Di-N-acetylchitobiase is a family 18 glycoside hydrolase that splits the reducing-end GlcNAc from chitooligosaccharides. The enzyme hydrolyzed only the α-anomer of five tested substrates, chitin di- through hexasaccharide. In all cases the glycosyl fragment retained its β-configuration while the split monosaccharide was α-D-GlcNAc. Chitobiose was hydrolyzed less than half as fast as the other larger substrates. All four of them, tri- to hexasaccharide, reacted at the same rate. The biochemical behavior of di-N-acetylchitobiase indicates it has three subsites, −2, −1, +1, in which the reducing-end trimer of any sized chitooligosaccharide is bound. The +1 site is specific for an α-anomer.

**Key words:** glycosidase family GH18; reducing-end glycoside hydrolase; chitinase

The glycosidase di-N-acetylchitobiase (“chitobiase”) was first observed in human liver. This enzyme showed unusual specificity, since it hydrolyzed the natural disaccharide chitobiose, but not synthetic p-nitrophenyl N-acetyl-β-D-glucosaminide. Chitobiase was later discovered to be localized in lysosomes. There it catalyzes physiological hydrolysis of the reducing-end GlcNAc from the core chitobiose unit that joins oligosaccharides to peptides in Asn-linked glycoproteins. The enzyme cannot act until the reducing-end GlcNAc is first exposed upon removal of the Asn by lysosomal glycosylasparaginase, and a 6-linked α-L-fucose that is sometimes present on this GlcNAc will also block the reaction. Lysosomal chitobiase purified from rat liver has been shown to be a true reducing-end glycoside hydrolase. Thus the enzyme hydrolyzed chitin di- to tetrasaccharide by releasing the reducing-end GlcNAc, but prior reduction of this terminal GlcNAc to an alditol inactivated these chitooligomers as substrates. Such exo-glycosidases that hydrolyze the reducing-end sugar have rarely been reported in comparison to those much more common ones that split off the residue at the non-reducing end. Chemically, these two end-residues are completely different components of the glycosidic bond undergoing hydrolysis. Thus the reducing-end sugar is the alcohol leaving group, *i.e.*, the aglycon, while the non-reducing end sugar is the aldehyde component, *i.e.*, the glycon. It is expected that glycoside hydrolases that split off a reducing-end sugar will have unique features for binding that residue of the substrate.

Chitobiase has remained a highly conserved enzyme throughout evolution and is present even in the slime mold *Dictyostelium discoideum*. Based on its amino acid sequence, it is a family GH18 member in the superfamily of glycoside hydrolases, which now has about 100 recognized groups of related enzymes (Carbohydrate-Active Enzymes Data Base, http://afmb.cnrs-mrs.fr/CAZY/). Family GH18, along with family GH19, which has a different protein fold and reaction mechanism, are the only two glycosidase families that are chitinases. Virtually all exo-chitinases in family GH18, other than chitobiase, are chitobiohydrolases that release a terminal disaccharide rather than GlcNAc. They also show varied specificity towards which end of a chitooligosaccharide they react. Some GH18 chitobiohydrolases act only at the reducing end while others act at the non-reducing end. In addition, there are GH18 enzymes that are endoglycosidases. To characterize the unique reducing-end mechanism of chitobiase, we studied the anomeric specificity it shows for its substrates and products during hydrolysis of chitooligosaccharides. The enzyme appears to have similar features to those described recently for another reducing-end specific glycosidase in family GH8a, an exo-oligoxyylanase from the bacterium *Bacillus halodurans*. Both of these exo-hydrolases require a specific anomer of the monosaccharide that is split from the reducing-end of their oligosaccharide substrates, and for both these enzymes it is the opposite anomer of that mechanistically formed on the glycosyl fragment during their hydrolysis reactions.

For the current project we used similar methods to those previously followed to isolate and characterize chitobiase from rat liver in order to obtain the enzyme...
from chicken liver. In addition, we produced human chitobiase by recombinant DNA methods using a baculovirus expression system. Based on a single major band observed in stained SDS–gels, the latter enzyme was judged to be approximately 90% pure. All three chitobiases, from chicken, human, and rat, were detectable by western blotting using the same peptide antirabbit IgG made against the human 14 amino acid sequence Lys161–Ser174. Hydrolysis reactions were done as previously reported, and products were analyzed by an isocratic HPLC method with a TSK-Gel Amide-80 column from TosoHaas (Montgomeryville, PA) that allowed determination of both $\alpha$- and $\beta$-anomers.

The anomeric equilibrium for N-acetylglucosamine and its chitooligosaccharides is approximately 60/40: $\alpha/\beta$. Chemical interchange of these isomers at C1 is greatly slowed at ice temperature (1.9 °C), and hence, we relied on this reaction condition to determine what anomers the enzyme required for its substrates and products. Chicken chitobiase was incubated with pentasaccharide at this low temperature (Fig. 1). The reaction was filtered immediately through a Centricon-10 membrane (Millipore, Bedford, MA) to remove enzyme, and both products and remaining substrate were analyzed by HPLC (Fig. 1B). The $\alpha$-anomer of each chitooligosaccharide (N2–N5) and GlcNAc (N1) has a shorter retention time than their $\beta$-anomer. One half of the reaction filtrate was post-incubated 90 min at 37 °C to equilibrate anomers.

