BBA - Proteins and Proteomics 1868 (2020) 140414

Contents lists available at ScienceDirect

BBA - Proteins and Proteomics

journal homepage: www.elsevier.com/locate/bbapap

Thermodynamic insights into the role of aromatic residues in chitooligosaccharide binding to the transglycosylating chitinase-D from *Serratia proteamaculans*

Jogi Madhuprakash[a,b,*], T. Swaroopa Rani[b], Appa Rao Podile[b], Vincent G.H. Eijsink[a], Morten Sørlie[a,*]

[a] Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences (NMBU), P.O. Box 5003, 1432 Ås, Norway
[b] Department of Plant Sciences, School of Life Sciences, University of Hyderabad, Gachibowli, Hyderabad, India

ARTICLE INFO

Keywords:
Chitinase
Thermodynamics
Substrate binding
Transglycosylation
Hydrolysis

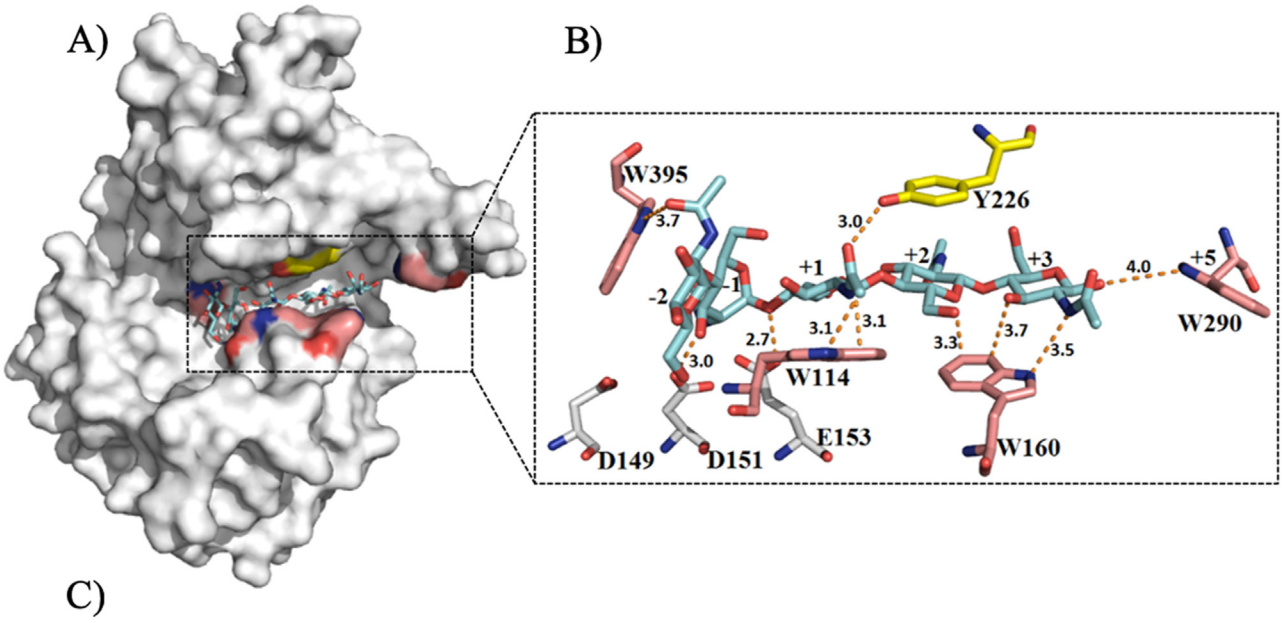
Chitin is crystalline polysaccharide made of β-1–4 linked *N*-acetyl glucosamine (GlcNAc) units. In α-chitin, the most abundant form of chitin, individual chains interact through three-dimensional hydrogen bond arrangements and stacking of the hydrophobic faces to form a crystal [1]. This makes chitin recalcitrant and difficult for enzymes to degrade. In Nature, degradation is catalyzed by family 18 chitinases that hydrolyze glycosidic linkages, primarily producing chitobiose [2]. Central for the ability of chitinases to degrade chitin is a series of surface exposed aromatic amino acids in the active site cleft that by strongly interacting with a single polysaccharide chain, promoting its dissociation from the crystal and guiding into the active site [3]. These interactions mediated by aromatic amino acids are also beneficial for the catalysis of transglycosylation [4,5]. *Sp*ChiD from *Serratia proteamaculans* is an interesting chitinase due to its ability to catalyze both hydrolysis and transglycosylation [6]. The active-site cleft of *Sp*ChiD contains five exposed aromatic amino acids stretching from subsites −1 to +5 (Fig. 1.). In this work, we have used isothermal titration calorimetry (ITC) and a series of engineered *Sp*ChiD variants to assess the contribution of each individual aromatic amino acid to the binding free energy of a single chitin chain, $(GlcNAc)_6$.

