Thermodynamic properties of chitinase interaction with oligosaccharides

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ABSTRACT

The intermolecular interactions involved in oligosaccharide-protein recognition, binding and cleavage are complex. Elucidation of these complex interactions is important in order to understand the biochemical role these proteins play. Fluorescence spectroscopy is a very good method to study the binding between a substrate and receptor. A non-native form (E315L) of the Family 18 Chitinase from the bacteria *Serratia marcescens* was produced and studied using fluorescence spectroscopy. Based upon the experimental data it is shown that the strength of binding ranges from –8.73 to –12.19 kcal/mol with the trend in binding as $GlcNAc_3 < GlcNAc_4 < GlcNAc_5 = GlcNAc_6$.

INTRODUCTION

Chitin, a homopolysaccharide of β-(1→4)-linked N-acetylglucosamine (GlcNAc) is structurally the 2-amino sugar analogue of cellulose. Chitin acts as a major structural component in the exoskeleton of arthropods (Blackwell, 1969; Minke & Blackwell, 1978), and with the exception of cellulose, it is the second most prominent biopolymer found in nature. Biological degradation of chitin is difficult because its flat, board-like chains are densely packed through interchain hydrogen bonding into microcrystalline substrates. Adjacent chitin chains can run either parallel (β-chitin) (Blackwell, 1969) or, most commonly, anti-parallel (α-chitin) (Minke & Blackwell, 1978). In a chitin chain each GlcNAc is rotated 180° relative to its neighbor.

Chitinases (EC 3.2.1.14) hydrolyze chitin β(1→4) linkages in order to make available the large quantities of carbon and nitrogen elements potentially trapped in the biosphere as insoluble GlcNAc (Keyhani & Roseman, 1999). Henrissat and Davies

(Henrissat & Davies, 2000) have classified glycosyl hydrolases into 91 evolutionary groups based on their homologous amino acid sequences. These enzyme families are further organized into clans that have common three-dimensional structures, but not necessarily common primary sequences. Glycosidase Family 18 and 19 encompass the chitin hydrolases. Many Family 18 chitinase crystal structures have been determined (Bortone, Monzingo *et al.*, 2002; Fusetti, Von Moeller *et al.*, 2002; Hollis, Monzingo *et al.*, 2000; Houston, Anneliese *et al.*, 2003; Houston, Eggleston *et al.*, 2002; Houston, Shiomi *et al.*, 2002; Matsumoto, 1999; Mohanty, Singh *et al.*, 2003; Orikoshi, Baba *et al.*, 2003; Perrakis, Tews *et al.*, 1994; Prag, 2000; Rao, 1999; Rao, 1995; Sun, Chang *et al.*, 2001; Terwisscha van Scheltinga, 1995; Terwisscha van Scheltinga, 1994; Vaaje-Kolstad, Vasella *et al.*, 2003; Van Aalten, Komander *et al.*, 2001; Van Roey, Rao *et al.*, 1994; Varela, LIera *et al.*, 2002; Waddling, Plummer *et al.*, 2000; Watanabe, Ishibashi *et al.*, 2001), and they consist of $(\beta/\alpha)_8$ barrels grouped in Clan GH-K. Glycosyl hydrolases work by general acid-base catalysis that requires two active-site residues, usually glutamic and/or aspartic acids (Zechel & Withers, 2001).

A unique biochemical feature of Family 18 chitinases is absence of any amino acid side chain that could serve as the reactive nucleophile. Catalysis instead occurs by a substrate-assisted mechanism whereby the carbonyl oxygen of the C-2 acetamido group of the glycosyl residue being hydrolyzed is the nucleophile (Brameld, Shrader *et al.*, 1998; Brameld & W. A. Goddard, 1998; Tews, S. *et al.*, 1997). A positive-charged oxazolinium intermediate is formed as this N-acetyl oxygen reacts at C1. It is stabilized through interaction with a conserved aspartic acid residue [D313 in *S. marcescens* chitinase A] (Bortone, Monzingo *et al.*, ; Lu, Zen *et al.*, 2002; Prag, 2000; Terwisscha van Scheltinga, 1995; Waddling, Plummer *et al.*, 2000). Hydrolysis is enhanced by the -

1 glycosyl GlcNAc being in a twisted boat conformation. This forces its glycosidic bond (C1 to O4 of the +1 GlcNAc) to be above and perpendicular to the plane of its pyranose ring, an orientation that is geometrically favorable for the Family 18 enzyme reaction (Brameld & W. A. Goddard, 1998). A glutamic acid just beyond the C-terminus of β-strand 4 (E315 in *Serratia marcescens* ChiA) is the single catalytic acid/base residue in the hydrolysis reaction performed by this group of chitinases (Brameld & W. A. Goddard, 1998; Tews, S. *et al.*, 1997; Watanabe, Kobori *et al.*, 1993).

