Expression and characterization of active site mutants of hevamine, a chitinase from the rubber tree *Hevea brasiliensis*

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Hevamine is a chitinase from the rubber tree *Hevea brasiliensis*. Its active site contains Asp125, Glu127, and Tyr183, which interact with the −1 sugar residue of the substrate. To investigate their role in catalysis, we have successfully expressed wild-type enzyme and mutants of these residues as inclusion bodies in *Escherichia coli*. After refolding and purification they were characterized by both structural and enzyme kinetic studies. Mutation of Tyr183 to phenylalanine produced an enzyme with a lower $k_{\text{cat}}$ and a slightly higher $K_m$ than the wild-type enzyme. Mutating Asp125 and Glu127 to alanine gave mutants with ≈ 2% residual activity. In contrast, the Asp125Asn mutant retained substantial activity, with an approximately twofold lower $k_{\text{cat}}$ and an approximately twofold higher $K_m$ than the wild-type enzyme. More interestingly, it showed activity to higher pH values than the other variants. The X-ray structure of the Asp125Ala/Glu127Ala double mutant soaked with chitotetraose shows that, compared with wild-type hevamine, the carbonyl oxygen atom of the N-acetyl group of the −1 sugar residue has rotated away from the C1 atom of that residue. The combined structural and kinetic data show that Asp125 and Tyr183 contribute to catalysis by positioning the carbonyl oxygen of the N-acetyl group near to the C1 atom. This allows the stabilization of a positively charged transient intermediate, in agreement with a previous proposal that the enzyme makes use of substrate-assisted catalysis.

**Keywords:** chitinase; site-directed mutagenesis; substrate-assisted catalysis; X-ray structure.
and 1 mM MgCl₂. At a D₆₀₀ of 0.8–1.0 expression was
membrane proteins. After three additional 1-min sonication
This mutant was made by two consecutive mutagenesis cycles using the Asp125Ala primer pair followed
cycles and subsequent centrifugation (15 min, 5000 g, 4 °C) inclusion bodies were obtained. The inclusion bodies were
washed once with 50 mM Tris, 40 mM EDTA pH 8.0, followed by centrifugation (15 min, 5000 g, 4 °C).

Reefolding of hevamine inclusion bodies
The method was adapted from Janssen et al. (1999) [19].

Sites-directed mutagenesis
Table 1 gives an overview of the primer pairs that were used
for site-directed mutagenesis. Mutants were made using the ‘Quikchange Site-directed Mutagenesis Kit’ (Stratagene), and according to the manufacturer’s specifications, with one
modification. Instead of Pfu polymerase, High fidelity PCR
mix (Roche) was used. After cloning in E. coli Top10F cells
and plasmid DNA isolation, the mutants were sequenced
and used X-ray analysis and enzyme kinetic experiments to
gain detailed insight in the role of these residues in
catalysis.

MATERIALS AND METHODS
Heterologous expression of hevamine in E. coli

For the heterologous expression of hevamine in E. coli, the T7 based expression vector pGELAF+ was used [18]. A construct, named pHEV, was made, which contained the mature wild-type hevamine sequence without the additional N- and C-terminal signal sequences. The primers used for its amplification were 5' TCTCATGGCCATGGGATCC
CATTGCC-3’ with an NcoI restriction site (in italic) for the 5’ end, and 5' AATGGGATCCATTATACACTATCCA
GAATGGAGG-3’ for the 3’ end with a BamHI restriction site. After the PCR, the product was digested with NcoI and BamHI and ligated in pGELAF+ treated with the same restriction enzymes. This gave a construct that was identical to mature hevamine, except for an extra methionine at the N-terminus.