Initially, $(GlcNAc)_6$ binding was investigated with the wild type *Sp*ChiD where the catalytic acid Glu^{153} was mutated to an alanine-residue to avoid hydrolysis [7], yielding a K_d of 0.35 ± 0.09 μM (ΔG_r° = −8.9 ± 0.1 kcal/mol, ΔH_r° = 3.2 ± 0.1 kcal/mol, and $-T\Delta S_r^\circ$ = −12.1 ± 0.1 kcal/mol) at t = 25 °C (Fig. 1). Exchange of Trp^{395} (−1 subsite) by alanine yielded a K_d of 16 ± 3 μM (ΔG_r° = −6.6 ± 0.4 kcal/mol, ΔH_r° = −3.5 ± 0.2 kcal/mol, and $-T\Delta S_r^\circ$ = −3.1 ± 0.4 kcal/mol). Exchange of Trp^{114} (+1 subsite) yielded a K_d of 147 ± 85 μM (ΔG_r° = −5.2 ± 0.3 kcal/mol, ΔH_r° = 5.7 ± 0.7 kcal/mol, and $-T\Delta S_r^\circ$ = −10.9 ± 0.8 kcal/mol). Exchange of Tyr^{226} (+2 subsite) yielded a K_d of 2.7 ± 0.4 μM (ΔG_r° = −7.6 ± 0.1 kcal/mol, ΔH_r° = 3.9 ± 0.2 kcal/mol, and $-T\Delta S_r^\circ$ = −11.5 ± 0.2 kcal/mol). Exchange of Trp^{160} (+3 subsite) yielded a K_d of 37 ± 14 μM (ΔG_r° = −6.0 ± 0.2 kcal/mol, ΔH_r° = 7.4 ± 0.7 kcal/mol, and $-T\Delta S_r^\circ$ = −13.4 ± 0.7 kcal/mol). Finally, exchange of Trp^{290} (+5 subsite) yielded a K_d of 0.7 ± 0.2 μM (ΔG_r° = −8.4 ± 0.2 kcal/mol, ΔH_r° = 5.3 ± 0.4 kcal/mol, and $-T\Delta S_r^\circ$ = −13.7 ± 0.4 kcal/mol).

Interestingly, the distribution between enthalpic and entropic contributions to the binding free energy to *Sp*ChiD-W395A is dramatically different compared to the other variants. The average ΔH_r° and $-T\Delta S_r^\circ$ are 5.4 ± 0.6 and − 12.6 ± 0.6 kcal/mol, respectively, for the other variants, while binding of $(GlcNAc)_6$ to *Sp*ChiD-W395A is accompanied by a (favorable) ΔH_r° of −3.5 kcal/mol and a $-T\Delta S_r^\circ$ of −3.1 kcal/mol, suggesting a very different binding mode. Structures of family 18 chitinases (Fig. 1; [9]) show that Trp^{395} is crucial for the energetically demanding conformational change of the GlcNAc moiety binding to the −1 subsite change from the $^4C_1$ chair conformation to the $^{1,4}B$ boat

* Corresponding authors at: Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences (NMBU), P.O. Box 5003, 1432 Ås, Norway.
E-mail addresses: madhuprakash25@gmail.com (J. Madhuprakash), morten.sorlie@nmbu.no (M. Sørlie).

https://doi.org/10.1016/j.bbapap.2020.140414
Received 30 January 2020; Received in revised form 17 March 2020; Accepted 19 March 2020
Available online 26 March 2020



Thermodynamic parameters for $(GlcNAc)_6$ binding to *Sp*ChiD mutants[a] at $t = 25$ °C, in 20 mM potassium phosphate, pH = 6.0.

Mutation	Subsite	K_d[b]	ΔG_r°[c]	ΔH_r°[b]	$-T\Delta S_r$°[c]	Protein concentration[d]	Ligand concentration[d]
Wild type[a]		0.35 ± 0.09	−8.9 ± 0.1	3.2 ± 0.2	−12.1 ± 0.1	23 μM	0.75 mM
W395A	−1	16 ± 3	−6.6 ± 0.4	−3.5 ± 0.2	−3.1 ± 0.4	15 μM	3 mM
W114A	+1	147 ± 85	−5.2 ± 0.3	5.7 ± 0.7	−10.9 ± 0.8	15 μM	3 mM
Y226A	+2	2.7 ± 0.4	−7.6 ± 0.1	3.9 ± 0.2	−11.5 ± 0.2	45 μM	1.5 mM
W160A	+3	37 ± 14	−6.0 ± 0.2	7.4 ± 0.7	−13.4 ± 0.7	23 μM	0.75 mM
W290A	+5	0.7 ± 0.2	−8.4 ± 0.2	5.3 ± 0.4	−13.7 ± 0.4	45 μM	1.5 mM

[a] all enzyme variants had the E153A mutation that abolishes hydrolytic activity, thus enabling ITC measurements. [b] μM, [c] kcal/mol, [d] The ITC experiments were carried out by titrating a solution of ligand into the enzyme solution; this column provides the concentrations used. All titrations were performed at 25 °C in 20 mM potassium phosphate, pH 6.0.