The crystal structures of complexes with chitin octa- and hexasaccharides show that the reducing-end disaccharide of a bound chitin oligosaccharide is the β-anomer and occupies subsites +1 and +2, while subsites -1 to -5 accommodate at least five more GlcNAc residues (Papanikolau, G. *et al.*, 2001; Van Aalten, Komander *et al.*, 2001; Watanabe, Ishibashi *et al.*, 2001). The predominant chemical interaction between substrate and enzyme within this surface groove is hydrophobic stacking of aromatic amino acids with every other GlcNAc residue. Hydrolysis occurs between sugars in the -1 and +1 subsites with the GlcNAc at -1 being the reacted glycosyl unit. The disaccharide aglycone released from +1 and +2 is virtually the exclusive product of Family 18 chitinases acting on chitin.

Crystal structures of complexes of inactive chitinase mutants with oligosaccharides ($GlcNAc_{6\text{-}8}$) show full occupancy of the binding-cleft subsites -5 (or -4) to +2 (Papanikolau, G. *et al.*, 2001; Van Aalten, Komander *et al.*, 2001; Watanabe, Ishibashi *et al.*, 2001). However, hydrolysis of chitopentaose and chitohexaose by the wild-type chitinase A occurs when these small oligosaccharides are bound only in sites -2 to +2, with one or two reducing-end GlcNAc residues extending into solution beyond the

protein (Aronson, Halloran *et al.*, 2003). Available subsites -3 or -3 and -4 are not utilized by these two substrates. Important questions about the mechanism of Family 18 chitinases involve the modularity of the binding sites and what chemical properties regulate the preference of subsites during binding and hydrolysis of oligosaccharide substrates. Fluorescence spectroscopy is an excellent method to measure the carbohydrate binding properties of lectin proteins, and we now have used this technique to determine the chemistry of interaction between an inactive mutant form of the Family 18 *S. marcescens* chitinase A and its chitin oligosaccharide substrates. Change of the catalytic acid/base residue E315 to a leucine (Protein Data Base *1NH6*) has provided an inactive lectin-mimic of the enzyme useful for this study. There are natural forms of the Family 18 chitinases, so-called "chitolectins", that are involved in tissue remodeling and have undergone this evolution of the catalytic acid/base (Fusetti, Pijning *et al.*, 2003; Houston, Anneliese *et al.*, 2003; Mohanty, Singh *et al.*, 2003; Sun, Chang *et al.*, 2001; Varela, LIera *et al.*, 2002). It is therefore important to understand the binding differences between the two types of evolutionary Family 18 proteins.

In order to elucidate the binding mechanism of Family 18 chitinases, binding studies via fluorescence techniques were developed. These studies were used to investigate the binding of different oligosaccharides to a non-native form of *Sm*ChiA. The binding free energy for the different oligosaccharides was experimentally determined using standard fluorescence titration techniques (Chipman, Grisaro *et al.*, 1967; Fukamizo, Ohkawa *et al.*, 1995; Fukamizo, Sasaki *et al.*, 2001; Hollis, Honda *et al.*, 1997).

MATERIALS AND METHODS

Materials

Chitin oligosaccharides $GlcNAc_{x=4, 5, 6}$ were purchased from Associates of Cape Cod (Seikagaku America, Falmouth, MA), whereas $GlcNAc_3$ was obtained from Sigma-Aldrich.

All solutions used in the fluorescence measurements were prepared using 0.1 M sodium acetate buffer, pH 5.0. Wild-type (WT) ChiA cDNA was isolated from the genomic DNA of *S. marcescens* QMB1466 (ATCC 990) by PCR amplification. Genetic engineering of the E315L mutant and its expression in *E.coli* were done as previously described (Aronson, Halloran *et al.*, 2003).