For the heterologous expression of hevamine and hevamine mutants E. coli BI21(DE3) trxB was used. The bacteria were grown at 37 °C in 500 mL Luria-Bertani medium supplemented with 0.2% glucose, 10 mM CaCl₂, and 1 mM MgCl₂. At an OD₆₀₀ of 0.8–1.0 expression was induced by addition of isopropyl thio-β-d-galactoside to a final concentration of 0.2 mM; 8 h after induction, bacteria were harvested by centrifugation (15 min, 4 °C, 5000 g). After centrifugation, the bacterial pellet was suspended in 30 mL 50 mM Tris, 40 mM EDTA pH 8.0. Cells were disrupted by lysozyme treatment (1 mg, 30 min), followed by osmotic shock in 30 mL 50 mM Tris, 40 mM EDTA pH 8.0, and sonication (1 min). After three sonication cycles, 750 µL Triton X-100 was added to solubilize membrane proteins. After three additional 1-min sonication

Table 1. Overview of primers used for site-directed mutagenesis.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Sense strand</th>
<th>Anti-sense strand</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp125Ala</td>
<td>S5’-GATGGTATTGATTTTGCCATAGACGATGTTCA-3’</td>
<td>S5’-GAACCATGCTCTAT6GCAAAATCAATACCATC-3’</td>
</tr>
<tr>
<td>Asp125Asn</td>
<td>S5’-TTGGATGTTATTTGATTTTACATAGAGCACTTGAACCAC-3’</td>
<td>S5’-GGTGAACCATGCTCTATGTTAAAATCATTACCATCCAA-3’</td>
</tr>
<tr>
<td>Glu127Ala</td>
<td>S5’-GGTGAACCATGCTCTATGTTAAAATCATTACCATCCAA-3’</td>
<td>S5’-GGTGAACCATGCTCTATGTTAAAATCATTACCATCCAA-3’</td>
</tr>
<tr>
<td>Asp125Ala/Glu127Ala</td>
<td>S5’-CAGGTGAACCATGCTCTATGTTAAAATCATTACCATCCAA-3’</td>
<td>S5’-CAGGTGAACCATGCTCTATGTTAAAATCATTACCATCCAA-3’</td>
</tr>
<tr>
<td>Tyr183Phe</td>
<td>S5’-CTGGCATGTTGATTTTGCTATTCTTAAACAATCTTACCATGCCAG-3’</td>
<td>S5’-CTGGCATGTTGATTTTGCTATTCTTAAACAATCTTACCATGCCAG-3’</td>
</tr>
<tr>
<td>Asp125Ala/Tyr183Phe</td>
<td>This mutant was made by two consecutive mutagenesis cycles using the Asp125Ala primer pair followed by the Tyr183Phe primer pair</td>
<td></td>
</tr>
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<td>Asp125Ala/Glu127Ala/Tyr183Phe</td>
<td>This mutant was made by two consecutive mutagenesis cycles using the Asp125Ala/Glu127Ala primer pair followed by the Tyr183Phe primer pair</td>
<td></td>
</tr>
</tbody>
</table>
according to the dideoxy chain termination method [20] to check for random PCR errors.

**Purification of hevamine from rubber latex**

Hevamine was purified as described before [7] with one modification. After CM32 column chromatography, hevamine was dialysed against 50 mM Bes buffer (2-[bis (tris-hydroxymethyl)amino]-2-(hydroxymethyl) propane-1,3-diol) pH 7.0. Subsequently, the protein was loaded on a Mono S FPLC column, equilibrated with the dialysis buffer, and eluted in 10 min using a linear gradient of 0–100 mM NaCl in 50 mM Bes buffer pH 7.0 at a flow rate of 0.5 mL min⁻¹. Hevamine A, the acid allelic variant of the protein [7], eluted from the column at a NaCl concentration of 80 mM. This material was used for the lysozyme and chitinase assays.

**Lysozyme assay**

*Micrococcus luteus* cells (Sigma) were suspended in 10 mM Na-acetate buffer pH 5.0, to an OD₆₀₀ of 0.7. Next, 3.3–33 pmol hevamine was mixed with 1 mL M. luteus suspension, depending on the activity of the hevamine mutants. The enzymatic activity was determined with a Uvikon 930 double beam spectrophotometer by measuring the decrease in absorbance at a wavelength of 600 nm. Activities were expressed in U mg protein⁻¹, one unit being the decrease of 0.001 absorbance units per min at 600 nm.

