Hevamine, a chitinase from the rubber tree *Hevea brasiliensis*, cleaves peptidoglycan between the C-1 of *N*-acetylglucosamine and C-4 of *N*-acetylmuramic acid and therefore is not a lysozyme

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Abstract Hevamine is a chitinase from the rubber tree *Hevea brasiliensis* and belongs to the family 18 glycosyl hydrolases. In this paper the cleavage specificity of hevamine for peptidoglycan was studied by HPLC and mass-spectrometry analysis of enzymatic digests. The results clearly showed that the enzyme cleaves between the C-1 of a *N*-acetylglucosamine and the C-4 of a *N*-acetylmuramate residue. This means that hevamine, and very likely also other family 18 glycosyl hydrolases which cleave peptidoglycan, cannot be classified as lysozymes.

Key words: Lysozyme; Chitinase; Cleavage specificity; Peptidoglycan

1. Introduction

Hevamine is a chitinase present in the lutoid bodies of the latex of the rubber tree *Hevea brasiliensis*. The primary and tertiary structure have been elucidated [1,2]. It has a $(\alpha/\beta)_8$ barrel fold and belongs to the family 18 glycosyl hydrolases [3]. The enzyme catalyzes the cleavage of β-1,4-glycosidic bonds of chitin, the main constituent of the fungal cell wall, and of the sugar moiety of peptidoglycan. Chitin is a homopolymer of *N*-acetylglucosamine, while the sugar moiety of peptidoglycan is an alternating polymer not only containing *N*-acetylglucosamine but also *N*-acetylmuramate. Hevamine is believed to play an important role in the self-defence of the rubber tree against pathogenic fungi. Previous experiments with short chitin fragments of 5–6 residues showed that cleavage occurs via a retaining mechanism [4]. Lysozymes cleave the β-glycosidic bond between the C-1 of a *N*-acetylmuramate and the C-4 of a *N*-acetylglucosamine of the bacterial peptidoglycan. In this paper, we present a detailed investigation of the cleavage specificity of hevamine for peptidoglycan and show that its cleavage specificity differs from that of lysozymes.

2. Material and methods

2.1. Isolation of bacterial cell walls

The method was adapted from that of Sharon and Jeanloz [5]. Two g of lyophilized *M. luteus* cells (SIGMA) were suspended in 50 ml of ice-cold water and sonicated with a Vibra Cell High Intensity Sonic Processor (Sonics and Materials Inc.) for 30 min to lyse the bacteria. After sonication, 200 ml of water was added to the suspension, followed by centrifugation at 1500×*g* for 15 min at 4°C. The precipitate was discarded and the supernatant was transferred to a new centrifuge tube and centrifuged again as described above. After the second transfer, cell wall material was precipitated by centrifugation at 10000×*g* for 30 min at 4°C. This pellet was suspended in 5% trichloroacetic acid (TCA) and incubated overnight at room temperature to remove teichoic acids [6]. The next day, cell walls were again precipitated by centrifugation at 10000×*g* as described. To remove residual TCA, the pellet was suspended in water and centrifuged again at 10000×*g* for 30 min. This procedure was repeated twice. After the final centrifugation, the cell wall material was lyophilized and stored at 4°C. 2 grams of lyophilized cells yielded approximately 100 mg of purified cell wall material

2.2. Determination of the cleavage specificity of hevamine for peptidoglycan

Fifty mg of cell wall material was suspended in 10 ml of a 0.01 M $(NH_4)_2SO_4$ buffer, pH 5.0. Fifty μg of hevamine was added and the reaction mixture was incubated at 37°C. The cleavage reaction was monitored by measuring the turbidity at 600 nm. When there was no decrease in turbidity any more, the sample was centrifuged at 10000×*g* for 15 min to remove insoluble debris. After centrifugation, the sample was loaded on a CM25 cation exchange column (10×2.5 cm) in the hydrogen form, to remove protein and glycopeptides. Peptide free cell wall material eluted in the void volume. After freeze-drying, the material was derivatized by reductive amination with ethyl-*p*-aminobenzoate (*p*-ABEE) [7]. This mixture was analyzed by HPLC. The HPLC column used was an analytical vydac wide pore (300 Å]) C-18 column(300×4.6 mm) and eluted with a water/acetonitrile gradient containing 0.01% trifluoroacetic acid ranging from 16% to 20% acetonitrile (v/v) in 25 min at a flow rate of 1.0 ml/min. The effluent was measured at 308 nm and peaks were further analyzed by Positive Ion Spray Mass Spectrometry, without further fragmentation and with fragmentation in such a way that fragmentation occurs at the glycosidic bond [8,9].