Fig. 1. A) Crystal structure of *Sp*ChiD (pdb 4nzc [8]) with $(GlcNAc)_5$ taken from pdb entry 1e6n [9]. B) A close-up view of aromatic amino acids in the active site of *Sp*ChiD highlighting key interactions with the substrate. C) The thermodynamic parameters of $(GlcNAc)_6$ binding to individual *Sp*ChiD mutants and their concentrations in the isothermal titration calorimetry experiments.

confirmation that is necessary to form the Michaelis complex [10]. It is thus conceivable that the W395A mutation reduces the enthalpic penalty of substrate binding.

All other mutations also resulted in reduced binding affinity. The largest reduction in binding free energy (3.6 kcal/mol) was observed for the mutations in subsite +1 (W114A) (Fig. 1). Mutation of Trp^{160} in the +3 subsite also had a large effect, whereas the effect of mutating the tyrosine in the +2 subsite was less profound. The relatively small contribution of the tyrosine is also apparent from previous studies, which showed that replacement of this Tyr or of an analogous Phe-residue ChiA from *S. marcescens* by tryptophan leads to improved binding and, consequently, increased transglycosylation [4,5]. Still, removal of the Phe residue in ChiA (exchange with an Ala-residue) reduced transglycosylation [4]. Similar observation has been made for a class V chitinase from cycad, *Cycas revoluta* where a Phe mutation to Ala in positive subsites reduced transglycosylation activity [11].

Finally, the lowest reduction in binding free energy (0.4 kcal/mol) among the mutants was observed for *Sp*ChiD-W290A (subsite +5). Interaction with this subsite would require the binding of $(GlcNAc)_6$ from subsites −1 to +5 in *Sp*ChiD. This may indeed happen since previous studies have shown that *Sp*ChiD can catalyze hydrolysis when only the −1 subsite is occupied. It has been shown that $(GlcNAc)_4$ productively binds to −1 to +3 (next to binding to–2 to +2) [6] and that *Sp*ChiD can cleave $(GlcNAc)_2$, which is a unique feature for family 18 chitinases [12]. Still, in reactions with $(GlcNAc)_6$ there is probably low occupancy for the +5 subsite, which may explain the limited effect of the W290A mutation.

Combined, our results demonstrate that all five investigated residues provide significant binding free energy to the carbohydrate substrate. The tryptophan residues seem essential in providing necessary binding free energy for the formation of a stable Michaelis complex. These results are in accordance with results from previous studies [3–5] showing that tryptophan-residues provide more binding free energy to GlcNAc-residues than phenylalanine- and tyrosine-residues. This is also a feature observed in other GH systems i.e. xylosaccharide binding to a family 10 xylanase where a Tyr-residue contributed less to

the binding free energy as well as the change in heat capacity than a Trp-residue [13].

Generation and expression of mutant enzymes and their purification has been thoroughly described previously [6]. Table S1 shows details of primers and templates used for generation of *Sp*ChiD variants. Execution of ITC experiments and the analysis of calorimetric data has been thoroughly described previously [14,15]. ITC experiments were performed with a VP-ITC system from Microcal, Inc. (Northampton, MA). Solutions of $(GlcNAc)_6$ were placed in the syringe whereas the reaction cell of the calorimeter contained solutions purified *Sp*ChiD variants; details are provided in Fig. 1. Titrations were performed at 25 °C in 20 mM potassium phosphate, pH 6.0. Next to the mutations used to denote the various enzyme variants, all variants carried a mutation of the catalytic acid (E153A), which abolishes hydrolytic activity, thus enabling ITC measurements. Errors in K_a and ΔH_r° were obtained as standard deviations from four individual experiments. K_d, ΔG_r°, ΔS_r°, and $-T\Delta S_r^\circ$ were calculated from Eq. 1, and errors in these parameters were obtained from propagation of error.

$$\Delta G_r^\circ = \Delta H_r^\circ - T\Delta S_r^\circ = -RT \ln K_a = RT \ln K_d \quad (1)$$

Credit author statement

JM designed and executed experiments, performed data analysis, wrote original draft.

TSR designed experiments, edited original draft.

ARP designed experiments, edited original draft.

VGHE designed experiments, edited original draft.

MS designed and executed experiments, performed data analysis, wrote original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbapap.2020.140414.

