Enzyme kinetics of hevamine, a chitinase from the rubber tree *Hevea brasiliensis*

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Abstract The enzyme kinetics of hevamine, a chitinase from the rubber tree *Hevea brasiliensis*, were studied in detail with a new enzyme assay. In this assay, the enzyme reaction products were derivatized by reductive coupling to a chromophore. Products were separated by HPLC and the amount of product was calculated by peak integration. Penta-*N*-acetylglucosamine (penta-nag) and hexa-*N*-acetylglucosamine (hexa-nag) were used as substrates. Hexa-nag was more efficiently converted than penta-nag, which is an indication that hevamine has at least six sugar binding sites in the active site. Tetra-*N*-acetylglucosamine (tetra-nag) and allosamidin were tested as inhibitors. Allosamidin was found to be a competitive inhibitor with a K_i of 3.1 μM. Under the conditions tested, tetra-nag did not inhibit hevamine. © 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Chitinase; Competitive inhibition; Chitin oligomer; *Hevea brasiliensis*

1. Introduction

Chitin, a β (1,4)-linked *N*-acetylglucosamine (GlcNAc)$_n$ polymer, is a major component of the exoskeleton of fungi and invertebrates. These organisms produce chitinases to remodel their exoskeleton during growth and cell division [1]. Other organisms make chitinases as well. For instance plants use chitinases as a defense against pathogenic fungi [2]. Hevamine, a chitinase from the rubber tree *Hevea brasiliensis* belongs to the family 18 glycosyl hydrolases. This class of chitinases has been found in a wide range of eukaryotes and prokaryotes [3,4]. Primary [5] and tertiary structures of hevamine [6] have been determined. The enzyme cleaves not only chitin, but also the sugar moiety of peptidoglycan between the C-1 of a *N*-acetylglucosamine and the C-4 of a *N*-acetylmuramate residue [7] and was the first enzyme found with this cleavage specificity.

The enzyme uses a retaining catalytic mechanism, which is also the mode of action of hen egg white lysozyme (HEWL), but there is no sequence homology between HEWL and hevamine. Structural data also indicate that the cleavage mechanisms of HEWL and hevamine are completely different [8,9]. In contrast to HEWL, which uses two carboxylate residues in its reaction mechanism, hevamine has only one carboxylate in its active site. Instead of an oxocarbenium ion, which is

formed during the HEWL reaction, structural and theoretical data suggest that in hevamine the reaction proceeds with anchimeric assistance of the neighboring *N*-acetyl group [10,11] and that an oxazolinium ion intermediate is formed. Support for this mechanism has been obtained from X-ray studies of the binding mode of allosamidin in the active site of hevamine. Allosamidin is thought to be a transition state analog (Fig. 1).

Although many chitinases have been isolated, detailed kinetic analyses of these proteins have been scarce. The assays for activity measurements mostly use colloidal chitin or chitin derivatives. Because of the heterogeneous nature of these substrates, it is not possible to determine k_{cat} and K_M values. An alternative assay uses short chitin oligomers coupled to a 4-methylumbelliferyl group and measures the fluorescence of the released umbelliferone group, which is a very sensitive method. A disadvantage is that with this substrate only the cleavage of the umbelliferyl group is measured and not cleavage reactions that occur between two sugar residues. Sometimes cleavage does not occur between the umbelliferone group and the oligomer, but between two sugar residues in the oligomer. In such a case, there is no fluorescence and no activity will be measured [12].

In this paper, we describe the production of short *N*-acetylglucosamine oligomers and the kinetics of hevamine with these substrates by a new assay, which does not suffer from the disadvantages of the existing assays. After incubation with enzyme, the products are coupled to an aromatic chromophore so that sensitive detection is possible in the near UV. Coupling to the chromophore also enhances the separation of the products by high performance liquid chromatography (HPLC). With this assay, the inhibition kinetics of hevamine with allosamidin and chitotetraose were studied in detail.
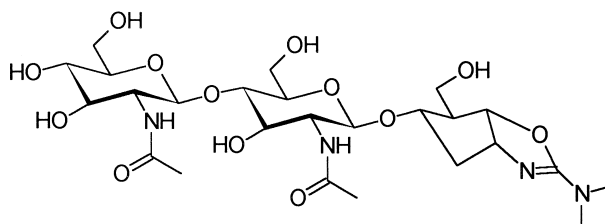

Fig. 1. Structure of allosamidin.

