Thermochimica Acta 527 (2012) 219–222

Contents lists available at SciVerse ScienceDirect

Thermochimica Acta

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

Short communication

Subsite binding energies of an exo-polygalacturonase using isothermal titration calorimetry

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ARTICLE INFO

Article history:
Received 12 September 2011
Received in revised form 19 October 2011
Accepted 27 October 2011
Available online 2 November 2011

Keywords:
Polygalacturonase
Isothermal titration calorimetry
Substrate binding
Thermodynamics

ABSTRACT

Thermodynamic parameters for binding of a series of galacturonic acid oligomers to an exo-polygalacturonase, RPG16 from *Rhizopus oryzae*, were determined by isothermal titration calorimetry. Binding of oligomers varying in chain length from two to five galacturonic acid residues is an exothermic process that is enthalpically driven and results in extremely tight binding of the substrate to RPG16. Binding energies in combination with prior biochemical data suggests that RPG16 has the potential for five subsites, −1 to +4, with the greatest contribution to binding energies arising from subsite −1/+1. While the enthalpic contribution to binding decreases substantially for subsites +2 to +4, beneficial entropic effects occur in subsites +3 and +4 leading to increased total free energy as the length of oligomer increases. This information will be useful for additional studies in determining the binding contributions of specific amino acids with mutant enzymes.

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1. Introduction

Pectin is a complex carbohydrate polymer found in the cell walls and middle lamella of plants that is generally classified by two distinct structures, the "smooth" regions consisting of long chains of homogalacturonan and the "hairy" regions that contain highly branched and substituted regions [1]. Polygalacturonases (PGs), exo-polygalacturonase (E.C. 3.2.1.67), and endo-polygalacturonase (E.C. 3.2.1.15) are members of the GH28 family of glycosyl hydrolase enzymes that catalyze the hydrolysis of the α-1,4 linked galacturonic acid residues of the homogalacturonan chains. Endo-polygalacturonases hydrolyze glycosidic bonds within the homogalacturonan chain and exo-polygalacturonases release D-galacturonic acid from the nonreducing ends of galacturonan chains with inversion of the anomeric configuration [2]. Efficient hydrolysis of the homogalacturonan chains by polygalacturonase (PG) enzymes is of substantial interest industrially in the clarification of juices and retting of flax where hydrolysis of pectin is desirable [1,3]. Conversely, PG enzymes are targets for inhibition since secretion of PGs by pathogenic bacterial and fungal species contribute to the invasion and maceration of economically important crops [1,4,5].

PG genes and enzymes have been isolated and characterized from a wide variety of microorganisms. The biochemical characteristics, catalytic constants along with pH and temperature stability/optima, have been reported for a number of endo- and exo-PG enzymes [6–8]. PG enzymes, much like glucoamylase, generally demonstrate a decreasing K_m and an increasing k_{cat} with increasing length of the carbohydrate oligomer [8–10]. Eventually, this trend does not continue with increased length of the carbohydrate oligomer, providing clues to the number of binding sites in the substrate binding pocket. While the kinetic constants help to define the number of binding sites in the binding pocket and theoretical methods based on these constants have been used to calculate individual subsite affinities [8,10], no direct measurement of free energy changes associated with binding in each of the subsites has been reported for PG enzymes.

Isothermal titration calorimetry (ITC) enables the direct measurement of free energy upon binding of substrate along with the enthalpic and entropic contributions providing some clues on the weak enzyme/substrate interactions [11–14]. ITC has been previously used to determine the thermodynamics of substrate binding for glycoside hydrolases such as chitinase and xylanase [12–14] and provides a common method for determining binding interactions for this class of enzymes. In this work, we report on the binding energetics of a series of galacturonic acid oligomers in the individual subsites of a *Rhizopus oryzae* exo-polygalacturonase, RPG16, using ITC.