3. Results and discussion

The products of peptidoglycan cleaved with hevamine separated by HPLC yielded three major peaks with retention times of 9.5, 10.5 and 13 min (Fig. 1). The M/Z values of the compounds were 1602, 1124 and 646, respectively, which correspond with $(NAG-NAM)_3-ABEE$, $(NAM-NAG)_2-ABEE$ and NAM−NAG−ABEE charged with a proton, respectively. The mass spectrum of peak C is presented in Fig. 2A and shows peaks at M/Z values of 646 and at 668; the latter is the Na^+-charged form of this disaccharide.

In the next step the compounds were fragmented in the mass-spectrometer. Fragmentation of the dimer yielded fragments with an M/Z of 276 and 371 (Fig. 2B). These values correspond to the masses of a single reduced muramate ion and a *N*-acetylglucosamine residue derivatized with *p*-ABEE,

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Abbreviations: NAG, *N*-acetylglucosamine; NAM, *N*-acetylmuramate; *p*-ABEE, ethyl-*p*-aminobenzoate

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respectively. Fragments with an M/Z of 443 and 204, corresponding to the a NAM−ABEE fragment and a free reduced *N*-acetylglucosamine residue, respectively, were not found in the spectrum. Fragmentation of the tetramer yielded a complex spectrum (Fig. 2C). The spectrum showed fragments with a M/Z of 276 and 371, the same fragments as shown in Fig. 2B, and fragments with a M/Z of 754, 849 and 871 u corresponding to the masses of reduced $(NAM-NAG-NAM+2H)^+$, $(NAG-NAM-NAG-ABEE+2H)^+$ and $(NAG-NAM-NAG-ABEE+H+Na)^+$, respectively. Fragments with a M/Z of 443, 682 and 921, corresponding to protonated NAM−ABEE, NAG−NAM−NAG and NAM−NAG−NAM−ABEE, respectively, were not found in the mass spectrum. Fragmentation of the hexamer fragment, gave two additional peaks with a M/Z of 1327 and 1349, respectively, when compared with the mass spectra described above. These values correspond to the pentamer NAG−NAM−NAG−NAM−NAG−ABEE charged with a H^+ and a Na^+, respectively (data not shown).

We performed a similar experiment on cell wall material digested with hen-egg-white lysozyme. A complex cleavage mixture was obtained that could not be properly separated by HPLC. Fragmentation of HEW lysozyme digested glycan chains in the mass-spectrometer yielded fragments with a M/Z value of 443 u, corresponding to the mass of a protonated NAM−ABEE, while the fragment with a M/Z value of 371 (protonated NAG−ABEE) was not found in the mass spectrum after fragmentation.

These results unambiguously show that after cleavage by hevamine the reducing end residue is an *N*-acetylglucosamine residue and never a muramate residue, indicating that the enzyme cleaves always at the reducing end of an *N*-acetylglucosamine residue in peptidoglycan and not at the reducing end of an *N*-acetylmuramate.

4. Discussion

In earlier work the structure of hevamine complexed with tetra-NAG was elucidated [4]. From subsequent modelling studies in which NAG residues were replaced by NAM residues, it was suggested that NAM does not fit in the −1 subsite, which is at the reducing end of the cleavage position (A.C. Terwisscha van Scheltinga, personal communication).

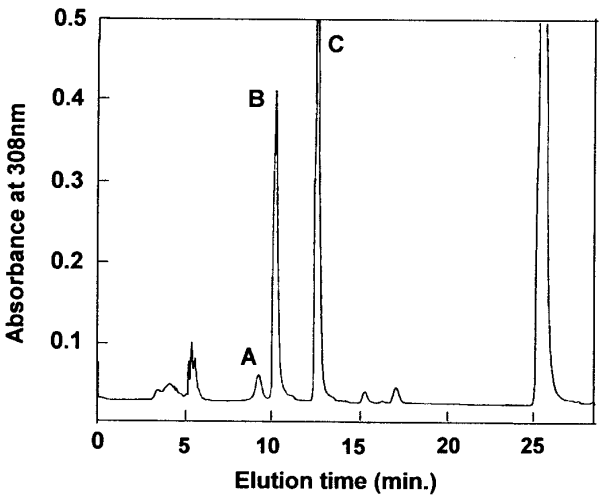

Fig. 1. Separation of glycan fragments by HPLC after derivatization with p-ABEE. Three peaks (A, B and C), were the hexamer, tetramer and dimer of glycan fragments, respectively.