For the binding experiments it is essential that the ligand remain bound to the protein and not be hydrolyzed or released. Hence, we used the E315L analog in this study, since it does not hydrolyze chitin oligosaccharides, but retains binding capability for the sugar ligands.

Methods

A Photon Technology International fluorometer was used to take all the fluorescence measurements. The excitation wavelength for all trials was 280 nm and the solutions were prepared in a 1 cm wide quartz cell. In order to determine the optimum concentration of *Sm*ChiA to be used during the fluorometric titrations, the fluorescence of four different concentrations (0.05, 0.10, 0.15 and 0.20 μM) of *Sm*ChiA with 0.1 M acetate buffer at pH 5 was measured at 15 °C.

For the $GlcNAc_x$ titrations, fluorescence was measured at 320 nm at room temperature. For each experiment, five to seven different concentrations of ligand were used within 0 - 50 μM range. At each concentration, three trials were done to obtain an average fluorescence value. For each ligand, the experiment was repeated three times to obtain an average value of the binding constant (K_a).

Binding isotherms were obtained as follows: $1/(F_0\text{-}F)$ is plotted as a function of 1/[S] where F_0 is the fluorescence of the chitinase in the absence of polysaccharide, F is the measured fluorescence at 320 nm and [S] is the concentration of the polysaccharide in the solution (Chipman et al., 1967). Based on this linear plot $1/(F_0\text{-}F_\infty)$ is extrapolated equal to $1/(F_0\text{-}F)$ when 1/[S] = 0 (Chipman, Grisaro *et al.*, 1967), and subsequently F_∞ is calculated. F_∞ is equivalent to the relative fluorescence intensity of the enzyme saturated with the polysaccharide.

$\text{Log}\left(\frac{F_0 - F}{F - F_\infty}\right)$ is plotted as a function of log [S], and the data is fit to a straight line yielding the parameters A and B for the equation:

$$\log\left(\frac{F_0 - F}{F - F_\infty}\right) = A + B*\log[S] \qquad (1).$$

The association constant (K_a) can be determined as follows:

$1/K_a = K_d$ = concentration where

$$F = (F_\infty + F_0)/2$$

$$\left(\frac{F_0 - F}{F - F_\infty}\right) = 1$$

$$\log\left(\frac{F_0 - F}{F - F_\infty}\right) = 0$$

Substituting this condition into equation (1);

$$0 = A + B*\log K_d$$

$$A = B*(\log K_a)$$

Therefore,

$-\log K_a = -A/B$

Furthermore, we calculated the binding free energy based on the equation,

$\Delta G^o = -RT\ln K_a + \Delta G_{mix}$

ΔG_{mix} is the free energy change of mixing, calculated at $-RT\ln 55.5 = -2.37$ kcal/mol (Fukamizo, Ohkawa *et al.*, 1995).

RESULTS

In order to determine the optimum concentration of *Sm*ChiA to be used during the fluorimetric titrations, the fluorescence of four different concentrations (0.05, 0.10, 0.15 and 0.20 μM) of *Sm*ChiA were measured. The results of these experiments are shown in **Figure 1**. These results show that the fluorescence value at 320 nm changes linearly with the concentration i.e. the protein concentrations used are in the linear range. For the subsequent experiments we used 0.10 μM *Sm*ChiA concentration since it is in the linear range and allowed us to perform all the experiments for this study.

For each of the ligands, the fluorescence intensity of *Sm*ChiA at a given wavelength varies with the concentration of the saccharide. **Figure 2** shows a typical plot for the binding of the $GlcNAc_4$ to *Sm*ChiA. $\mathrm{Log}\left(\frac{F_0 - F}{F - F_\infty}\right)$ is plotted as a function of log[S], where F_0, F and F_∞ are the fluorescence intensities of solutions of enzyme alone, enzyme in presence of a concentration [S] of saccharide, and enzyme saturated with

saccharide respectively. The equation of the linear fit is described on the plot. The slope of this plot is unity indicating the formation of a one-to-one complex. As described earlier, $-\log K_a$ and K_d can be calculated from the linear fit parameters.

The results of different saccharides are summarized in **Table 1**. These results show that the binding constants (K_a) for the oligosacharrides increase as their number of GlcNAc units or length increases. The trend is obvious from $GlcNAc_3$ to $GlcNAc_4$ to $GlcNAc_5$. However, this trend stops at hexasaccharide i.e. $GlcNAc_5$ and $GlcNAc_6$ are equally strong binders.