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0040-6031/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.tca.2011.10.029

2. Experimental

2.1. Reagents

All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise noted. Di-galacturonic acid (Gal*p*A)$_2$ and tri-galacturonic acid (Gal*p*A)$_3$ of 90% purity were purchased and used without further purification. The remaining galacturonic acid oligomers, tetra-galacturonic acid (Gal*p*A)$_4$, and penta-galacturonic acid (Gal*p*A)$_5$ were isolated and purified using a multi-step procedure. Polygalacturonic acid (Fluka Biochemical, St. Louis, MO) (1% (w/v) at pH 4.4) solution was autoclaved at 121 °C for 60 min [15]. The reaction was dried and enrichment of (Gal*p*A)$_{2-10}$ was achieved by ethanol precipitation in 400 mg aliquots (ethanol 50% (v/v), −20 °C, 12 h) to precipitate the large oligomers. The (Gal*p*A)$_{2-10}$ oligomers were then recovered by precipitation through increasing the ethanol concentration to 80% (v/v) (−20 °C, 12 h). The precipitate enriched in (Gal*p*A)$_{2-10}$ oligomers was pelleted by centrifugation, supernatant discarded, the pellet dried, and resuspended in 500 μL of distilled and deionized H_2O (ddH_2O). The (Gal*p*A)$_{2-10}$ oligomer fraction was applied to a 22 mm × 250 mm Carbo-Pac PA-100 column (Dionex, Sunnyvale, CA) running at 10 mL/min with 100 mM NaOH as the buffer and the oligomers separated with a 5–45% 1 M sodium acetate gradient. Fractions containing the individual (Gal*p*A)$_{3-5}$ oligomers were collected and the pH adjusted to 6.0 by addition of 1 M HCl. Oligomer fractions were ethanol precipitated (80% ethanol, −20 °C, 12 h) three times, to remove the salts from the chromatographic separation. The final pellet was resuspended in 200 μL 25 mM sodium acetate, pH 4.0. An aliquot of the purified (Gal*p*A) oligomers was diluted 100-fold and subjected to High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) to determine the purity of the oligomers following conditions previously reported [16]. The remainder of the diluted oligomers was digested to completion using *R. oryzae* RPG16 and subjected to HPAEC-PAD for quantification using galacturonic acid as the standard. This multi-step process routinely resulted in the production of (Gal*p*A) oligomers with 90% or greater purity and concentrations in excess of 5 mM.

R. oryzae RPG16 D209N was created using the Quick-Change II site directed mutagenesis kit according to manufacturer's instructions (Stratagene, La Jolla, CA) using RPG16 as the template [16]. The RPG16 D209N construct was sequenced to ensure the fidelity of the construct. *Pichia pastoris* strain X-33 (Life Technologies, Carlsbad, CA) was transformed with the mutant construct and the enzyme expressed and purified as previously reported [15]. Protein concentrations were determined at 280 nm using an extinction coefficient of $\varepsilon_m = 3.39 \times 10^4\ M^{-1}\ cm^{-1}$ according to Gill and von Hippel [17]. The RPG16 D209N mutant was found to have a 4000-fold reduced specific activity relative to the wild type rendering the enzyme essentially inactive.

2.2. ITC binding experiments

The VP-ITC system was used with VP viewer software and Origin 7.0 for graphing functions (Microcal, Northampton, MA) [11]. The volume of the calorimeter cell was 1.3519 mL and experiments were performed at 20 °C. Sodium acetate (25 mM) pH 4.0 was used for all substrate and enzyme dilutions and was also used in the ITC reference cell. All solutions were thoroughly degassed immediately before use. For each experiment, the purified (Gal*p*A) oligomers at the desired concentration were placed in the syringe with the stir rate set to 310 rpm. RPG16 D209N in 25 mM sodium acetate pH 4.0 was placed in the cell. The concentration of enzyme used, with the exception of the (Gal*p*A)$_5$ experiments, was such that the Wiseman *c* value was between 10 and 1000 and was calculated according to equation 1 [11].

$$c = nK_a(M)_t \quad (1)$$

where n is the stoichiometry of the reaction, K_a is the equilibrium binding association constant, and $(M)_t$ is the protein concentration. After equilibration of the calorimeter at 20 °C, the experiments were set to record a baseline for 60 s followed by 20 injections every 120 s for (Gal*p*A)$_{2-3}$ and every 180 s for (Gal*p*A)$_{4-5}$ to ensure a return to the baseline. Binding experiments performed with (Gal*p*A)$_3$ obtained in the same way as (Gal*p*A)$_4$ and (Gal*p*A)$_5$, to ensure against potential differences of the purchased substrate and the prepared substrates, resulted in thermodynamic parameters within experimental error (data not shown). Data were corrected for heat of dilution, which was minimal, before deconvolution of the data. The values for n, ΔH, K_a, and ΔS were obtained from the thermograms using Origin 7 and ΔG was calculated using Eq. (2).

$$\Delta G = -RT \ln K_a = \Delta H - T\Delta S \quad (2)$$

Error for ΔG, ΔH, and K_d ($1/K_a$) was obtained as the standard deviation of at least three independent experiments and error for $T\Delta S$ and ΔS was calculated by propagation of errors.

