Assuming one hevamine molecule of molecular mass 29,000 per asymmetric unit the crystal volume per unit mass, $V_m$, is 2·2 Å$^3$/dalton. This is well within the range 1·6 to 3·6 found to be typical for protein crystals (Matthews, 1977). Using a standard partial specific volume for globular proteins of 0·736 cm$^3$/g (Smith, 1968), the solvent content of these crystals can be calculated to be 44%. Hevamine B was crystallized in the same way as hevamine A from 2·5 M to 4·3 M-sodium chloride (pH 5·0 to 8·0). The crystals show the same morphology as the hevamine A crystals, and have the same space group and cell dimensions. No differences in the diffraction pattern could be detected in $\mu = 16°$ precession pictures so far.

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