DISCUSSION

There is a large difference in the value of binding constants (K_a) between $GlcNAc_3$, $GlcNAc_4$, and $GlcNAc_5$. The results show that the oligosacharrides bind stronger as the number of GlcNAc units or length increases until $GlcNAc_5$. $GlcNAc_5$ and $GlcNAc_6$ bind equally well which suggests a similar mode of interaction for the two oligosaccharides.

These results are consistent with a previous study by Hollis et al. (Hollis, Honda *et al.*, 1997), where they found that dissociation constants for binding of $GlcNAc_3$ to barley chitinase is 19 μM, which is in excellent agreement with the 21.96 ± 3.24 μM obtained in this study. For $GlcNAc_4$ binding they found the dissociation constant to be 6 μM which is double the value of 3.04 ± 0.73 μM obtained in this study.

In a separate study Fukamizo et al. (Fukamizo, Sasaki *et al.*, 2001) calculated the dissociation constant for binding of $GlcNAc_4$ to *Coccidioides immitis* Chitinase-I using

similar fluorescence techniques. They determined the value of K_d for this binding to be 50 μM, which is higher compared to 3.04 ± 0.73μM obtained in this study.

It should be noted that Hollis et al. (Hollis, Honda *et al.*, 1997) and Fukamizo et al. (Fukamizo, Sasaki *et al.*, 2001) used an active chitinase, whereas, in this study we used a chitinase which was rendered inactive by a mutation. Hence, although there might be only negligible hydrolysis during the course of the experiment, there is a potential for an overestimation of K_d when an active enzyme is used to determine the binding properties.

Fukamizo et al. (Fukamizo, Ohkawa *et al.*, 1995) in a separate study measured the binding between $GlcNAc_3$ and lysozyme (not a Family 18 chitinase). They found the affinity constant K_a to be 1.07×10^5 M^{-1}, which is similar to 1.1×10^5 M^{-1} obtained by Chipman et al. (Chipman, Grisaro *et al.*, 1967) and twice the value of $4.67 \times 10^4 \pm 7.6 \times 10^3$ M^{-1} obtained in this study for binding of $GlcNAc_3$ to *Sm*ChiA.

In the present study we observe that $GlcNAc_5$ and $GlcNAc_6$ bind *Sm*ChiA with the same affinity. One possible explanation is that the $GlcNAc_6$ binding involves –3 to +3 subsites, and the interaction between sugar and +3 subsite is minimal. Another interpretation of similar affinity for $GlcNAc_5$ and $GlcNAc_6$ binding to SmChiA is that $GlcNAc_6$ binding involves –4 to +2 subsite is minimal. Based on the crystal structure of the protein, we observe that Tyr390 is close to the +3 subsite. Hence, if $GlcNAc_6$ binds –3 to +3, then it would bind more strongly compared to $GlcNAc_5$ bound in –3 to +2 subsites. On the other hand, the sugar at –4 position has the hydrophobic surface exposed and the subsite is not lined with a hydrophobic residue. Thus, binding of sugar to –4 subsite would not provide substantial favorable interaction. In other words,

$GlcNAc_6$ binding at –4 to +2 subsites would be energetically similar to $GlcNAc_5$ binding –3 to +2 subsites, supporting the binding of $GlcNAc_6$ to –4 to +2 subsites.

HPLC analysis indicates that there is a significant amount of –2 to +3 binding for $GlcNAC_5$ and –2 to +4 binding for $GlcNAC_6$ respectively (Aronson, Halloran *et al.*, 2003). Furthermore, results from HPLC studies show a significant amount of $GlcNAc_6$ is hydrolyzed to $GlcNAc_3$ which would occur when the hexasaccharide is bound –3 to +3. The HPLC proposed binding modes for $GlcNAC_6$ (-2 to +4 and –3 to +3) is in contrast to the present fluorescence spectroscopy results as well as crystallographic studies of complexes of inactive mutants with hexasaccharides, which show –4 to +2 binding. Thus, it would appear that three techniques yield different results for $GlcNAc_6$; X-ray and fluorescence spectroscopy indicate -4 to +2 binding more, whereas HPLC results indicate -2 to +4 (and –3 to +3) binding. It should be noted that an inactive enzyme (E315L) was employed for fluorescence and x-ray studies, whereas an active enzyme was used in the HPLC studies. These results indicate that the sugar binds differently to the enzymatic chitinase compared to the non-enzymatic chitolectin.