**Chitinase assays**

To determine chitinase activity, two different assays were used. The first used coloured colloidal chitin as a substrate [21]. To 200 μL 0.1 M sodium acetate buffer (pH 4.0–6.0) or 0.1 M Tris/sodium acetate buffer (pH 6.0–9.0) 100 μL of a 2 mg mL⁻¹ CM chitin–RBV suspension (Loewe Biochemica GmbH, München) was added. After preincubation at 37 °C 0.1 μg hevamine was added to the solution and the incubation was continued for 30 min. The reaction was stopped by the addition of 100 μL 1.0 N HCl, followed by cooling on ice for at least 10 min. After cooling, the samples were centrifuged in an Eppendorf centrifuge for 10 min at maximum speed. Then 200 μL of the supernatant was transferred to a cuvette and 800 μL of water was added. The absorbance was measured at 550 nm and corrected for absorption by a control, containing no hevamine. Enzyme activities were given as 1 mM chitin–RBV suspension (Loewe Biochemica GmbH, München) per assay for mature and recombinant over a wide range [7]. To obtain reliable values, we used These values are not proportional to enzyme concentrations

**Expression of hevamine in *E. coli***

Initially, we tried to use an expression protocol in which hevamine is translocated to the periplasm of *E. coli*. To do this, we coupled hevamine N-terminally to the C-terminus of the *E. coli* phosphatase A signal sequence. Although this construct could be transformed to *E. coli* Top10F⁻ without any problems, transformation to the *E. coli* expression strain BI21(DE3) trxB gave no transformants. In contrast, the nearly inactive Glu127Ala mutant could be transformed to *E. coli* BI21(DE3) trxB, but its expression was very low and no expressed protein could be detected by SDS/PAGE or Western blotting. Possibly, hevamine interferes with the peptidoglycan metabolism of the bacterium, even despite its

**RESULTS**

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low activity on peptidoglycan at physiological ionic strength [7]. Therefore, we investigated a system that expresses mature hevamine in the E. coli cytoplasm. This seemed particularly promising, as E. coli BL21(DE3) trxB does not express thioredoxin reductase, which results in enhanced formation of correct disulphide bonds in heterologously expressed proteins in the cytoplasm [31]. Unfortunately, under all conditions investigated, we could obtain only inclusion bodies of hevamine. Also lowering the growth temperature to 20°C did not yield soluble protein. As the expression levels were sufficiently high, we decided to refold these inclusion bodies.

The procedure yielded pure protein as judged by SDS/PAGE. The activity of the pure recombinant protein was 80% of that of the wild-type protein in both the lysozyme and chitinase assays. Attempts to further purify the recombinant hevamine on a Mono S column, similar to the procedure for wild-type hevamine, failed because the recombinant hevamine did not bind to the column, probably because of the high amount of arginine present in the refolding buffer. Even after repeated, extensive dialysis the recombinant hevamine was not retained on the Mono S column. Nevertheless, the recombinant hevamine and hevamine mutants crystallized under similar conditions to wild-type hevamine. The crystals have the same space group (P2₁2₁2₁) and similar cell dimensions. The resulting X-ray structures are indistinguishable from the wild-type hevamine structure. No density is present for the extra N-terminal methionine residue. As the \(\alpha-NH₃^+\) group of Gly₁ forms a salt bridge with the enzyme’s C terminus [28], and no space for an additional amino-acid residue is available, the extra N-terminal methionine residue resulting from the cloning procedure has apparently been cleaved off during the maturation of the enzyme.

**Enzyme activity studies**

The lysozyme activities of the various hevamine variants are shown in Table 3. No enzyme activity was detectable for the Asp₁₂₅Ala/Glu₁₂₇Ala and Asp₁₂₅Ala/Tyr₁₈₃Phe double mutants, and the Asp₁₂₅Ala/Glu₁₂₇Ala/Tyr₁₈₃Phe triple mutant. The single Asp₁₂₅Ala and Glu₁₂₇Ala mutants had approximately 2% of the wild-type hevamine activity. Mutants Tyr₁₈₃Phe and Asp₁₂₅Asn had 65% and 72% activity, respectively, compared with recombinant hevamine. The mutants with > 50% relative activity were used for further characterization.