Reactions were at ice temperature (1.9 °C) for the indicated times in 40 µl of pH 4.0 buffer containing 0.5 mM chitooligosaccharide and 0.08 mg/ml human chitobiase. Total substrate disappearance was analyzed by HPLC (Fig. 2). The dashed line through the data points estimates an equivalent disappearance rate for all substrates except (GlcNAc)2.
In another experiment, di- through hexasaccharide were hydrolyzed in a 2 h reaction at ice temperature to test the effect of substrate length on the chitobiase reaction. The disaccharide was hydrolyzed the slowest, while tri- through hexasaccharide disappeared more than twice as fast, and notably, at virtually the same rate (Fig. 2). Hydrolysis of these five substrates was limited to their $\alpha$-anomer (data not shown). To test this specificity for the one isomer further, the trisaccharide was reacted with recombinant human chitobiase (Fig. 3). Again only the $\alpha$-anomer of the substrate underwent hydrolysis, which was completed in 1 h. The slight decrease in $\beta$-anomer over the 105-min reaction period must have been due to its slow mutarotation to the reactive $\alpha$-anomer at ice temperature. The rates of release of the two expected products, GlcNAc (aglycon) and chitobiose (glycon), were equivalent, but again GlcNAc was its $\alpha$-anomer and chitobiose was its $\beta$-anomer.

Based on the experimental results, the reaction pathway for chitobiase with chitotriose as the model substrate is: (1) the $\alpha$-anomer binds to the enzyme; (2) the glycosyl fragment retains its $\beta$-anomeric structure due to the substrate-assisted mechanism of family 18 chitinases; and (3) the reducing-end GlcNAc is released as its prebound $\alpha$-anomer. In regard to the predicted geometry of the chitobiase active-site region (Fig. 4), a three-subsite model accounts for the slowed hydrolysis of chitobiase versus the faster and equivalent reaction rate for chitotriose through chitohexaose (Fig. 2). This overall structure for substrate binding is similar to the predicted three subsites for xylose residues in a reducing-end exo-oligoxylanase ("Rex") from Bacillus halodurans, an architecture confirmed recently by x-ray crystallography.

There are notable similarities, as well as differences, between the two reducing-end exo-glycosidases. Rex is a disordered ($\alpha/\alpha$)$_6$ barrel, while chitobiase, being a family GH18 member, is likely to have a ($\beta/\alpha$)$_8$ barrel fold based on its sequence homology to those chitinases. Family GH18 $\beta$-strands 4–8 are clearly recognizable in the C-terminal portion of the chitobiase sequence. The amino third of the protein that should contain $\beta$-strands 1–3, however, is not homologous to the amino-end region in the many family 18 chitinases for which there are crystal structures. In a typical GH18 chitinase, e.g., chitinase A from Serratia marcescens, Trp275 in this segment of the amino acid sequence is highly conserved, and it stacks hydrophobically with the $\beta$-hydroxyl group at C1 of the reducing-end xylose. Related enzyme-substrate interactions that might occur at putative subsite +1 in chitobiase should be revealed once its crystal structure is solved.
in the boat conformation required to perform substrate-assisted hydrolysis. Neither chitobiase nor Rex classic hydrolyzes a synthetic p-nitrophenyl glycoside, and a disaccharide is not as effective a substrate for them as are longer oligosaccharides. Indeed, Rex is barely reactive with xylolbiose, hydrolyzing it at only 0.01% of its rate with xylotriose. The reaction mechanisms and specific anomor requirements for substrates and products by these two reducing-end exoglycosidases are different. The reducing-end exo-oligoxylanase has an invariant mechanism of hydrolysis, and therefore its glycosyl moiety is converted from a starting β-linkage into an α-xylene at its reducing end. Rex, however, requires the reducing-end of its substrate to be the β-anomer bound at its +1 subsite, and this anomer of xylose is released as the aglycon moiety during each hydrolytic cycle.

An interesting observation about both these glycoside exo-hydrolases is that the glycon product fragment has an anomeric structure opposite of that required to become the next one-sugar smaller substrate bound in subsite +1. As is evident from the stability of an initial glycon product, the β-anomer of (GlcNAc)2, to hydrolysis by chitobiase (Fig. 3), this alternating specificity for β-anomer +1 might prevent these two enzymes from reacting processively. Interconversion of the anomers, however, is sufficiently fast in most biological environments for sequential hydrolysis to occur. We are trying to obtain a crystal structure of chitobiase to confirm the three-subsite proposal for its active site, and to compare molecular details of its overall structure to those present in both the family GH8a reducing-end exo-oligoxylanase and the broader group of family GH18 chitinases.

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**References**

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