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2. Materials and methods

2.1. Preparation of chitin oligomers

Chitin oligomers were prepared according to the method of Aiba et al. [13] by partial acetylation of chitosan, followed by cleavage with a chitinase and complete acetylation of the product. 1 g of chitosan was dissolved in 20 ml of 3% acetic acid. After one night of solubilization, 80 ml of methanol was slowly added under stirring until a homogeneous solution was obtained. Subsequently 100 μl of acetic acid anhydride was added to 100 ml reaction mixture in a 5 min period under vigorous mixing conditions. Mixing continued for at least 1 h. Methanol and acetic acid were removed by evaporation in a film evaporator. The partially acetylated chitosan was dissolved in 20 ml 0.2 M ammonium acetate, pH 5.4. 1.5 U chitinase from *Streptomyces griseus* (Sigma) was added and the solution was incubated for 1 week at 37°C. After 1 week, another 1.5 U of chitinase was added and incubation continued for another week. After drying, the sample was fully acetylated as described above, by using 1.5 ml instead of 100 μl acetic acid anhydride. Again the sample was dried and dissolved in 20 ml of water. 100–150 mg of oligomers were loaded on a Biogel P4 extra fine grade (Bio-Rad) gel filtration column of 98×3.2 cm. Several peaks were collected and a second purification step was performed by HPLC on a semi preparative (30×1.0 cm) Vydac C18 wide pore (300 Å) column with a 5 μM bead diameter. A guard column (3×0.5 cm) filled with the same column material was used to protect the main column. 0.01% trifluoroacetic acid in water was used as an eluent. UV detection of the peaks was done at 210 nm. Identity and purity of the peaks were checked by comparing the samples with known standards (Seikagaku Corporation, Japan) which were available in small quantities. In this way di-, tri-, tetra-, penta- and hexasaccharides were obtained with a >95% purity as judged by HPLC.

2.2. Enzyme assay

The enzyme reactions were carried out in 1.5–3.0 ml 0.2 M citrate buffer, pH 4.2, at 30°C. Substrate concentrations were chosen in the range of approximately 0.3–5 times the K_M. Reaction velocities were measured in duplicate or triplicate per substrate concentration. Approximately 1 pmol of hevamine was added to 1.5–3 ml reaction mixture in Greiner tubes. After 30 min, the reaction was stopped, by cooling the samples in liquid nitrogen. After freeze drying, the samples were derivatized by reductive coupling of *p*-aminoethylbenzoate (*p*-ABEE) to the 1′-site of the reducing sugars [14]. To the freeze-dried material, 10 μl of water and 50 μl of reagent solution were added. The reagent solution was prepared by dissolving 165 mg *p*-ABEE and 35 mg of sodium cyanoborohydride in 41 μl glacial acetic acid and 350 μl of methanol. The tubes were capped and heated at 80°C for 30 min.

After drying, the samples were dissolved in 350 μl of water containing approximately 0.50 μM of internal standard and extracted with chloroform to remove unreacted ABEE. The water phase was analyzed by HPLC. With penta-*N*-acetylglucosamine (penta-nag) as a substrate, di-*N*-acetylglucosamine was used as an internal standard. When hexa-*N*-acetylglucosamine (hexa-nag) was used as a substrate, *N*-acetylglucosamine was used as an internal standard.

The HPLC column used was an analytical Vydac wide pore (300 Å) C18 column (300×4.6 mm) with a guard column (50×4.6 mm) and eluted with a linear 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 column temperature was 40°C. The effluent was measured at 308 nm. The amount of product was measured by peak integration and comparison with standards, that were treated the same way as the samples. K_M and k_{cat} values were calculated with the program 'Enzfitter' [15], using robust statistical weighting.