3. Results and discussion

To aid in the determination of the binding thermodynamics, a catalytic mutant of *R. oryzae* exo-polygalacturonase, RPG16 D209N, was used. This aspartic acid residue, conserved in PG enzymes acts as a general acid during catalysis [18,19] and the RPG16 D209N mutant enzyme was found to be essentially inactive with a catalytic rate 4000-fold less than the wild-type enzyme (data not shown). The thermograms for (Gal*p*A)$_{2-5}$ oligomers showed that all binding reactions were exothermic (Fig. 1). The thermodynamic parameters and the binding constants determined from the thermograms are summarized in Table 1. The titration curves all fit to the single site binding model with calculated n values of 1 ± 0.08. Binding for all substrates is enthalpically driven as the enthalpy values determined from binding experiments were negative for all substrates, with ΔH values ranging from −15 kcal/mol for (Gal*p*A)$_2$ to −18.5 kcal/mol for (Gal*p*A)$_5$. The highly favorable enthalpy values offset the negative entropy values resulting in negative ΔG values that increased with increasing length of the (Gal*p*A) oligomers. The K_d values also decreased with increasing length of the oligomer, although the difference between the values obtained for K_d, ΔH, and ΔS for (Gal*p*A)$_4$ and (Gal*p*A)$_5$ are minimal. This trend for K_d is similar to that seen previously with the glycoside hydrolases chitinase and xylanase [12–14]. The extremely tight binding of the (Gal*p*A)$_4$ and (Gal*p*A)$_5$ substrates resulted in Wiseman *c* values that were at the upper edge of the *K* window in the case of (Gal*p*A)$_4$ and slightly outside the *K* window for (Gal*p*A)$_5$. While this is less than ideal, it is possible to measure *K* values in this range if the enthalpy is greater than 10 kcal/mol [11] and is the case for all of the substrates used in this work.

Catalysis of substrates by RPG16 was previously shown to result in the production of galacturonic acid with no detection of other oligomers suggesting that RPG16 has a single subsite toward the reducing end (Fig. 2) [16]. While RPG16 demonstrates a preference for cleavage at a single subsite toward the reducing end, the decreasing K_d values with oligomer length suggests that RPG16 potentially has 5 subsites, labeled −1 to +4 according to convention [20], that contribute to substrate binding (Fig. 2). The potential for 5 subsites is further strengthened by a plot of free energy vs. number of (Gal*p*A) units that demonstrates a linear relationship with an average increase of −0.9 kcal/mol from (Gal*p*A)$_2$ to (Gal*p*A)$_5$ (data not shown). Combining the information on substrate binding