CONCLUSION

In this study we have calculated binding parameters for different lengths of GlcNAc to the inactive E315L analog of *Sm*ChiA. Most importantly, the binding strength of oligosaccharide increases with the increasing number of units. However, there is a limit to this phenomenon, this trend stops at 5-mers i.e. $GlcNAc_3 < GlcNAc_4 < GlcNAc_5 = GlcNAc_6$. Finally, we propose based upon our fluorescence results that $GlcNAc_6$ binds in the –4 to +2 subsites and there exists different binding modes for

chitinases and chitolectins.

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ABBREVIATIONS

N-acetylglucosamine (GlcNAc); *Seratia marcescens* chitinase A, *Sm*ChiA

REFERENCES

Aronson, N. N., Halloran, B. A., Alexyev, M. F., Amable, L., Madura, J. D., Pasupulati, L., & Van Roey, P. (2003). Family 18 chitinase-oligosaccharide substrate interaction: subsite preference and anomer selectivity of Serratia marcescens chitinase A. *Biochem J.*, **376,** 87-95.

Blackwell, J. (1969). Structure of β- Chitin or parallel chain systems of poly-beta-(1-4)-N-acetyl-D-glucosamine. *Bioplymers*, **7,** 281-298.

Bortone, K., Monzingo, A. F., Ernst, S., & Robertus, J. D. (2002). The structure of an allosamidin complex with the *Coccidioides immitis* chitinase defines a role for a second acid residue in substrate-assisted mechanism. *J.Mol. Biol*, **320,** 293-302.

Brameld, K. A., Shrader, W. D., Imperiali, B., & Goddard, W. A., III (1998). Substrate assistance in the mechanism of family 18 chitinases: theoretical studies of potential intermediates and inhibitors. *Journal of Molecular Biology*, **280,** 913-923.

Brameld, K. A., & W. A. Goddard, I. (1998). Substrate distortion to a boat conformation at subsite –1 is critical in the mechanism of family 18 chitinases. *J. Am. Chem. Soc.*, **120,** 3571-3580.

Chipman, D. M., Grisaro, V., & Sharon, N. (1967). The binding of oligosaccharides containing N-acetylglucosamine and N-acetylmuramic acid to lysozyme. *J. Biol. Che.*, **242,** 4388-4394.

Fukamizo, T., Ohkawa, T., Ikeda, Y., Torikata, T., & Goto, S. (1995). Binding mode of N,N',N",N'"-tetraacetylchitotetraitol to hen egg white lysozyme. *Carbohydrate Research*, **267,** 135-142.

Fukamizo, T., Sasaki, C., Schelp, E., Bortone, K., & Robertus, J. D. (2001). Kinetic properties of chitinase-1 from the fungal pathogen *Coccidioides immitis. Biochemstry*, **40,** 2448-2454.

Fusetti, F., Pijning, T., Kalk, K. H., Bos, E., & Dijkstra Bauke, W. (2003). Crystal structure and carbohydrate binding properties of the human cartilage glycoprotein-39. *J. Biol. Chem.*, **278,** 37753-37760.

Fusetti, F., Von Moeller, H., Houston, D., Rozeboom, H. J., Dijkstra, B. W., Boot, R. G., Aerts, J. M., & Van Aalten, D. M. (2002). Structure of human chiototriosidase. Implications for specific inhibitor design and function of mammalin chitnase-like lectins. *J. Biol. Chem.*, **277,** 25537-25544.

Henrissat, B., & Davies, G. J. (2000). Glycoside hydrolases and glycosyltransferases. Families, modules, and implications forgenomics. *Plant Physiol*, **124,** 1515-1519.

Hollis, T., Honda, Y., Fukamizo, T., Marcotte, E., Day, P., J., & Robertus, J. D. (1997). Kinetic analysis of barley chitinase. *Archives of Biochemistry and Biophysics*, **344,** 335-342.

Hollis, T., Monzingo, A. F., Bortone, K., Ernst, S., Cox, R., & Robertus, J. D. (2000). The X-ray structure of a chitinase fromthe pathogenic fungs *Coccidioides immitis*. *Protein Sci.*, **9,** 544-551.