3. Results

In Fig. 2, the result of the separation of *N*-acetylglucosamine oligomers, prepared as described, is shown. The amount of hexamer is less than 10% of the total amount of oligomers. The amount of shorter oligomers increases with decreasing molecular weight. These results are consistent with the results of Aiba [13]. This procedure yielded oligomers
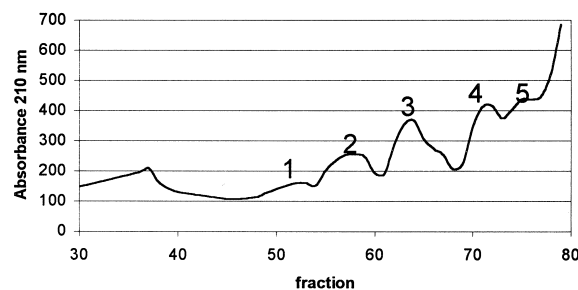

Fig. 2. Separation of *N*-acetylglucosamine oligomers by gel filtration on a Biogel P4 column (extra fine grade (98×3.2) cm) with water as an eluent. 1: Hexa-nag, 2: penta-nag, 3: tetra-nag, 4: tri-*N*-acetylglucosamine (tri-nag), 5: di-*N*-acetylglucosamine (di-nag).

with a purity of more than 80%. Further purification of the oligomers by reversed phase HPLC yielded sufficient pure products (>95%) for enzyme kinetic measurements.

Hevamine cleaves oligomers that are larger than four residues. Penta-nag is cleaved into a tetramer and a monomer, hexa-nag is cleaved in a tetramer and a dimer [10]. In Fig. 3, a chromatogram is shown of the digestion of pentamer by hevamine. Dimer is added as an internal standard. The substrate, product and internal standard are baseline separated, meaning that the product peak can be measured accurately by peak integration. The retention time of derivatized oligosaccharides decreases with increasing degree of polymerization. Tetra-*N*-acetylglucosamine (tetra-nag)-ABEE and penta-nag-ABEE hardly differ in retention time. Therefore the small tetra-nag-ABEE peak is completely overlapped by penta-nag-ABEE and cannot be measured.

Hevamine shows Michaelis–Menten behavior with penta-nag and hexa-nag as substrates (Fig. 4). The k_{cat} and K_M of hevamine with penta-nag and hexa-nag are given in Table 1. Hexa-nag was converted more efficiently than penta-nag, as hevamine has a higher k_{cat} and a lower K_M value. From modelling studies, it was already proposed that hevamine can bind six sugar residues in the active site. The kinetic data show that this indeed is the case. Adding 3.13 μM of allosamidin to the incubation mix gave a K_M of (27.6±2.7) μM and a k_{cat} of (0.385±0.016) s^{-1} (Fig. 4).

There was no significant change in k_{cat}, but a significant change in K_M. The K_i value was 3.1 μM. Adding 15 μM of
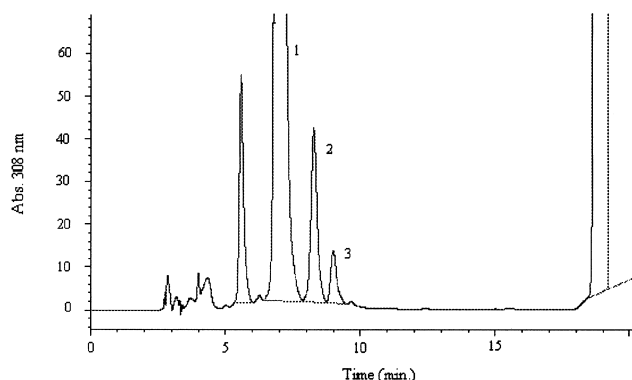

