Enzyme kinetics of hevamine, a chitinase from the rubber tree

*Hevea brasiliensis*

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Received 30 May 2000; revised 3 July 2000; accepted 4 July 2000

Edited by Pierre Jolles

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 derivitized 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 $\mu$M. 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) 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 invertebrates. These organisms produce chitinases to remodel their exoskeleton during growth and cell division [1].

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.

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PHI: S0014-5793(00)01833-0
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 overnight of solubilization, 80 ml of methanol was slowly added under stirring until a homogenous 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 of 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 was 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 observed in duplicate or triplicate per substrate concentration. Reaction velocities were measured in duplicate or triplicate per substrate concentration. Approximate 1 pmol of hevamine was added to 1.5 ml of water. The amount of product was measured in duplicate or triplicate per substrate concentration. Reaction velocities were measured in duplicate or triplicate per substrate concentration.

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-salmine with sodium cyanoborohydride was added to 100 ml reaction mixture in a 5 min period under vigorous mixing conditions. Mixing continued for another week. After freeze drying, the samples were dissolved in 350 µl of water and 50 µl of reagent solution was added. The reagent solution was prepared by dissolving 165 mg (Seikagaku Corporation, Japan) which were available in small quantities. In this way dis-, tri-, tetra-, penta- and hexasaccharides were obtained with a >95% purity as judged by HPLC.

3. Results

In Fig. 2, the result of the separation of N-acetylgalactosamine oligomers, prepared as described, is shown. The amount of hexamer is lower 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 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-acetylgalactosamine (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. 2. Separation of N-acetylgalactosamine 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-acetylgalactosamine (tri-nag), 5: di-N-acetylgalactosamine (di-nag).](image1)

![Fig. 3. Chromatogram of penta-nag cleaved by hevamine. 1 = Penta-nag-ABEE (substrate), 2 = di-nag-ABEE (internal standard), 3 = N-acetylgalactosamine-ABEE (product), tetra-nag-ABEE is completely overlapped by penta-nag-ABEE.](image2)
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 M. 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]:

$$\frac{(k_{cat}/K_M)A}{(k_{cat}/K_M)B} = \exp\left(\frac{-\Delta G_6}{RT}\right)$$

The additional binding energy $\Delta G_6$ of the sixth residue is approximately $-6 \text{ kJ/mol}$.

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 M, which contrasts with the $K_i$ value of 20 M 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.

References