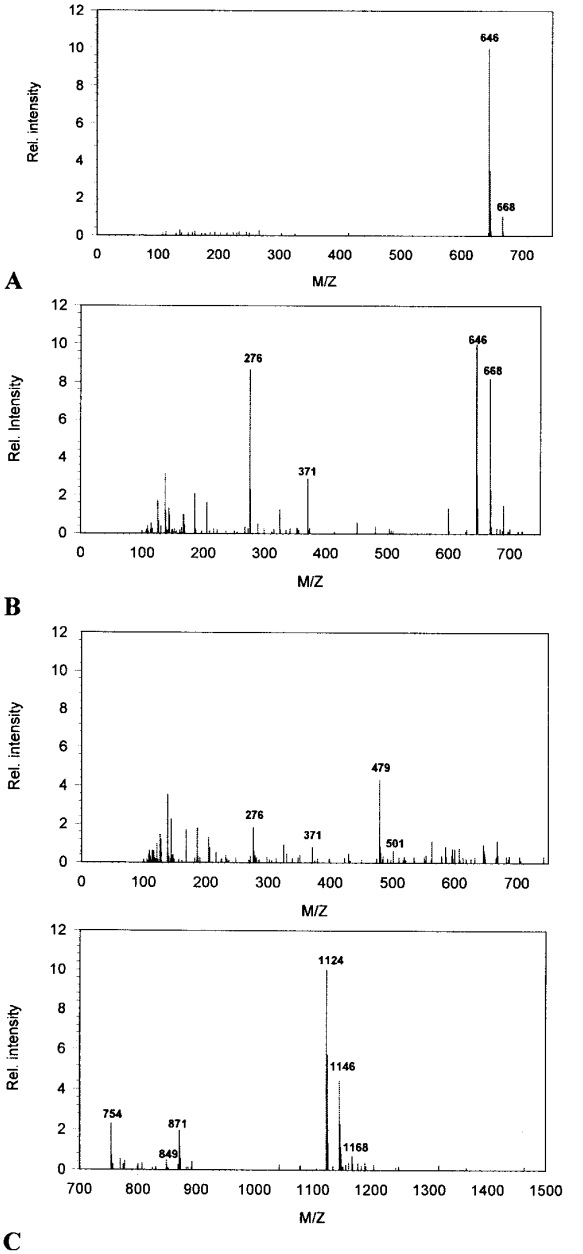

Fig. 2. Mass spectra of the three main peaks of Fig. 1 with and without fragmentation. (A) Mass spectrum of peak C without fragmentation (nozzle = 70 V). (B) Mass spectrum of peak C after fragmentation (nozzle = 200 V). (C) Mass spectrum of peak B without fragmentation (nozzle = 200 V).

This study shows that the definition of a lysozyme as being an enzyme that cleaves between the C-1 of a muramate residue and the C-4 of an *N*-acetylglucosamine [10] does not hold for hevamine. Hevamine is not a lysozyme, because this enzyme cleaves peptidoglycan between the C-1 of a *N*-acetylglucosamine and the C-4 of a *N*-acetylmuramate. Cleavage of the β-glycosidic bond between the C-1 of NAM and the C-4 of NAG of the peptidoglycan has been shown for hen egg-white [11], papaya [12], wheat germ [13] and T-4 [14] lysozyme by either chemical identification of the reducing end after cleavage or by X-ray studies. Other peptidoglycan cleaving en-

zymes can only be tentatively classified as lysozymes on the basis of homologous primary structures and similarities of polypeptide folding in the substrate-binding and active-site regions. Structurally characterized peptidoglycan cleaving plant chitinases are enzymes with a basic iso-electric point belonging to either family 18 or family 19 glycosyl hydrolases [15]. Holm and Sander [16] demonstrated that not only c-type lysozymes, g-type lysozymes and bacteriophage T4 lysozyme have a similar polypeptide fold in the substrate-binding and active-site regions, but also barley chitinase, which belongs to the family 19 chitinases. Family 18 and 19 glycosyl hydrolases not only have completely different polypeptide folds, but differ also in catalytic mechanism with substrate assisted catalysis and retention of configuration in family 18 [4] and inversion of configuration in family 19 glycosyl hydrolases [17]. No or few structural data are known for the lysozymes from papaya [12] and wheat germ [13]. Papaya lysozyme cleaves its substrate with inversion of configuration. It is tempting to speculate about the possibility that the structurally uncharacterized plant lysozymes from papaya and wheat germ are family 19 enzymes, and that peptidoglycan hydrolyzing enzymes in this family have the correct specificity to be classified as lysozymes.

However, we may expect that peptidoglycan cleaving chitinases that are homologous to hevamine, like basic chitinases of *Citrus sinensis* [18], tobacco [19] and *Parthenocissus quinquifolia* [20], which also have optimal peptidoglycan hydrolyzing activities at a pH around 5 and at low ionic strength, have the same substrate specificity as hevamine and cannot be classified as lysozymes as well.

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