**PH dependency of hevamine activity**

Figs 1 and 2 show the pH dependency of the various hevamine variants on chitopentaose and colloidal chitin as substrate, respectively. With chitopentaose all hevamine variants have their maximum activity at pH 2.0–3.0. Enzyme activity decreases rapidly at pH 5.0 and above. At pH 8.0 and above, there is no activity remaining. An
exception is the Asp125Asn mutant, which shows a somewhat lesser decrease in activity at higher pH values. Nevertheless, at pH 8.0 this mutant also has hardly any activity left.

The pH profile is rather different with colloidal chitin as the substrate. As this substrate precipitates at low pH, it could not be used for the activity measurements at pH 2–3 where hevamine has its highest activity on chitopentaose (Fig. 1). The pH optimum is rather broad, with, surprisingly, considerable activity at pH 9.0, as found earlier [7]. Absolutely no activity could be detected at this pH with chitopentaose as the substrate. It is interesting that at higher pH values the relative differences in activity between wild-type and Asp125Asn and Tyr183Phe hevamine are smaller with colloidal chitin than with the pentasaccharide. Evidently, the interaction between colloidal chitin and the enzyme influences the active site properties. The cause of these differences is not known.

Table 3. Relative lysozyme activity of hevamine and hevamine mutants at pH 5.0. ND, no detectable activity.

<table>
<thead>
<tr>
<th>Hevamine variant</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type hevamine</td>
<td>123</td>
</tr>
<tr>
<td>Recombinant hevamine</td>
<td>100</td>
</tr>
<tr>
<td>Tyr183Phe</td>
<td>65</td>
</tr>
<tr>
<td>Asp125Asn</td>
<td>72</td>
</tr>
<tr>
<td>Asp125Ala</td>
<td>2</td>
</tr>
<tr>
<td>Glu127Ala</td>
<td>2</td>
</tr>
<tr>
<td>Asp125Ala/Glu127Ala</td>
<td>ND</td>
</tr>
<tr>
<td>Asp125Ala/Tyr183Phe</td>
<td>ND</td>
</tr>
<tr>
<td>Asp125Ala/Glu127Ala/Tyr183Phe</td>
<td>ND</td>
</tr>
</tbody>
</table>

Fig. 1. Enzyme activity of hevamine and hevamine mutants as a function of pH with 50 μM chitopentaose as substrate. The enzyme concentration was 5.6 pmol·mL\(^{-1}\).

Fig. 2. Enzyme activity of hevamine and hevamine mutants at various pH using colloidal chitin as substrate. The enzyme concentrations were 11 pmol·mL\(^{-1}\) for wild-type and recombinant hevamine, and 17 pmol·mL\(^{-1}\) and 21 pmol·mL\(^{-1}\) for the Asp125Asn and Tyr183Phe mutants, respectively.

Table 4. Kinetic parameters of hevamine and selected mutants with chitopentaose as substrate at pH 4.2.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>(K_m) (μM)</th>
<th>(k_{cat}) (s(^{-1}))</th>
<th>(k_{cat}/K_m) (s(^{-1})μM(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hevamine</td>
<td>14.3 ± 2.3</td>
<td>0.77 ± 0.050</td>
<td>(5.4 ± 1.1) × 10(^4)</td>
</tr>
<tr>
<td>Recombinant hevamine</td>
<td>16.3 ± 0.7</td>
<td>0.61 ± 0.011</td>
<td>(3.7 ± 0.3) × 10(^4)</td>
</tr>
<tr>
<td>Asp125Asn</td>
<td>27.6 ± 2.3</td>
<td>0.278 ± 0.16</td>
<td>(1.0 ± 0.12) × 10(^4)</td>
</tr>
<tr>
<td>Tyr183Phe</td>
<td>19.9 ± 2.4</td>
<td>0.116 ± 0.08</td>
<td>(5.8 ± 1.0) × 10(^3)</td>
</tr>
</tbody>
</table>

shows that the Tyr183Phe mutant has the lowest \(k_{cat}\) value (Table 4). Its \(K_m\) value is increased only slightly, demonstrating that substrate binding is hardly affected by this mutation. The Asp125Asn mutant has \(\approx 50\%\) of the wild-type hevamine activity, while its \(K_m\) value is approximately twice as high. These data indicate that both reactivity and substrate binding are affected in this mutant.

