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.

Chitin, β-(1,4)-linked poly(N-acetylglucosamine), is one of the most abundant polymers in nature. It is a major component of the cell wall of yeast and other fungi, and the exoskeleton of arthropods. Although chitin is not abundant in organisms such as bacteria, plants and vertebrates, all have chitinases that can cleave the β-(1,4)-glycosidic bond in chitin.

Chitinases have many different functions in these organisms. Bacteria, for instance, produce chitinases to be able to use chitin as a carbon source for growth [1]. In yeast and other fungi, chitinases are important for cell division [2]. Finally, in plants and mammals, chitinases are believed to play a role in defense against pathogenic fungi by disrupting their cell wall [3–6].

Hevamine is a chitinase from the rubber tree *Hevea brasiliensis*. It is located in so-called lutoid bodies, which are low pH vacuolar organelles filled with hydrolytic enzymes and lectins [7]. These lutoid bodies are believed to play an important role in the protection of the rubber tree against fungal infection. It has been shown that upon wounding, the lutoid bodies burst and release antifungal proteins like the lectin hevain, β-(1,3)-glucanase and hevamine [7]. In this way the lutoid bodies act as a first line of defense against fungal pathogens. The primary [8] and tertiary structures [9] of hevamine have been elucidated. The protein belongs to glycosyl hydrolase family 18 [10,11] and has an (α/β)$_8$ fold, which is one of the most abundant protein folding motifs. Recently, the DNA sequence of hevamine was determined [12]. It appeared that the hevamine gene has no introns, but has extensions at the N- and C-termini, which are absent in the amino-acid sequence of the mature protein. At the N-terminus there is a 26 amino-acid signal sequence for protein export, while at the C-terminus a sequence of 12 additional amino acids is present that is most probably a vacuolar targeting signal.

Hevamine cleaves chitin with retention of the configuration at the C1 atom [13]. X-ray studies suggested the importance of several amino-acid residues for catalysis [13,14]: Glu127 is in a suitable position to donate a proton to the scissile glycosidic bond between the sugar residues bound at the −1 and +1 subsites (for sugar binding site nomenclature see [15]). Its side chain has also a hydrogen bond interaction with the Asp125 side chain, which, in turn, is hydrogen bonded to the nitrogen atom of the N-acetyl group of the −1 sugar residue, orienting the carbonyl oxygen towards the C1 atom. Tyr183 is believed to assist Asp125 in this function by hydrogen bonding to the carbonyl oxygen of the N-acetyl group. In this specific orientation the N-acetyl carbonyl oxygen atom is in an optimal position to stabilize the positively charged reaction intermediate [14]. From this observation it has been concluded that hevamine makes use of substrate-assisted catalysis to catalyse the hydrolysis reaction [13,14].

Previous protein engineering studies of other family 18 chitinases have already shown that mutation of the amino-acid residues equivalent to Asp125 and Glu127 in hevamine abolished enzyme activity almost completely.

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and 1 mM MgCl2. At a

O.D600 of 0.8–1.0 expression was

membrane proteins. After three additional

1-min sonication cycles, 750 µL

pH 8.0, and sonication (1 min). After three sonication

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). The bacterial pellet was suspended in

after centrifugation, the bacterial pellet was suspended in 30 mL 50 mM Tris, 40 mM EDTA

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by osmotic shock in 30 mL 50 mM Tris, 40 mM EDTA

pH 8.0, and sonication (1 min). After three sonication

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).

Refolding of hevamine inclusion bodies

The method was adapted from Janssen et al. (1999) [19]. The

protein pellet was dissolved in 30 mL 7 mM guanidine

HCl, 0.3 mM Na2SO3 pH 8.4, and sulphonated by adding

9 mL 50 mM disodium-2-nitro-5(sulphothio)benzoate over

a 5-min period. After acidicification with 5 mM glacial acetic

acid, 200 mL water was added and a pellet with the fully

sulphonated protein was obtained by centrifugation

(30 min, 8000 g, 4°C). The pellet was washed twice with

water and dissolved in an 8 µm urea solution in 10 mM Tris

buffer pH 8.0.

The denatured protein (2.5 mg) was refolded at 4°C by

rapid dilution in 500 mL 50 mM borate buffer pH 8.9,

containing 0.5 mM arginine/HCl, 2 mM reduced glutathione,

and 0.3 mM oxidized glutathione. After stirring the

suspension for 8 h, a further 2.5 mg denatured protein

were added, and the suspension was stirred for another

8 h. Subsequently, the protein concentration was increased

to a final concentration of 25 mg mL−1 by addition of small

amounts of denatured protein. After one additional night

of refolding, the protein suspension was concentrated to

≈ 25 mL by ultrafiltration through an Amicon diaflow

membrane (10 kDa exclusion pore) fitted in an Amicon

apparatus. After concentration, the sample was dialysed at

least twice against 1 L 50 mM Na acetate, pH 5.0, to

precipitate any incorrectly folded protein. In this way, ≈ 5 mg

correctly folded protein was obtained (40% recovery).