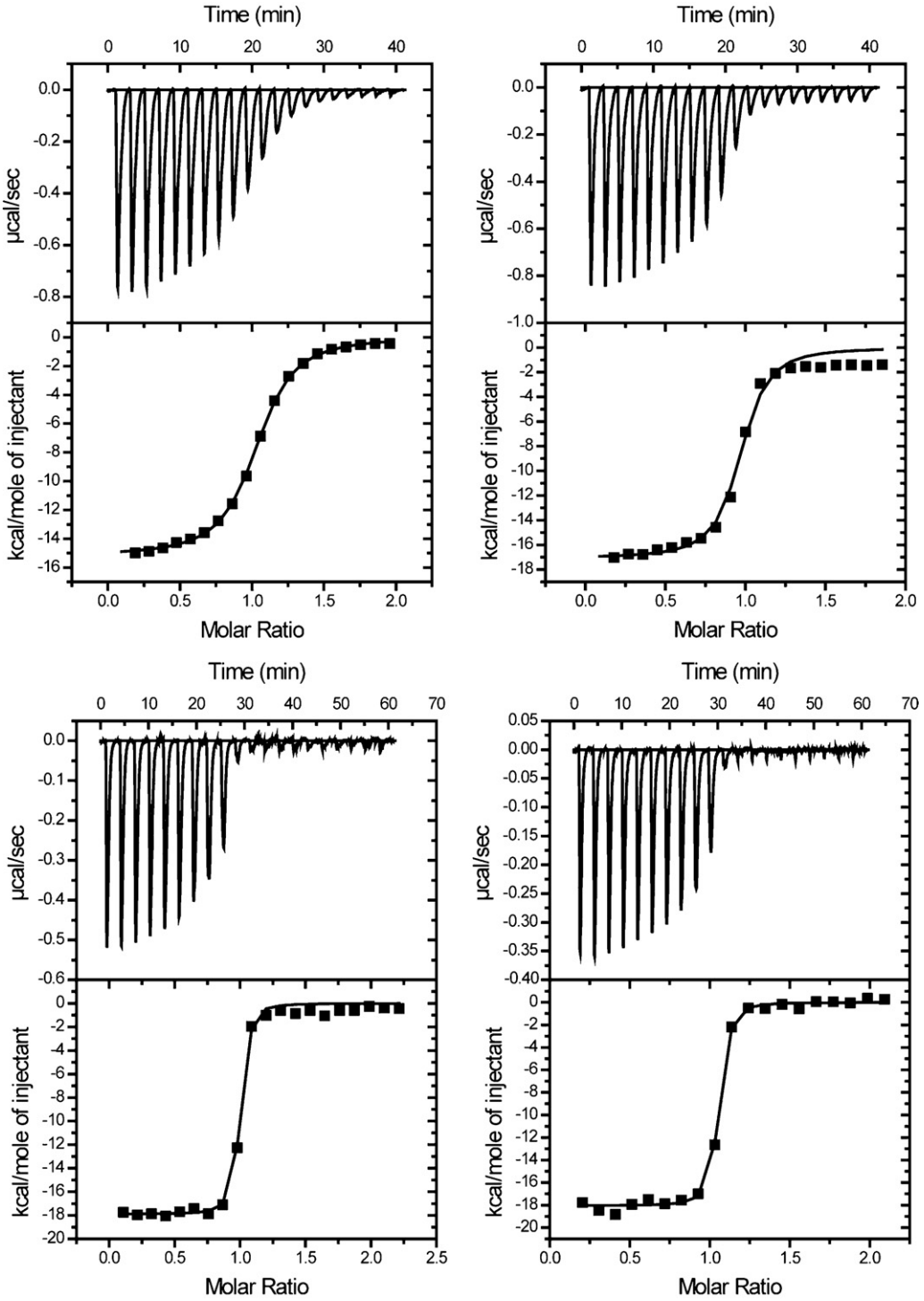

Fig. 1. Binding isotherms (lower panels) with theoretical fits and thermograms (upper panels) obtained from binding experiments for the binding of (Gal*p*A)$_2$ (top left), (Gal*p*A)$_3$ (top right), (Gal*p*A)$_4$ (bottom left), and (Gal*p*A)$_5$ (bottom right) to exo-polygalacturonase RPG16 D209N. Binding experiments were performed in triplicate, with a typical thermogram and binding isotherm with fit shown for each (Gal*p*A) oligomer. Binding experiments were performed at 20 °C in 25 mM sodium acetate pH 4.0.

Table 1
Thermodynamic parameters of galacturonic acid oligomers binding to the inactive mutant RPG16 D209N determined using ITC. Experiments were performed at T = 20 °C in 25 mM sodium acetate, pH 4.0.

Substrate	K_d (nM)	ΔG° (kcal/mol)	ΔH° (kcal/mol)	$T\Delta S^\circ$ (kcal/mol)	ΔS° (cal/K mol)
(Gal*p*A)$_2$	244 ± 2.7	−8.9 ± 0.05	−15.1 ± 0.04	−6.2 ± 0.1	−21.2 ± 0.3
(Gal*p*A)$_3$	98.2 ± 0.4	−9.4 ± 0.08	−17.2 ± 0.3	−7.8 ± 0.5	−26.7 ± 1.0
(Gal*p*A)$_4$	7.5 ± 0.4	−10.9 ± 0.01	−18.1 ± 0.2	−7.2 ± 0.3	−24.5 ± 0.8
(Gal*p*A)$_5$	2.2 ± 0.5	−11.6 ± 0.1	−18.5 ± 0.7	−6.9 ± 0.9	−23.6 ± 2.2