Crystal structures of hevamine mutants with bound oligosaccharides

Table 2 shows that the use of chitohexaose in the cocrystallization experiments resulted only in a chitotetraose molecule being bound in the active site (at subsites \(–1\) to \(–4\)). In contrast, the cocrystallization experiment with chitopentaose resulted in a bound pentasaccharide, with four \(N\)-acetylglucosamine residues bound at subsites \(–1\) to \(–4\), and the fifth \(N\)-acetylglucosamine residue protruding out into the solvent. This latter residue does not make close contacts with hevamine. Nevertheless, its average \(B\)-factor is only 18.5 Å\(^2\), compared with 15.5, 13.5, 12.0, and 13.5 Å\(^2\) for the \(–4\), \(–3\), \(–2\), and \(–1\) \(N\)-acetylglucosamine residues. Presumably, even in the triple mutant chitohexaose, but not chitopentaose, is degraded slowly during the crystallization.
process. This is in agreement with previous observations that chitohexaose is a better substrate for hevamine than chitopentaose [22].

Comparison of the Asp125Ala/Glu127Ala and Asp125Ala/Tyr183Phe double mutants with bound chitotetraose (Table 2) with wild-type hevamine complexed with chitotetraose [14] showed that the overall structures of mutants and wild-type hevamine are virtually identical. The only difference occurs in the active site, where the −1 N-acetylglucosamine residue shows somewhat different interactions. In wild-type hevamine, the N-acetyl oxygen atom of this sugar is positioned close to the residue’s C1 atom. The conformation of the N-acetyl group is stabilized by hydrogen bonds between its carbonyl oxygen atom and the Tyr183 hydroxyl group, and between its amide nitrogen atom and Asp125. In the mutants, the hydrogen bond of the amide nitrogen with the Asp125 side chain is not possible anymore, and the −1 N-acetyl group points away from the C1 atom of the −1 sugar (Fig. 3). Apparently, as witnessed by the structure of the Asp125Ala/Glu127Ala mutant, the interaction with Tyr183 alone is not strong enough to keep the N-acetyl carbonyl oxygen in the correct orientation. Thus, Asp125 is important to orient the N-acetyl group, and to position the carbonyl oxygen atom close to the C1 atom of the −1 N-acetylglucosamine residue. In this way, Asp125 is instrumental in facilitating substrate-assisted catalysis [13,14].

An additional difference is observed for the Glu127 side chain. In the complex of wild-type hevamine with chitotetraose the Glu127 side chain Oe1 atom is hydrogen bonded to the O1 atom of the −1 N-acetylglucosamine residue, as well as to the Asp125 side chain [14]. In the Asp125Ala/Tyr183Phe mutant (as well as in the Asp125Ala single mutant; data not shown) the Glu127 side chain has a different rotameric conformation. As a consequence, the hydrogen bond with Asp125 is absent because of the Asp125Ala mutation (Fig. 3C). Instead, the new rotamer of Glu127 is stabilized by a water-mediated hydrogen bond of the Glu127 side chain with the carbonyl oxygen atom of the −1 N-acetylglucosamine group. Thus, the Asp125Ala mutation has also induced a less effective position for catalysis of the side chain of the proton donor residue.

**DISCUSSION**

We have investigated the role of the hevamine active site residues Asp125, Glu127, and Tyr183. Previously, their function in catalysis was deduced from crystallographic studies of the wild-type enzyme [9,14]. Here we complement those studies with crystallographic and kinetic investigations of several heterologously expressed variants of these residues.