References

[1] P. Sikorski, R. Hori, M. Wada, Revisit of α-chitin crystal structure using high resolution X-ray diffraction data, Biomacromolecules 10 (2009) 1100–1105, https://doi.org/10.1021/bm801251e.

[2] G. Vaaje-Kolstad, S.J. Horn, M. Sørlie, V.G.H. Eijsink, The chitinolytic machinery of *Serratia marcescens* – a model system for enzymatic degradation of recalcitrant polysaccharides, FEBS J. 280 (2013) 3028–3049, https://doi.org/10.1111/febs.12181.

[3] S. Jana, A.G. Hamre, P. Wildberger, M.M. Holen, V.G.H. Eijsink, G.T. Beckham, M. Sørlie, C.M. Payne, Aromatic-mediated carbohydrate recognition in processive *Serratia marcescens* chitinases, J. Phys. Chem. B 120 (2016) 1236–1249, https://doi.org/10.1021/acs.jpcb.5b12610.

[4] H. Zakariassen, M.C. Hansen, M. Jøranli, V.G.H. Eijsink, M. Sørlie, Mutational effects on transglycosylating activity of family 18 chitinases and construction of a hypertransglycosylating mutant, Biochemistry 50 (2011) 5693–5703, https://doi.org/10.1021/bi2002532.

[5] J. Madhuprakash, B. Dalhus, T.S. Rani, A.R. Podile, V.G.H. Eijsink, M. Sørlie, Key residues affecting transglycosylation activity in family 18 chitinases: insights into donor and acceptor subsites, Biochemistry 57 (2018) 4325–4337, https://doi.org/10.1021/acs.biochem.8b00381.

[6] J. Madhuprakash, K. Tanneeru, P. Purushotham, L. Guruprasad, A.R. Podile, Transglycosylation by chitinase D from *Serratia proteamaculans* improved through altered substrate interactions, J. Biol. Chem. 287 (2012) 44619–44627, https://doi.org/10.1074/jbc.M112.400879.

[7] J. Madhuprakash, B. Dalhus, G. Vaaje-Kolstad, S. Sakuda, A.R. Podile, V.G.H. Eijsink, M. Sørlie, Structural and thermodynamic signatures of ligand binding to the enigmatic chitinase D of *Serratia proteamaculans*, J. Phys. Chem. B 123 (2019) 2270–2279, https://doi.org/10.1021/acs.jpcb.8b11448.

[8] J. Madhuprakash, A. Singh, S. Kumar, M. Sinha, P. Kaur, S. Sharma, A.R. Podile, T.P. Singh, Structure of chitinase D from *Serratia proteamaculans* reveals the structural basis of its dual action of hydrolysis and transglycosylation, Int J Biochem Mol Biol 4 (2013) 166–178.

[9] D.M.F. van Aalten, D. Komander, B. Synstad, S. Gåseidnes, M.G. Peter, V.G.H. Eijsink, Structural insights into the catalytic mechanism of a family 18 exo-chitinase, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 8979–8984.

[10] H.B. Mayes, L.J. Broadbelt, G.T. Beckham, How sugars pucker: electronic structure calculations map the kinetic landscape of five biologically paramount monosaccharides and their implications for enzymatic catalysis, J. Am. Chem. Soc. 136 (2014) 1008–1022, https://doi.org/10.1021/ja410264d.

[11] T. Taira, M. Fujiwara, N. Dennhart, H. Hayashi, S. Onaga, T. Ohnuma, T. Letzel, S. Sakuda, T. Fukamizo, Transglycosylation reaction catalyzed by a class V chitinase from cycad, Cycas revoluta: a study involving site-directed mutagenesis, HPLC, and real-time ESI-MS, Biochim. Biophys. Acta, Proteins Proteomics 1804 (2010) 668–675.

[12] P. Purushotham, A.R. Podile, Synthesis of long-chain chitooligosaccharides by a hypertransglycosylating processive endochitinase of *Serratia proteamaculans* 568, J. Bacteriol. 194 (2012) 4260–4271, https://doi.org/10.1128/jb.06473-11.

[13] G. Zolotnitsky, U. Cogan, N. Adir, V. Solomon, G. Shoham, Y. Shoham, Mapping glycoside hydrolase substrate subsites by isothermal titration calorimetry, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 11275–11280.

[14] A.L. Norberg, V. Karlsen, I.A. Hoell, I. Bakke, V.G.H. Eijsink, M. Sørlie, Determination of substrate binding energies in individual subsites of a family 18 chitinase, FEBS Lett. 584 (2010) 4581–4585.

[15] F.H. Cederkvist, S.F. Saua, V. Karlsen, S. Sakuda, V.G.H. Eijsink, M. Sørlie, Thermodynamic analysis of allosamidin binding to a family 18 chitinase, Biochemistry 46 (2007) 12347–12354.