Kinetic characterization of Aspergillus niger chitinase CfcI using a HPAEC-PAD method for native chitin oligosaccharides
van Munster, Jolanda M.; Sanders, Peter; ten Kate, Geralt A.; Dijkhuizen, Lubbert; van der Maarel, Marc J. E. C.

Published in:
Carbohydrate Research

DOI:
10.1016/j.carres.2015.01.014

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2015

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment.

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.
Kinetic characterization of Aspergillus niger chitinase CfcI using a HPAEC-PAD method for native chitin oligosaccharides

Jolanda M. van Munster a,*, Peter Sanders a, y, Geralt A. ten Kate a, Lubbert Dijkhuizen a, Marc J.E.C. van der Maarel a, b

a Microbial Physiology Research Group, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, Nijenborgh 7, 9747 AG Groningen, The Netherlands
b Aquatic Biotechnology and Bioproduct Engineering Department, Institute for Technology and Management (ITM), University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands

**A R T I C L E   I N F O**

Article history:
Received 17 November 2014
Accepted 23 January 2015
Available online 3 February 2015

Keywords:
HPAEC-PAD
Method validation
Chitin oligosaccharide
Chitinase
Aspergillus niger
Carbohydrate binding module

**A B S T R A C T**

The abundant polymer chitin can be degraded by chitinases (EC 3.2.1.14) and β-N-acetyl-hexosaminidases (EC 3.2.1.52) to oligosaccharides and N-acetyl-glucosamine (GlcNAc) monomers. Kinetic characterization of these enzymes requires product quantification by an assay method with a low detection limit, preferably compatible with the use of native, non-labeled substrates. Here we report a quantitative HPAEC-PAD method that allows fast separation of chitin oligosaccharides (COS) ranging from (GlcNAc)1 to (GlcNAc)6 at detection limits of 1–3 pmol and a linear range of 5–250 pmol. Quantification under intra- and interday precision conditions was performed with 2.1–5.4% relative standard deviation (RSD) and 1.2–10.3% RSD, respectively. This method was successfully used for the determination of the kinetic parameters of the Aspergillus niger chitinase CfcI with native COS. CfcI was recently shown to release GlcNAc from the reducing end of COS, a new activity for fungal chitinases. A Carbohydrate Binding Module of family 18 (CBM18) is inserted in the CfcI catalytic domain. Site directed mutagenesis was used to assess the functionality of this CfcI-CBM18: four of its key amino acids were replaced by glycine residues, yielding CfcISYNF. Comparison of the kinetic parameters of CfcI and CfcISYNF confirmed that this CBM18 is functionally involved in catalysis.

© 2015 Elsevier Ltd. All rights reserved.

1. Introduction

Chitin, a β-(1,4) linked N-acetyl-glucosamine polymer, is abundantly present as a structural component of the arthropod exoskeleton and fungal cell walls. Chitinases (EC 3.2.1.14) and β-N-acetylhexosaminidases (EC 3.2.1.52) hydrolyze chitin to chitin oligosaccharides (COS) or N-acetyl-glucosamine GlcNAc monomers. Several methods are available to characterize chitinase activity, employing chromatographic substrates,1 HPLC,2–4 mass spectrometry5,6 or capillary electrophoresis.7–9 The use of chromogenic substrates is convenient but kinetic parameters determined using these substrates may differ significantly from those found with native substrates.6 Although advances have been made in recent years, the number of methods for analysis of native COS in low concentrations is still limited. Most methods are semi-quantitative,1 require derivatization,1,8 have a relatively high detection limit9 or require specialized equipment.5