Site-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

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>5'-GATGGTATTGATTCTTCCATAGACGTGTTCA-3'</td>
<td>5'-GAACCATCTGCTCTGAAATACATACATC-3'</td>
</tr>
<tr>
<td>Asp125Asn</td>
<td>5'-TTGGATGGATTTGCTTTTAAATACAGGCTGTTCAACC-3'</td>
<td>5'-GATGGTTAATCGTCACTGAAATCACATCC-3'</td>
</tr>
<tr>
<td>Glu127Ala</td>
<td>5'-GGGTAGCAACATGCTCTGAAATACATACATCC-3'</td>
<td>5'-GATGGTATTGTTGTCACTGAAATACATACATCC-3'</td>
</tr>
<tr>
<td>Asp125Ala/Glu127Ala</td>
<td>5'-GATGGTATTGTTGTCACTGAAATACATACATCC-3'</td>
<td>5'-GATGGTATTGTTGTCACTGAAATACATACATCC-3'</td>
</tr>
<tr>
<td>Tyr183Phe</td>
<td>5'-GATGGTATTGTTGTCACTGAAATACATACATCC-3'</td>
<td>5'-GATGGTATTGTTGTCACTGAAATACATACATCC-3'</td>
</tr>
<tr>
<td>Asp125Ala/Tyr183Phe</td>
<td>5'-GATGGTATTGTTGTCACTGAAATACATACATCC-3'</td>
<td>5'-GATGGTATTGTTGTCACTGAAATACATACATCC-3'</td>
</tr>
<tr>
<td>Asp125Ala/Glu127Ala/Tyr183Phe</td>
<td>5'-GATGGTATTGTTGTCACTGAAATACATACATCC-3'</td>
<td>5'-GATGGTATTGTTGTCACTGAAATACATACATCC-3'</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. 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 Δ550-pmole protein⁻¹min⁻¹ values. These values are not proportional to enzyme concentrations over a wide range [7]. To obtain reliable values, we used 3.3 pmol enzyme per assay for mature and recombinant hevamine and 6.6 pmol and 4.9 pmol for the Tyr183Phe and Asp125Asn mutants, respectively. At these protein concentrations, there is a reasonable linear relationship between the absorbance and the enzyme activity.

The second method used chitopentaose as the substrate [22]. The enzyme reactions were carried out with 1 pmol hevamine in 1.5 mL 0.2 M citrate buffer, pH 4.2, at 30 °C. Substrate concentrations were chosen in the range of 0.5-fold to fivefold the *Kₐₚ*. Reaction velocities were measured in duplicate or triplicate per substrate concentration. After 30 min the reaction was stopped by freezing the samples in liquid nitrogen, and the substrate and reaction products were derivatized by reductive coupling to p-aminobenzoic acid-ethylester (p-ABEE) [23]. *Kₚ* and *kₐₚ* values were calculated with the program ENZFITTER [24], using robust statistical weighting. For a pH-activity profile, activity was measured at a substrate concentration of 50 μM. Enzyme activities were measured in 0.1 M citrate/phosphate buffer (pH 2 and 3), 0.1 M citrate buffer (pH 3–5) or in 0.1 M phosphate buffer (pH 6–9).

Crystallization and X-ray data collection

Crystals of hevamine were prepared as described by Rozeboom et al. [25]. A wide screen of conditions for the recombinant hevamine and its mutants revealed that in addition to the previously used ammonium sulphate and sodium chloride conditions, crystals could be grown from sodium citrate, potassium-sodium tartrate, potassium-sodium phosphate, ammonium phosphate, PEG8000, PEG3350, and PEG2000MME. In the present study we used (co)crystals grown from 1.1 to 1.4 M ammonium sulphate or PEG3350 solutions [pH 7.0, 10–30% (w/v)].

For soaking experiments crystals were transferred to synthetic mother liquor containing the oligosaccharide. The Asp125Ala/Tyr183Phe mutant was successfully soaked overnight in 1.5 M ammonium sulphate, pH 7.5, containing 2 mM chitotetraose. Soaking with chitopentaose and chitohexaose was not feasible because crystal contacts obstruct substrate binding at the +1 and +2 subsites. Therefore, we carried out co-crystallizations with chitopentaose and chitohexaose (see Table 2). Crystals appeared after 1–2 weeks.

Data were collected in house on MacScience DIP2000 or DIP-2030H Image Plate detectors with Cu Kα X-rays from a rotating anode generator. The data sets were integrated and merged using the DENZO&SOLVE/SCALEPACK package [26]. Data processing statistics are given in Table 2.