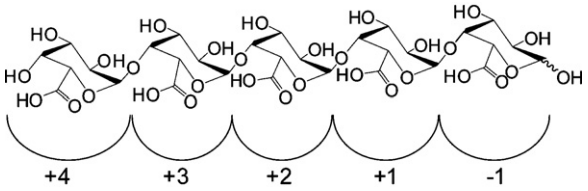

Fig. 2. Schematic depiction of substrate (Gal*p*A)$_5$ binding in the subsites of RPG16 exo-polygalacturonase. RPG16 catalyzes the scission of a single galacturonic acid molecule from the reducing end of (Gal*p*A) oligomers.

Table 2
Thermodynamic parameters associated with binding of galacturonic acid oligomers to individual subsites of RPG16 D209N. Parameters were calculated based on data presented in Table 1 and previously reported biochemical data [16].

Subsite	−1/+1	+2	+3	+4
ΔG (kcal/mol)	−8.9	−0.5	−1.5	−0.7
ΔH (kcal/mol)	−15.1	−1.8	−0.9	−0.4
$T\Delta S$ (kcal/mol)	−6.2	−1.6	0.6	0.3
ΔS (cal/K mol)	−21.2	−5.5	2.2	0.9

determined through biochemical assays with the thermodynamic parameters allows estimation of binding energy for individual subsites [12]. The calculated thermodynamic parameters for (Gal*p*A) oligomers to subsites +2/+4 along with the values for the combined −1/+1 subsite are shown in Table 2. The free energy, largely due the favorable enthalpy contribution, is greatest for the −1/+1 subsite. The enthalpy drops substantially for subsite +2 and decreases an additional 0.9 kcal/mole at subsite +3. The entropic penalty at subsite +2 decreases dramatically from the −1/+1 combined subsite. While there is an entropic penalty for binding in subsite −1/+1 and subsite +2, there is an entropic contribution at subsites +3 and +4, that leads to an increase in free energy at these two subsites relative to subsite +2 (Table 2). The individual subsite binding energies would suggest that binding of substrate is largely the result of interactions at the −1/+1 subsites and is in general agreement with the theoretical subsite affinity calculations performed with endo-polygalacturonase enzymes [8,10]. The catalytic residues along with two additional conserved charged residues previously shown to be involved in substrate binding are found in the −1/+1 subsite. Although binding at subsite +4 appears to be relatively weak and the existence of the +3 subsite has been questioned for *Aspergillus* endo-polygalacturonase enzymes [18,21], the data presented here suggests that at least some contribution is made to substrate binding beyond subsite +2. An amino acid residue proposed to be involved in binding substrate in subsite +3, Y326 in *Aspergillus niger* Endo II, but mutation of this residue had no effect on substrate binding [21]. In some respects, it may not be all that surprising that tyrosine or other aromatic residues are not involved in binding (Gal*p*A) substrates in PG enzymes. Galacturonans are acid sugars and might be expected to also bind through ionic interactions unlike the neutral sugars such as xylans that have been shown to bind to xylanases through stacking interactions [13]. Binding of (Gal*p*A) oligomers to RPG16 appears to be more similar to chitooligosaccharides binding to GH19 chitinase enzymes rather than xylans binding to xylanases [13,14]. In addition, while the Y326 residue found in the *A. niger* Endo II enzyme is not conserved in RPG16, it does not preclude the possibility that there are other residues potentially unique to RPG16 that may contribute to binding in this subsite. Unfortunately, a crystal structure is not available for RPG16 making this last point difficult to discern.

The data presented here on the thermodynamics of (Gal*p*A) oligomers binding to RPG16 provide insight into the dynamics of substrate binding in polygalacturonase enzymes. The determination of the individual subsite binding energies, not only provides a baseline for binding energies, it acts as the starting point for additional mutational studies to more fully understand the binding of substrates to this family of enzymes. Additionally, the use of ITC with this family of enzymes could also potentially provide an avenue, in addition to crystal structures, for the study of determinants involved in binding of plant polygalacturonase-inhibiting proteins to PG enzymes.

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