**Role of Glu127 in catalysis**

Crystal structures of hevamine have shown that the carboxyl side chain of Glu127 is in a suitable position to donate a proton to the glycosidic oxygen of the scissile bond [13,14]. In agreement with such an essential function in catalysis is the strict conservation of this residue in family 18 chitinases [28,33]. Moreover, mutation of the homologous residues resulted in strongly decreased activities of the chitinases from *Bacillus circulans* [33,34], *Alteromonas* sp. [16], *Aeromonas caviae* [17], and *Coccidioides immitis* [35]. Mutation of Glu127 in hevamine also strongly reduced the activity (Table 3). Nevertheless, the Glu residue is not equally important for activity in all chitinases. Glu → Gln and Glu → Asp mutations in the *B. circulans* and *Alteromonas* sp. chitinases resulted in mutants that had ≤ 0.1% residual activity. In contrast, the same mutations in *A. caviae* chitinase yielded mutants that retained 5% of the wild-type activity. The Glu127Ala mutant of hevamine has also marked residual activity (2%). An explanation for this latter observation is obvious from the crystal structure of **Fig. 3. Stereo representation of (A) wild-type hevamine complexed with the degradation product chitotetraose in the active site [14], compared with (B) the Asp125Ala/Glu127Ala and (C) the Asp125Ala/Tyr183Phe double mutants with bound chitotetraose.** Only the carbohydrate residue bound at subsite −1 is shown. Hydrogen bonds are indicated with dashed lines. In wild-type hevamine, the oxygen atom of the N-acetyl group of the −1 sugar is positioned close to the C1 atom of the −1 sugar, and is hydrogen bonded to Tyr183. Asp125 makes a hydrogen bond to the nitrogen atom of the N-acetyl group. In the double mutants, the N-acetyl group points away from the C1 atom, and its hydrogen bonding interactions are lost. In addition, in the Asp125Ala/Tyr183Phe mutant, the Glu127 side chain has rotated away from the scissile bond glycosidic oxygen and is therefore in a less favourable position for its function as catalytic acid. HOH in Fig. 3B is a well-defined water molecule. This figure was made with the program MOLSCRIPT [32].
enhancing the reactivity of the carbonyl oxygen atom (Fig. 4). Alternatively, the Asp125Asn mutation may affect the pK_a of the Glu127 side chain. The Asp125Asn mutant has a somewhat higher K_m than wild-type hevamine. This is probably caused by a slight rearrangement of the Asn125 side chain due to the loss of the hydrogen-bonding interaction with the side chain amide nitrogen of Asn181 [39]. This may cause less effective substrate binding in the –1 subsite. Interestingly, in the family 18 Arabidopsis thaliana chitinase, which is ≈75% identical in amino-acid sequence to hevamine, an asparagine residue occurs naturally at this position [40]. Figures 1 and 2 show that Asp125Asn hevamine has a broader pH optimum than the wild-type enzyme. Although the A. thaliana chitinase has not yet been expressed and characterized, the lack of a vacuolar targeting signal in its sequence indicates that it is an extracellular enzyme, functioning in a less acidic environment than the vacuole-located hevamine. The Asp → Asn mutation in this enzyme may thus be important to shift its pH optimum to higher pH. In the nonrelated glycosyl hydrolase family 11 xylanase it has also been shown that exchanging an aspartate for an asparagine near the catalytic glutamate raises the pH optimum of the enzyme [41].

The kinetic properties of Asp125Asn hevamine are similar to those found of A. caviae chitinase (50% activity [17]). They are quite different from the Alteromonas sp. [16] and B. circulans [33] chitinases, where the Asp → Asn mutants retained only 0.03% and 0.2% of the wild-type activity, respectively. This suggests that in the B. circulans and the Alteromonas sp. chitinases a negatively charged catalytic aspartate residue is absolutely essential, while in the hevamine and A. caviae chitinases the catalytic aspartate can be replaced by a neutral asparagine residue. From these observations and those on the essentiality of the catalytic Glu (see above) it can be concluded that at least two classes of family 18 chitinases exist: one group containing hevamine and A. caviae chitinase retains ≈50% residual activity when the catalytic aspartate is mutated; the other group contains B. circulans and Alteromonas sp. chitinase, which become virtually inactive upon mutation of the catalytic glutamate and aspartate residues. Unfortunately, no X-ray structures are known yet of the B. circulans or Alteromonas sp. chitinases that allow an atomic explanation for the differences between these two classes.