Houston, D., R., Anneliese, D. R., Joanne, C. K., & Van Aalten , D. M. (2003). Structure and ligand-induced conformational change of the 39 kD glycoprotein from human articular chondrocytes. *J. Biol. Che.*, **278,** 30206-30212.

Houston, D. R., Eggleston, I., Synstad, B., Eijsink, V. G., & Van Aalten , D. M. (2002). The cyclic dipeptide C1-4[cyclo-(L-Arg-D-Pro)] inhibits family 18 chitinases by structural mimicry of a reaction intermediate. *Biochem. J.*, **368,** 23-27.

Houston, D. R., Shiomi, K., Arai, N., Omura, S., Peter, M. G., Turberg, A., Synstad, B., Eijsink, V. G. H., & Van Aalten, D. M. F. (2002). High-resolution structures of a chitinase complexed with natural product cyclopentapeptide inhibitors: mimicry of carbohydrate substrate. *Proceedings of the National Academy of Sciences of the United States of America*, **99,** 9127-9132.

Keyhani, N. O., & Roseman, S. (1999). Physiological aspects of chitin catabolism in marine bacteria. *Biochim. Biophys. Acta*, **1473,** 108-122.

Lu, Y., Zen, K.-C., Muthukrishnan, S., & Kramer, K. J. (2002). Site-directed mutagenesis and functional analysis of active site acidic amino acid residues D142, D144 and E146 in Manduca sexta (tobacco hornworm) chitinase. *Insect Biochemistry and Molecular Biology*, **32,** 1369-1382.

Matsumoto, T., Nonaka . T., Hashimoto, M., Watanabe, T. and Mitsui, Y. (1999). Three-dimensional structure of the catalytic domain of chitinase A1 from *Bacillus circulans* WL-12 at a very high resolution. *Proc. Japan Acad.*, **75,** 269-274.

Minke, R., & Blackwell, J. (1978). The structure of α-chitin. *J.Mol. Biol*, **120,** 167-181.

Mohanty, A. K., Singh, G., Paramasivam, M., Saravanan, K., Jabeen, T., Sharma, S., Yadav, S., Kaur, P., Kumar, P., Srinivasan, A., & Singh, T. P. (2003). Crystal structure of a novel regulatory 40 kDa mammary gland protein (MGP-40) secreted during involution. *J. Biol. Chem.*, **278,** 14451-14460.

Orikoshi, H., Baba, N., Nakayama, S., Kashu, H., Miyamoto, K., Yasuda, M., Inamori, Y., & Tsujibo, H. (2003). Molecular analysis of the gene encoding a novel cold-adapted chitinase (ChiB) from a marine bacterium, Alteromonas sp. strain O-7. *Journal of Bacteriology*, **185,** 1153-1160.

Papanikolau, Y., G., P., Tavlas, G., Vorgias, C. E., Oppenheim, A. B., & Petratos, K. (2001). High resolution structrural analysis of mutant chitinase A complexs with substrates provide new insight into the mechanism of catalysis. *Biochemstry*, **40,** 11338-11343.

Perrakis, A., Tews, I., Dauter, Z., Oppenheim, A. B., Chet, I., Wilson, K. S., & Vorgias, C. E. (1994). Crystal structure of a bacterial chitinase at 2.3A resolution. *Structure*, **2,** 1169-1180.

Prag, G., Papanikolau, Y., Tavlas, G., Vorgias, C.E., Petratos, K. and Oppenheim, A.B. (2000). Structures of chitobiase mutants complexed with the substrate Di-N-acetyl-D-glucosamine: the catalytic role ofthe consrved acidic pair, aspartate 539 and glutamate 540. *J.Mol. Biol*, **300,** 611-617.

Rao, V., Cui. T., Guan , C. and Van Roey, P. (1999). Mutations of endo-beta-N-acetylglucoseaminidase H active site residue Asp 130 and Glu 132: activities and conformations. *Protein Sci.*, **8,** 2338-2346.

Rao, V., Guan , C. and Van Roey, P. (1995). Crystal structure of endo-beta-N-acetylglucoseaminidase H at 1.9 A resolutio: active-site geometry and substrate recognition. *Structure*, **3,** 449-457.