Refinement was achieved with the CNS program-suite [27], starting from the wild-type hevamine structure with all water molecules removed [28]. Initial σA-weighted 2Fo-Fc and Fo-Fc electron density maps [29] clearly showed density for a chitotetraose or chitopentaose when present (see Table 2 for details). After initial rounds of rigid body refinement, the models were subjected to positional and B-factor refinement of all atoms. At all stages σA-weighted 2Fo-Fc electron density maps were calculated and inspected with O [30] to check the agreement of the model with the data.

RESULTS

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
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 disulfide 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 (P2_12_1) 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 α-NH_3^+ 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

<table>
<thead>
<tr>
<th>Mutant</th>
<th>D125A/Y183F</th>
<th>D125A/E127A</th>
<th>D125A/E127A/Y183F</th>
<th>D125A/E127A/Y183F</th>
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<tbody>
<tr>
<td>Crystallization agent</td>
<td>(NH_4)_2SO_4</td>
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<td>(NH_4)_2SO_4</td>
<td>(NH_4)_2SO_4</td>
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<td>Derivatizing method</td>
<td>Soak</td>
<td>Cocrystallization</td>
<td>Cocrystallization</td>
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<td>Ligand (substrate)</td>
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<td>Chitohexaose</td>
<td>Chitopentaose</td>
<td>Chitohexaose</td>
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<td>Complex in crystal</td>
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<td>Chitohexaose</td>
<td>Chitopentaose</td>
<td>Chitohexaose</td>
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<tr>
<td>Data collection temperature (K)</td>
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<td>120</td>
<td>293</td>
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<td>Cryoprotection agent</td>
<td>15% glycerol</td>
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<td>Space group</td>
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<td>Cell dimensions [a,b,c(Å)]</td>
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<td>51.48, 56.94, 81.34</td>
<td>51.75, 57.60, 82.51</td>
</tr>
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<td>Resolution range (Å)</td>
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<td>34.4–2.00</td>
<td>43.5–1.92</td>
<td>28.8–1.92</td>
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<td>Highest resolution shell</td>
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<td>1.95–1.92</td>
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<td>Total number of observations</td>
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<td>83761</td>
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<td>Number of unique reflections</td>
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<tr>
<td>Completeness (%)</td>
<td>99.8 (97.4)</td>
<td>97.1 (96.8)</td>
<td>99.8 (99.2)</td>
<td>98.5 (97.3)</td>
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<tr>
<td>Rmerge (%)</td>
<td>14.8 (4.5)</td>
<td>10.2 (3.1)</td>
<td>14.7 (5.7)</td>
<td>12.9 (5.5)</td>
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<td>2079</td>
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<td>Number of carbohydrate atoms</td>
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<td>71</td>
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<td>3</td>
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<tr>
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<tr>
<td>Number of water molecules</td>
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<td>256</td>
<td>299</td>
<td>143</td>
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<td>R-factor (%)</td>
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<td>16.7</td>
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</tr>
<tr>
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<td>0.005</td>
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<tr>
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<td>1.3</td>
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<td>&lt;B&gt; overall (Å^2)</td>
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<td>16.5</td>
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<td>&lt;B&gt; protein (Å^2)</td>
<td>20.2</td>
<td>14.9</td>
<td>13.7</td>
<td>18.6</td>
</tr>
</tbody>
</table>

Values in parentheses are for the highest resolution bin.
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.

### $K_m$ and $k_{cat}$ measurements of hevamine and mutants

Comparison of the steady-state kinetic parameters of hevamine and the Tyr183Phe and Asp125Asn mutants 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.

### 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>

### 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) $\times 10^4$</td>
</tr>
<tr>
<td>Rec. hevamine</td>
<td>16.3 ± 0.7</td>
<td>0.61 ± 0.011</td>
<td>(3.7 ± 0.3) $\times 10^4$</td>
</tr>
<tr>
<td>Asp125Asn</td>
<td>27.6 ± 2.3</td>
<td>0.278 ± 0.16</td>
<td>(1.0 ± 0.12) $\times 10^4$</td>
</tr>
<tr>
<td>Tyr183Phe</td>
<td>19.9 ± 2.4</td>
<td>0.116 ± 0.08</td>
<td>(5.8 ± 1.0) $\times 10^3$</td>
</tr>
</tbody>
</table>

Crystal structures of hevamine mutants with bound oligosaccharides

Table 2 shows that the use of chitohexaose in the cocystalization experiments resulted only in a chitotetraose molecule being bound in the active site (at subsites $-1$ to $-4$). In contrast, the cocystalization 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

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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.
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. 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 anomic 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 kcat 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 enhancing the reactivity of the carbonyl oxygen atom (Fig. 4). Alternatively, the Asp125Asn mutation may affect the pKα of the Glu127 side chain. The Asp125Asn mutant has a somewhat higher Km 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.

Role of Tyr183 in catalysis

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 a family 18 chitinase a mutant of this residue. While our kinetic data show that Tyr183 is not important for substrate binding, as the Km value of the Tyr183Phe mutant hardly differs from that of the wild-type enzyme (Table 4), the Kcat 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