Fig. 4. Stabilization of the putative oxazolinium ion reaction intermediate. Hydrogen bonding interactions with Asp125 and Tyr183 are indicated.

the Asp125Ala/Glu127Ala mutant: between the Cβ of Ala127 and the N-acetyl oxygen and O1 atoms of the –1 sugar residue a cavity is present that accommodates a water molecule (Fig. 3B). If an intact substrate is bound, this water molecule would be at hydrogen bonding distance from the scissile bond oxygen atom, and may thus take over the proton donating function of Glu127, especially at low pH. A similar explanation has been suggested for the Glu540Ala mutant of the family 20 chitobiase from Serratia marcescens [36]. Similarly, the capacity to accommodate a protonating water molecule in the active site could explain the high residual activity of some of the chitinases mentioned above. Unfortunately, as yet no structural information is available on those chitinases to support this notion.

**Role of Asp125 in catalysis**

Information on the catalytic role of Asp125 has also been deduced from crystal structures. The side chain O1 atom of Asp125 is at hydrogen bonding distance from the amide nitrogen of the N-acetyl group of the –1 sugar residue. This orients the N-acetyl group such that its carbonyl oxygen atom is in close proximity to the C1 atom of the –1 sugar, allowing it to stabilize the positively charged anomeric carbon atom at the transition state during the hydrolysis reaction [13,14]. This stabilization may either occur via an electrostatic interaction or via an intermediate in which the N-acetyl carbonyl oxygen atom is covalently bound to the C1 atom of the –1 sugar residue. The covalent oxazolinium ion intermediate is believed to be energetically more favourable [37,38].

Our kinetic data show that replacement of Asp125 with an asparagine yields a protein with a high residual activity (Tables 3 and 4). The (relatively small) decrease in k_cat of the Asp125Asn mutant of hevamine could be the result of the replacement of the negatively charged aspartate by a neutral asparagine residue. A negatively charged amino-acid residue polarizes the N-acetyl group to a greater extent, thereby

In previous crystallographic studies it was shown that the hydroxyl side chain of Tyr183 is within hydrogen bonding distance of the N-acetyl carbonyl oxygen of the sugar residue bound at subsite –1 (Fig. 3A [14]). From this observation it was proposed that, together with Glu127 and Asp125, Tyr183 plays a role in catalysis. Here, we characterize for the first time for a family 18 chitinase a mutant of this residue. While our kinetic data show that Tyr183 is not important for substrate binding, as the K_m value of the Tyr183Phe mutant hardly differs from that of the wild-type enzyme (Table 4), the K_cat value of this mutant has dropped by 80% (Table 4). From the structural data it can be concluded that Tyr183 helps in stabilizing the transition state by hydrogen bonding to the –1 N-acetyl carbonyl oxygen atom. This hydrogen bond stabilizes the partially negative charge on the carbonyl oxygen, thereby facilitating
the formation of the oxazolinium intermediate. Our kinetic and structural data also show that Tyr183 alone is not sufficient for efficient catalysis, because it is not capable on its own to bring the N-acetyl group carbonyl oxygen atom towards the C1 atom (Fig. 3b). Nevertheless, its contribution to catalysis is obvious, as the Asp125Ala/Tyr183Phe double mutant is inactive, whereas the single Asp125Ala mutant still has 2% activity (Table 3).

CONCLUSIONS

In this study we investigated the catalytic role of Asp125, Glu127 and Tyr183 in hevamine by X-ray crystallographic and kinetic analysis of several mutants. We show that Glu127 is the proton-donating residue, in agreement with previous proposals. However, mutation of Glu127 to alanine does not abolish the activity completely, probably because a water molecule can take over the proton donating function.

Mutation of Asp125 to alanine yields an enzyme with only 2% residual activity. The crystal structures show that this residue is important for positioning the N-acetyl group of the −1 sugar residue close to the sugar’s C1 atom in this way, the sugar is able to form an oxazolinium intermediate. Furthermore, Asp125 interacts with Glu127. Mutating Asp125 to an asparagine yields an enzyme with more than 50% residual activity, which shows that in hevamine the negative charge of this residue is not absolutely essential.

Tyr183 is also beneficial for catalysis, albeit to a lesser extent than Asp125 and Glu127. Our kinetic and structural data show that it contributes to the formation of the oxazolinium intermediate in concert with Asp125, but not to the binding of the substrate.

Comparison of our kinetic data with data obtained from other family 18 chitinases shows that there are at least two classes of family 18 chitinases. The molecular basis for these differences in kinetic properties needs further investigation.

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REFERENCES