High performance anion exchange chromatography (HPAEC) combined with pulsed amperometric detection (PAD) is a powerful tool for the separation, sensitive detection and quantitation of a range of underivatized carbohydrates.10 HPAEC-PAD is widely used in carbohydrate analysis and a wide range of protocols and
methods is available.\textsuperscript{11} Separation of COS by HPAEC-PAD has previously been reported, but neither of these methods has found wide application.\textsuperscript{10,12–15} Separation of COS on a CarboPac PA1 column with 18 mM sodium hydroxide allowed detection of GlcNAc and (GlcNAc)$_2$, but failed to separate (GlcNAc)$_3$ to (GlcNAc)$_4$.\textsuperscript{15} Better separation of longer COS has been described with 25 mM sodium hydroxide using a MA1 column,\textsuperscript{12} but the separation time of 3 h per sample is highly inefficient for analysis of multiple samples. Furthermore, the alkaline pH conditions usually employed during HPAEC are detrimental for the reducing end GlcNAc (as discussed in \textsuperscript{10}). In this study, a HPAEC-PAD method developed for the separation and sensitive detection of non-labeled COS in the range of at least (GlcNAc)$_1$ to (GlcNAc)$_3$ is described and validated. The separation of COS was performed in water, preventing high pH induced epimerization of COS on the column. Post column addition of sodium hydroxide allowed detection and quantification by PAD. Alternatively, the use of water as low salt, neutral pH liquid phase would allow direct coupling to on line mass spectrometry equipment. This powerful combination of analytical techniques has already been applied to successfully separate and identify components of a complex mixture of lignocellulose-derived oligosaccharides.\textsuperscript{16}

To demonstrate the suitability of the validated HPAEC-PAD method in chitinase activity assays, it was applied to the determination of the kinetic parameters of Aspergillus niger chitinase CfcI. \textit{A. niger} is a common saprophytic fungus of significant industrial importance. The genome of this fungus contains 13 putative chitinases belonging to glycoside hydrolase family 18 (GH18) (http://www.cazy.org). One of these chitinases, CfcI, is a representative of a phylogenetic cluster conserved within Aspergilli. We recently showed that CfcI differs from previously characterized fungal family GH18 members in displaying a novel reaction specificity: CfcI releases GlcNAc monomers from the reducing end of COS.\textsuperscript{17}

As is the case for other exo-chitinases, the catalytic domain of CfcI consists of a TIM-barrel with an additional $\alpha$-$\beta$ domain inserted between $\beta$-sheet 7 and $\alpha$-helix 7. However, only in CfcI and its homologs an additional carbohydrate binding module of family 18 (CBM18) is integrated in this $\alpha$-$\beta$ domain. As the position of the CBM18 insertion is in close proximity to the CfcI active site, this CBM18 may be functionally involved in catalysis. CBM18 domains are present in plant lectins, anti-microbial peptides and are found attached to fungal chitinases.\textsuperscript{18–21} Their characterization has shown that these modules can bind COS. Aromatic amino acids Trr$_{21}$ and Tyf$_{23}$ (numbering as in the lectin hvenin) stack to the bound GlcNAc residues, resulting in three binding subsites.\textsuperscript{12,27} Mutation of these aromatic amino acids to alanine in the \textit{Amaranthus caudatus} antimicrobial peptide resulted in a decrease in substrate affinity.\textsuperscript{13} Mutational analysis indicated that the hydrogen bond between Ser$_{19}$ and the acetamide group of the substrate significantly contributes to stability and specificity of COS binding. Furthermore, the acetamide group of the substrate interacts with the aromatic ring of Tyf$_{30}$.\textsuperscript{25} Ser$_{19}$ is conserved in CfcI-CBM18 (Ser$_{122}$) and aromatic residues are present at positions 21 (Tyf$_{324}$) and 30 (Phe$_{333}$), but an Asn residue is found at position 23 (Asn$_{326}$). Replacement of the aromatic residue at position 23 with Asn, Thr or Ser, is conserved in, but not limited to, Aspergillus chitinases of the phylogenetic cluster containing CfcI. To determine whether CfcI-CBM18 is functionally involved in catalysis, we constructed a mutant enzyme in which these 4 amino acids were replaced by Gly residues (CfcI$_{SYNF}$). Comparison of the kinetic parameters of the CfcI wild type and mutant enzyme CfcI$_{SYNF}$ with native COS, using the newly developed HPAEC-PAD method, confirmed that CBM18 is functionally involved in catalysis.

\section*{2. Results}

\subsection*{2.1. Chromatography conditions}

To determine kinetic parameters of chitinases with native substrates, a sensitive and fast assay is needed to separate, identify and quantify reaction products. HPAEC-PAD has proven to be an effective, sensitive method, which allows separation and quantification of a broad range of carbohydrates without the need for labeling. We have investigated whether HPAEC-PAD also can be used as assay method for the separation and quantification of COS.

