Summary

Chitin is a biopolymer that consists of β(1,4)-linked *N*-acetylglucosamine (GlcNAc) residues. It confers rigidity to the cell wall of fungi and the exoskeleton of arthropods, such as insects or crustaceans. In order to grow or to moult, these organisms need to be able to partially degrade the chitin fibrils, for which purpose they produce chitinases and *N*-acetylglucosaminidases.

Chitinolytic enzymes are also produced by organisms that do not contain any chitin. For example, plants and mammals produce chitinases to weaken the fungal cell wall, in order to prevent or fight fungal infections. Some bacteria are able to use chitin fragments as a source of energy and carbon, and octopuses use chitinases to partly dissolve the shell of crustaceans and get to the meat.

This thesis focuses on hevamine: a family 18 chitinase which is secreted by the rubber tree when latex is tapped and the tree is vulnerable to infections. We have determined the three-dimensional structure of hevamine, and studied the interactions with chitin fragments and specific chitinase inhibitors. Comparison with structures of related proteins identifies the amino acid residues that are essential for maintaining the overall structure or the chitinolytic activity.

Summary of the chapters

Chapter 1

In previous years, chitin and chitinolytic enzymes have been studied intensively. For many organisms, the physiological function and the characteristics of chitinases have been described. On the basis of their sequences, chitinolytic enzymes have been classified in four sequence families, which are unrelated. Chitinases belong to the

families 18 or 19. Whereas family 19 enzymes are mostly found in plants, family 18 chitinases occur in fungi, insects, bacteria, mammals, plants and viruses.

Enzymatic hydrolysis of chitin may proceed through several mechanisms, and it is most likely that the different families do not use the same mechanism. Most of the ideas evolved in the last thirty years are summarised in this chapter. Ironically, the mechanism of hen egg-white lysozyme, the most studied glycosyl hydrolase, is still a subject of much debate.

Chapter 2

The three-dimensional structure of hevamine has been determined through multiple isomorphous replacement, using anomalous scattering. To complex the protein with heavy atom scatterers, the NaCl in the mother liquor was replaced by the non-ionic PEG-6000. Suitable complexes were obtained with a silver, a lead, and two mercury compounds, which resulted in an interpretable electron density map.

The enzyme consists of one single domain, which has a $(\beta\alpha)_8$-barrel ("TIM barrel") folding motif. Two regions that are conserved throughout family 18 roughly correspond to the third and fourth barrel strands. Binding studies with the chitin fragment $(GlcNAc)_3$ indicate that loops at the carboxy-terminal ends of the barrel β-strands form the substrate binding cleft, like observed for all other $(\beta\alpha)_8$ barrel enzymes. This cleft contains six sugar binding sites, named -4 to +2, and the conserved catalytic acid Glu127, belonging to the second consensus sequence, is positioned between the sites -1 and +1.

Chapter 3

The structure of hevamine has been refined to a resolution of 1.8 Å. We compared it with the structures of five related proteins: a bacterial chitinase, two bacterial endo-β-*N*-acetylglucosaminidases, which cleave between two GlcNAc residues in branched saccharides of glycoproteins, and two plant proteins which have no known enzymatic activity. Although the sequences of the six proteins generally have a very low identity level, the cores of the structures superimpose very well. The two conserved sequence regions have similar positions in all structures, and appear to be important both for stability of the fold and for catalytic activity. The substrate specificity of the enzymes is determined by the loops following the barrel strands, which

form the substrate-binding site. All enzymes have an aspartic acid and a glutamic acid residue in positions identical to Asp125 and the catalytic Glu127 of hevamine. The lack of chitinase activity of the two inactive plant proteins can be explained by the absence of one of these two carboxylate groups, and by differences in the loops that form the substrate-binding cleft in hevamine.

Chapter 4

HPLC analysis of reaction products showed hevamine to utilise a retaining mechanism. Such a mechanism requires, besides a catalytic acid, a catalytic nucleophile. Whereas the conserved Glu127 of hevamine is in an excellent position to act as the catalytic acid, no side chain could be identified which could function as a nucleophile. This may be explained by a mechanism in which the carbonyl oxygen of the substrate's acetamido group assumes the role of the catalytic nucleophile, which might result in an oxazoline intermediate. Such an intermediate is mimicked by the allosamizoline moiety of allosamidin, a specific inhibitor of family 18 chitinases. Allosamidin binds in the active site of hevamine, confirming the role of Glu127 as the catalytic acid and the role of the acetamido group as the nucleophile. As allosamidin inhibits all family 18 chitinases tested so far, it may be assumed that the mechanism is conserved throughout the family.

Chapter 5

The mechanism of substrate-assisted catalysis is confirmed by the complex of hevamine with $(GlcNAc)_4$. Both hydrogen bonds and van der Waals interactions force the acetamido group of the residue at site -1 in the orientation needed. The mechanism is not unique for family 18, as the structure of the complex of a family 20 chitobiase with its intact substrate $(GlcNAc)_2$ implies a similar mode of action. Moreover, these studies unambiguously demonstrate the distortion of the sugar ring towards a sofa conformation, long postulated as being close to that of the transition state in glycosyl hydrolysis.

Chapter 6

As fungi need chitinases for growth and cell division, inhibitors of these enzymes may be useful for the design of drugs against fungal infections. The amino acid

sequences of fungal chitinases show a significant level of identity to that of hevamine, so that the hevamine structure may be used as a model to design fungicidal chitinase inhibitors. The complexes of hevamine with two designed chitinase inhibitors show that their low inhibition constants can be explained by the low number of interactions with the enzyme. Some suggestions are made to design improved inhibitors.

Chapter 7

This chapter evaluates the research described in this thesis. The structure of hevamine and its complexes with chitin fragments and inhibitors may be used to understand the properties of related proteins. Of special interest are the human proteins: not only human chitinase, but also proteins involved in rheumatoid arthritis and fertility.

Comparison of the interactions made by the catalytic Glu127 at several pH values gives a possible explanation for the observation that hevamine is active at a pH well above the pKa of this amino acid residue. The conformations of the carboxylate group at different pH values indicate that, as soon as the substrate binds, Glu127 might abstract a proton from Asp125.

All in all, besides answering some existing questions, the research described in this thesis evokes plenty of new ones. Hopefully, this thesis will also contribute to the finding of their answers.