Sun, Y. J., Chang, N. C., Hung, S. I., Chou, C. C., & Hsiao, C. D. (2001). The crystal structure of a novel mammalian lectin, Ym1, suggests a saccharide binding site. *J. Biol. Chem.*, **276,** 17507-17514.

Terwisscha van Scheltinga, A. C., Armand, S., Kalk, K.H., Isogai, A., Henrissat, B. and Dijkstra, B.W. (1995). Stereochemistry of chitin hydrolysis by a plant chitinase/lysozyme and X-ray structureof a complex with allosamidin: evidence for substrate assisted catalysis. *Biochemistry*, **34,** 15619-15623.

Terwisscha van Scheltinga, A. C., S., Kalk, K.H., Beintema, J.J. and Dijkstra, B.W. (1994). Crystal structures of hevamine, a plant defence protein with chitinase and lysozyme activity, and its complex with inhibitor. *Structure*, **2,** 1181-1189.

Tews, I., S., T. v., Anke, C., Perrakis, A., Wilson, K. S., & Dijkstra, B. W. (1997). Substrate assisted catalysis unifies two families of chitnolytic enzymes. *J. Am. Chem. Soc.*, **119,** 7954-7959.

Vaaje-Kolstad, G., Vasella, A., Peter Martin, G., Netter, C., Houston Douglas, R., Westereng, B., Synstad, B., Eijsink Vincent, G. H., & Van Aalten , D. M. (2003). Interactions of a family 18 chitinase with the designedinhibitor HM508, and its degradation product,chitobiono-delta-lactone. *J. Biol. Che.*, **in press**.

Van Aalten, D. M., Komander, D., Synstad, B., Gaseidnes, S., Peter, M. G., & Eijsink, V. G. (2001). Structural insights into the catalytic mechanism of a family 18 exo-chitinase. *Proc. Natl. Acad. Sci. U.S.A*, **98,** 8979-8984.

Van Roey, P., Rao, V., Jr., P. T. H., & Tarentino, A. L. (1994). Crystal stucture of endo-beta-N-acetylglucoseaminidase F1, an alpha/beta-barrel enzyme adapted for a complex substrate. *Biochemstry*, **33,** 13989-13996.

Varela, P. F., LIera , A. S., Mariuzza, R. A., & Tormo, J. (2002). Crystal structure of imaginal disc growth factor-2. A member of a new family of growth-promoting glycoproteins from *Drosophila melanogaster. J. Biol. Chem.*, **277,** 13229-13236.

Waddling, C. A., Plummer, T. H. J., Tarentino, A. L., & Van Roey, P. (2000). Structural basis for the substratespecificity of endo-beta-N-acetylglucoseaminidase F(3). *Biochemistry*, **39,** 7878-7885.

Watanabe, T., Ishibashi, A., Ariga, Y., Yashimoto, M., Nikaidou, N., Sugiyama, J., Matsumoto, T., & Nonaka, T. (2001). Trp122 and Trp134 on the surface of the catalytic domain are essential for crystalline chitine hydrolysis by *Bacillus circulans* chitinase A1. *FEBS Lett.*, **494,** 74-78.

Watanabe, T., Kobori, K., Fujii, T., Sakai, H., Uchida, M., & Tanaka, H. (1993). Identification of glutamic acid 204 and aspartic acid 200 in chitinase A1 of *Bacillus circulans* WL-12 as essential residues for chitinase activity. *J. Biol. Chem.*, **268,** 18567-18572.

Zechel, D. L., & Withers, S. G. (2001). Dissection of nucelophilic and acid-base catalysis in glycosidases. *Curr. Opini. Chem. Biol.*, **5,** 643-649.

FIGURE LEGENDS

Figure 1: Fluorescence measurements of *Sm*ChiA. The x-axis represents the *Sm*ChiA concentration in μM and y-axis represents the fluorescence value in arbitrary units.

Figure 2 Binding of the $GlcNAc_4$ to *Sm*ChiA. $\mathrm{Log}\left(\frac{F_0 - F}{F - F_\infty}\right)$ is plotted as a function of log[S]. The variables, F_0, F and F_∞ (F_{inf}) are described in the text. The data are plotted as filled squares and the linear fit is shown as a line. The equation for the fit is shown at the top of the plot.