After testing various conditions, we found that COS of DP2-6 could be separated reasonably well within 6 min at 15 °C and 20 °C using 25 mM sodium hydroxide supplemented with 22.2 mM sodium acetate, but complete baseline separation was not achieved. Separation was dependent on preconditioning of the column using steps 2, 3 and 4 as described in experimental section 4.2. At 10 °C, also decacetylated chitin oligosaccharides (GlcN oligosaccharides) of DP2-6 could be separated. However, during COS separation additional peaks were found. Alkaline conditions above pH 9 are known to induce epimerization of GlcNAc residues to ManNAc.\textsuperscript{26,27} Incubation of COS in sodium hydroxide before analysis increased the area of the additional peaks from 6% to 30% of the total peak area, indicating epimerization of COS may indeed occur under the chromatography conditions used. Because the peaks of COS are adjacent in the 6 min chromatogram, the additional peaks partly overlap with the COS peaks. The described chromatography conditions are therefore suitable for the fast separation and detection of COS present in roughly equal amounts, but less so for quantification of COS during kinetic studies where the substrate peak is high compared to the product peak.

To improve separation and avoid interference of epimerization peaks, we optimized the above protocol using separation in ultrapure water, followed by post-column addition of base. Optimized preconditioning and temperature, as detailed in the experimental section 4.2, allowed baseline separation of COS in the range of at least (GlcNAc)$_1$ to (GlcNAc)$_3$. Although small additional peaks (2–4% of total peak area) were still observed for some COS, these did not interfere with quantification. Injection of each COS individually allowed determination of typical retention times. The order of elution of COS observed here is unusual compared to other oligosaccharides.
(Fig. 1). Oligosaccharides with the highest DP eluted first, followed by COS with a lower DP, corresponding to previously described HPAEC separations of COS.11 The elution order, however, was dependent on column temperature. At 25 °C, GlcNAc eluted between (GlcNAc)$_3$ and (GlcNAc)$_2$. At lower temperatures the retention time increased and at 20 °C or 15 °C the monomer peak overlapped with (GlcNAc)$_3$, either partially or completely, respectively (data not shown).

2.2. Method validation

To investigate the selectivity of the method, a range of monomers was injected. Baseline separation of COS in the range of GlcNAc to at least (GlcNAc)$_6$ or the 4-nitrophenol labeled GlcNACP$_{pNP}$, (GlcNAc)$_2$pNP and (GlcNAc)$_3$pNP was successfully performed. N-Acetyl-galactosamine eluted at a retention time similar to N-acetyl-glucosamine. Elution of glucosamine oligosaccharides GlcN(−GlcN)$_6$ or galactosamine (GalN) was not detected up to 30 min after injection, suggesting that these carbohydrates are retained on the column. Taken together, within the group of amino sugars, the assay is specific for acetylated carbohydrates. In addition, various monomers without an amino group, such as glucose, mannose and galactose, were detected.

Precision of the optimized method was determined by measuring known amounts of analytes under sample conditions. Variations in peak areas (as ratio of I.S. peak area) and retention times were determined to establish repeatability (intraday) and intermediate (interday) precision (Table 1). Under repeatability conditions, the retention time of all tested analytes was stable with 0.1–0.4% RSD over 6 h and 0.5–0.6% RSD over 10 h. The detected analyte amounts varied between 1.2 and 5.4% RSD with a mean of 2.8% RSD. Measurements for intermediate precision were performed by two individuals on five separate days using five separately prepared batches of buffer. The variation in detected analyte amounts was with a mean of 3.3% and range of 1.2–10.3% RSD higher than found under repeatability conditions. The accuracy of the method, determined under intermediate precision conditions, is satisfactory with recovery rates of 88.2–98.7% (Table 2).

