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 to 3.4 M-NaCl (pH 5.0 to 9.0) for the major isozyme, and by vapor diffusion against 2.5 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 $a = 82.3 \AA$, $b = 58.1 \AA$ and $c = 52.5 \AA$ ($1 \AA = 0.1$ 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, 1961a,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 & Aih, 1983). The functions of these proteins are largely unknown, although both chitinase (Legrand et al., 1987) and $\beta$-1,3-glucoanase 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-$[\beta$-1,4-$\alpha$-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-$\beta$-d-muramate (NAM) and C-4 of N-acetyl-$\beta$-D-glucosamine (NAG). Bacterial cell walls consist of poly-$[\beta$-1,4-$\alpha$-NAG-$\beta$-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 cytoplasm 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 (Pujaarniscle, 1990). 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 bactericides/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 dried Hevea brasiliensis latex bottom fraction, for a gift of freeze dried Hevea brasiliensis latex bottom fraction.

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