Fig. 3. Chromatogram of penta-nag cleaved by hevamine. 1 = Penta-nag-ABEE (substrate), 2 = di-nag-ABEE (internal standard), 3 = *N*-acetylglucosamine-ABEE (product), tetra-nag-ABEE is completely overlapped by penta-nag-ABEE.
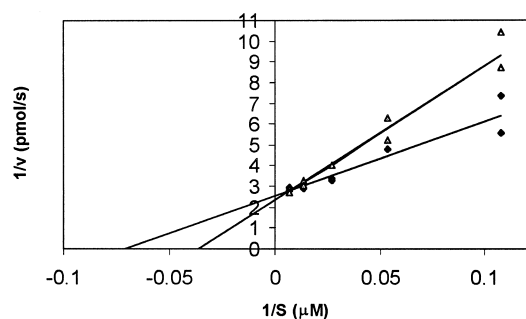
Fig. 4. Lineweaver–Burke plot of enzyme kinetics of hevamine with penta-nag as substrate. ♦: Incubation without allosamidin, ▲: Incubation with 3.13 μM allosamidin.

tetra-nag to the incubation mixture did not inhibit the enzyme reaction, showing that tetra-nag, although it is binding in the active site, is not a high affinity inhibitor.

4. Discussion

The kinetic data indicate that the active site of hevamine contains at least six sugar binding sites. This was shown with enzyme kinetic experiments with short chitin oligomers. The results showed that hevamine has a higher k_{cat} and a lower K_M with hexa-nag compared to penta-nag as substrate, meaning that binding of a sixth residue enhances catalysis. This was already proposed for hevamine from modelling studies. Soaking of penta-nag or hexa-nag in the active site of hevamine was unsuccessful, because these substrates were degraded in the hevamine crystals (unpublished results). It was not possible to accurately determine the K_M and k_{cat} values of hevamine for hexa-nag, because the method used is not sensitive enough to measure product peaks accurately below concentrations of 0.2 μM. These kinetic studies also show that binding of the sixth sugar residue in the active site enhances the enzyme reaction. The additional binding energy of hexa-nag compared to penta-nag can be calculated with the following formula [16]:

$$\frac{(k_{cat}/K_M)A}{(k_{cat}/K_M)B} = \exp\left(-\frac{\Delta\Delta G_b}{RT}\right)$$

The additional binding energy $\Delta\Delta G_b$ of the sixth residue is approximately −6 kJ/mol.

Of the two compounds tested, only allosamidin gave a clear competitive inhibition. In the literature, there are many studies of inhibition of chitinases by allosamidin and allosamidin analogues [17]. In most cases reported, allosamidin gave a competitive inhibition [18–21]. The K_i values found were approximately one order of magnitude lower than the value found for hevamine, meaning that hevamine has a relatively low binding affinity for allosamidin, compared to other family

18 chitinase. Only a cytosolic chitinase from *Neurospora crassa* gave non-competitive inhibition [22]. Milewski et al. [21] studied inhibition of *Candida albicans* chitinase, using *N*-umbelliferone chitooligomers as substrates, and found that the mode of inhibition also depends on the buffer system used. In PIPES, MES and phosphate buffers, allosamidin was a competitive inhibitor. In citrate containing buffers, the mode of inhibition was mixed or even non-competitive. Our buffer system also contained citrate but we did not observe such a buffer effect.

These experiments also showed, that tetra-nag is not a high affinity inhibitor. Our results indicate that its K_i value is much higher than 20 μM, which contrasts with the K_i value of 20 μM of tetra-nag bound to HEWL [23]. This was somewhat surprising, because soaking studies showed that tetra-nag binds in positions −4 to −1 in the active site [6]. Allosamidin binds in the active site in positions −3 to −1 [10]. This mode of binding combined with the low K_i value of allosamidin is an indication that the oxazoline ring is essential for the binding in the active site of hevamine.

This new enzyme assay is very useful for accurate determination of K_M and k_{cat} values and will be used in the future to characterize the kinetic constants of hevamine mutants.

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Table 1
Kinetic parameters, with their standard deviations, of hevamine with penta- and hexa-nag as substrates

Substrate	K_M	k_{cat}	K_M/k_{cat}
Penta-nag	(13.8 ± 0.7) μM	(0.355 ± 0.010) s^{-1}	$(2.57 \pm 0.21) \times 10^4$ M^{-1} s^{-1}
Hexa-nag	(3.2 ± 0.8) μM	(1.0 ± 0.06) s^{-1}	$(3.1 \pm 1.0) \times 10^5$ M^{-1} s^{-1}

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