The linear range for (GlcNAc)$_4$− was 5–500 pmol (corresponding to 25 μl injections with 0.2–20 μM COS). For (GlcNAc)$_5$ the range was 5–250 pmol and for (GlcNAc)$_6$, 2.5–250 pmol. Coefficients of determination ($R^2$) were at least 0.9994. The limits of detection (LOD) (Table 3), defined as the ‘signal level which may be a priori expected to lead to detection’ was in the range of 1–4 pmol (0.04–0.16 μM). The quantification limits (LOQ (Table 3), defined as the smallest amount of analyte that can be quantified with a limit of error of 10% RSD, were calculated to be in the range of 1–10 pmol (0.02–0.4 μM). Experimental verification gave values for LOD and LOQ of the same order of magnitude.

2.3. Determination of kinetic parameters of CfcI and CfcI SYNF

The suitability of the method was evaluated by determining kinetic parameters ($K_m$ and $k_{cat}$) of A. niger CfcI chitinase. CfcI was produced in E. coli with an N-terminal 6xHis-tag, and purified to near homogeneity. CfcI was obtained with a specific activity of 5.6±0.2 U mg$^{-1}$, determined with (GlcNAc)$_3$−pNP, with a yield of 2.1±0.3 mg protein per liter culture (mean±SE, n=2). This is comparable to the 4–9 U mg$^{-1}$ and 2.1 mg protein per liter culture reported previously for CfcI-MBP.12 Kinetic parameters of CfcI were determined with three substrates using the validated HPAEC-PAD method. The results (Table 4) indicate that the catalytic efficiency $k_{cat}/K_m$ of CfcI increased with the chain length of the substrate. This increase was mainly caused by a higher affinity for the substrate, as the $K_m$ decreased with substrate chain length but $k_{cat}$ remained stable at 12–16 s$^{-1}$.

To investigate whether the CBM18 in CfcI has a function in catalysis, we modified key amino acid residues by mutagenesis. From sequence alignments it appeared that Ser322, Tyr324, Asn326 and Trp21, Trp23 and Tyr30 in the lectin hevein. These four amino acids in CfcI CBM18 were replaced by glycine residues, resulting in CfcI SYNF. CfcI SYNF protein was produced as described in Methods with a yield of 1.1±0.3 mg protein per liter culture, showing a specific activity of 5.8±0.2 U mg$^{-1}$ with (GlcNAc)$_3$−pNP, similar to the activity of wild type CfcI. Separation and identification of CfcI SYNF reaction products showed a product profile similar to that of the wild type CfcI, with GlcNAc as the main reaction product. Comparison of the kinetic parameters of CfcI SYNF and CfcI (Table 4) revealed that the affinity of CfcI SYNF for substrates (GlcNAc)$_4$ and (GlcNAc)$_3$ is lower, since these $K_m$ values had significantly (p<0.01 and p=0.03, respectively) increased with approximately 150%. Although not significant, the $k_{cat}$ values of CfcI SYNF for substrates (GlcNAc)$_4$ and (GlcNAc)$_3$ appeared to increase as well, resulting in a catalytic efficiency $k_{cat}/K_m$ of CfcI SYNF similar to that of CfcI. At relatively low substrate concentrations the activity of CfcI SYNF thus is markedly lower than observed for the

| Table 1 Method precision, given as mean ratio of analyte peak area (Aa)/IS peak area (A0) and RSD (%). Results given under repeatability are representative values from one of two performed sets of measurements |
|---|---|---|---|---|---|---|---|---|
| Analyte | Repeatability (n=6) | Intermediate precision (n=5) |
| | Peak area (Aa/A0) | Retention Time (min) | Peak area (Aa/A0) | Retention Time (min) |
| 50 pmol | 250 pmol | Mean | RSD | Mean | RSD | Mean | RSD |
| (GlcNAc)$_3$ | 0.19 | 2.9 | 1.20 | 4.3 | 10.4 | 0.5 | 0.24 | 14.6 | 1.37 | 8.6 | 10.4 | 2.7 |
| (GlcNAc)$_2$ | 0.30 | 3.2 | 1.56 | 4.0 | 13.5 | 0.5 | 0.35 | 14.8 | 1.78 | 7.3 | 13.5 | 3.5 |
| (GlcNAc)$_3$ | 0.32 | 2.1 | 1.77 | 3.9 | 9.4 | 0.6 | 0.37 | 10.3 | 1.97 | 6.6 | 9.4 | 3.8 |
| (GlcNAc)$_4$ | 0.35 | 2.9 | 1.84 | 4.8 | 7.2 | 0.6 | 0.42 | 5.7 | 2.10 | 7.8 | 7.2 | 4.0 |
| (GlcNAc)$_5$ | 0.40 | 5.3 | 1.96 | 4.2 | 5.8 | 0.6 | 0.44 | 7.8 | 2.27 | 8.6 | 5.8 | 4.2 |
| (GlcNAc)$_6$ | 0.38 | 2.9 | 1.87 | 4.0 | 4.8 | 0.6 | 0.43 | 8.6 | 2.18 | 9.4 | 4.8 | 4.3 |