Table 1: Calculated $-\log K_a$, K_a, K_d and ΔG values for differing length saccharides binding to *Sm*ChiA.

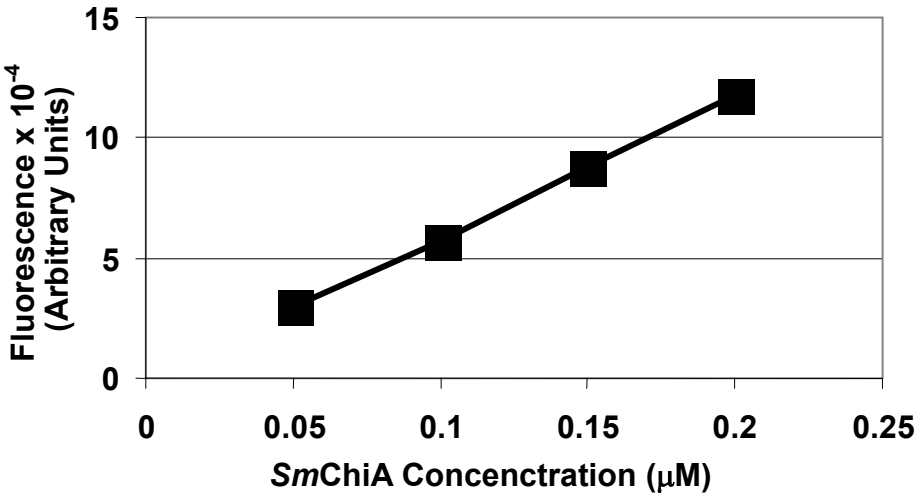

Figure 1: Fluorescence measurements of SmChiA.

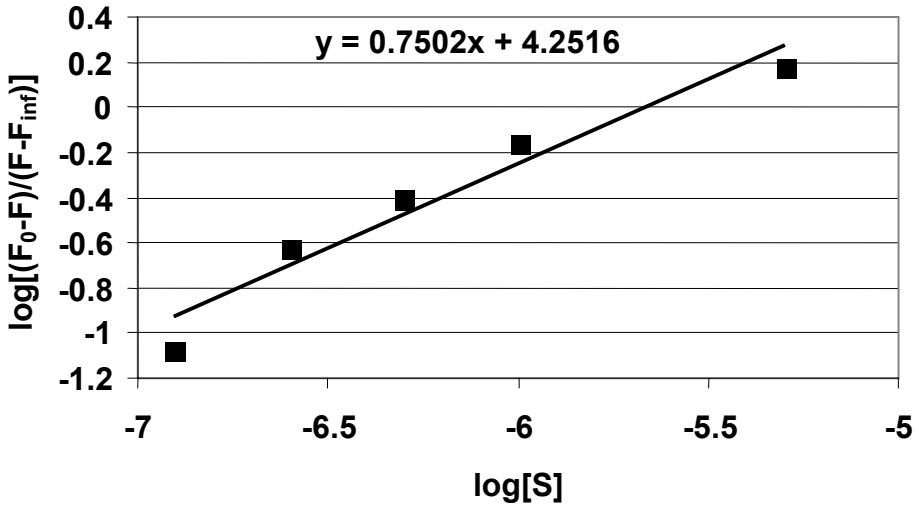

Figure 2: Binding of the $GlcNAc_4$ to *Sm*ChiA.

Ligand	-logK_a	K_a (M^{-1})	K_d (μM)	ΔG° (kcal/mol)
$GlcNAc_3$	-4.66 ± 0.07	$4.67 \times 10^4 \pm 7.6 \times 10^3$	21.96 ± 3.24	-8.73 + 0.09
$GlcNAc_4$	-5.53 ± 0.11	$3.49 \times 10^5 \pm 8.7 \times 10^4$	3.04 ± 0.73	-9.91 ± 0.14
$GlcNAc_5$	-7.20 ± 0.11	$1.58 \times 10^7 \pm 3.9 \times 10^6$	0.06 ± 0.01	-12.19 ± 0.15
$GlcNAc_6$	-7.16 ± 0.17	$1.45 \times 10^7 \pm 5.9 \times 10^6$	0.07 ± 0.02	-12.13 ± 0.23

Table 1: Thermodynamics properties for interaction between *Sm*ChiA and $(NAG)_{3-6}$