The suitability of the method was evaluated by determining kinetic parameters ($K_m$ and $k_{cat}$) of A. niger CfcI chitinase. CfcI was produced in E. coli with an N-terminal 6xHis-tag, and purified to near homogeneity. CfcI was obtained with a specific activity of 5.6±0.2 U mg$^{-1}$, determined with (GlcNAc)$_3$−pNP, with a yield of 2.1±0.3 mg protein per liter culture (mean±SE, n=2). This is comparable to the 4–9 U mg$^{-1}$ and 2.1 mg protein per liter culture reported previously for CfcI-MBP. Kinetic parameters of CfcI were determined with three substrates using the validated HPAEC-PAD method. The results (Table 4) indicate that the catalytic efficiency $k_{cat}/K_m$ of CfcI increased with the chain length of the substrate. This increase was mainly caused by a higher affinity for the substrate, as the $K_m$ decreased with substrate chain length but $k_{cat}$ remained stable at 12–16 s$^{-1}$.

To investigate whether the CBM18 in CfcI has a function in catalysis, we modified key amino acid residues by mutagenesis. From sequence alignments it appeared that Ser322, Tyr324, Asn326 and Trp21, Trp23 and Tyr30 in the lectin hevein. These four amino acids in CfcI CBM18 were replaced by glycine residues, resulting in CfcI SYNF. CfcI SYNF protein was produced as described in Methods with a yield of 1.1±0.3 mg protein per liter culture, showing a specific activity of 5.8±0.2 U mg$^{-1}$ with (GlcNAc)$_3$−pNP, similar to the activity of wild type CfcI. Separation and identification of CfcI SYNF reaction products showed a product profile similar to that of the wild type CfcI, with GlcNAc as the main reaction product. Comparison of the kinetic parameters of CfcI SYNF and CfcI (Table 4) revealed that the affinity of CfcI SYNF for substrates (GlcNAc)$_4$ and (GlcNAc)$_3$ is lower, since these $K_m$ values had significantly (p<0.01 and p=0.03, respectively) increased with approximately 150%. Although not significant, the $k_{cat}$ values of CfcI SYNF for substrates (GlcNAc)$_4$ and (GlcNAc)$_3$ appeared to increase as well, resulting in a catalytic efficiency $k_{cat}/K_m$ of CfcI SYNF similar to that of CfcI. At relatively low substrate concentrations the activity of CfcI SYNF thus is markedly lower than observed for the
wild type enzyme. We conclude that CBM18 has a functional role in catalysis by the wild type CfcI protein, increasing affinity of binding at low substrate concentrations.

3. Discussion

Specific and sensitive separation, identification and quantification methods for COS are essential to assess their functions and potential applications in both glycobiology and enzymology. The HPAEC-PAD method described here provides such a sensitive, relatively fast, quantitative assay for these acetylated carbohydrates. The range of 2.5 pmol to at least 250 pmol in which quantitative detection is possible, and the complete baseline separation of a series of (GlcNAc)1–6 in 20 min per sample, outperforms both previously described and commonly used HPLC methods for underivatized COS as well as HPAEC-PAD methods for COS separation. The described HPAEC-PAD method is suitable for the analysis of clean mixtures of GlcNAc oligosaccharides, which are for example used during enzyme assays. The method allows selective detection and quantification of acetylated COS in mixtures of N-acetyl-glucosamine and glucosamine monomers or oligosaccharides. The observed separation of COS when using water as mobile phase depends on preconditioning of the column. It must be noted that the assay is less suitable for the analysis of samples with a matrix containing large amounts of salt or buffers: injection of acetate or sodium hydroxide is detrimental to the repeatability of retention times and peak areas. Furthermore, as the system is sensitive to carbonate accumulation on the column, it is essential to prepare CO2 free eluent. This is consistent with the fact that after measuring an extended number of samples (70–140 in our system), removal of the carbonate accumulated on the column, by rinsing with solution B, improved method precision.

We demonstrated the suitability of the method by identifying differences in the kinetic properties of A. niger CfcI wild type and mutant enzymes acting on native substrates. Determination of kinetic parameters of CfcI revealed Km values in the range of 15–50 μM and kcat values in the range of 12–16 s⁻¹. These kinetic parameters of CfcI are in the same range as those of other fungal chitinases. Reports on kinetic constants of several fungal chitinases, obtained with labeled COS, show that Km values are in the lower μM range. Based on these values, we calculated the percentage of binding in the CfcI enzyme: mutagenesis of key amino acids in this enzyme revealed Km values in the range of 0.2 mM and 2.0 mM for the substrates (GlcNAc)2-pNP and (GlcNAc)2-pNP, respectively. Evidence has been reported previously that enzyme kinetic data obtained with labeled substrates may differ significantly from that obtained with native substrates. For example, for Serratia marcescens ChiB a kcat of 57 s⁻¹ and Km of 7 μM were reported using (GlcNAc)2-4MU but with the native (GlcNAc)4 a kcat of 28 s⁻¹ and Km of 4 μM were found.21,32 Our data shows that CfcI-CBM18 is important for substrate binding in the CfcI enzyme: mutagenesis of key amino acids in this CBM18 caused a decrease in substrate affinity. Rather surprisingly, and not understood at present, CfcI SYN appears to have a higher turnover number kcat without a functional CBM18. Nevertheless, the overall catalytic efficiency kcat/Km of the two enzymes remained similar. In conclusion, comparison of the kinetic parameters of the CfcI wild type and mutant enzyme CfcI SYN with native COS, using the newly developed HPAEC-PAD method, showed that CBM18 is functionally involved in catalysis at low substrate concentrations.

4. Experimental

4.1. Chemicals and instruments

GlcNAc and l-Fucose were obtained from Acros Organics (Geel, Belgium), and (GlcNAc)2–(GlcNAc)6 from Megazyme (Bray, Ireland). The 4-nitrophenyl labeled GlcNAc-pNP, (GlcNAc)2-pNP and (GlcNAc)2-pNP were from Sigma Aldrich (Zwijndrecht, the Netherlands). Sodium hydroxide was obtained from J.T. Baker (Deventer, the Netherlands) as 50% (w/v) solution and sodium acetate was from Sigma Aldrich. HPAEC-PAD was performed on an ICS-3000 system ( Dionex, Sunnyvale, CA, USA) consisting of a detector-chromatography module, dual pump module, an autosampler and a thermal compartment. The system was equipped with a 4×250 mm CarboPac PA-1 analytical column (Dionex) and 4×50 mm CarboPac PA1 guard column (Dionex). A home-made device for mixing eluent with post column added solutions was used, consisting of a 3 m peek tubing with an internal diameter of 0.0254 cm (0.01 inches), and internal volume of 152 μl, wound around a plastic cylinder of 16.8 mm diameter. The mixer was installed in line between the mixing tee and the electrochemical detector. The detector was equipped with an Au electrode and a combined pH-Ag/AgCl reference electrode. Reagents were kept under constant helium pressure to ensure CO2 exclusion.

4.2. HPAEC-PAD conditions

The CarboPac PA-1 column was operated at 25 °C. Solution A consisted of 0.1 M sodium hydroxide, solution B of 0.1 M sodium hydroxide with 0.6 M sodium acetate, and solution C consists of ultrapure water (0.22 μm filtered, deionized water, 18.2 MΩ cm⁻¹, Milli-Q, Millipore, Amsterdam, the Netherlands). The column was preconditioned in 5 steps by running at a flow speed of 1 ml.min⁻¹ for 10 min in 100% solution A (step 1), then 10 min in 100% solution B (step 2) followed by 10 min running with 3.7% B, 96.3% A, (step 3) and 20 min with 3.7% B, 21.3% A, 75% C (step 4). The column was then equilibrated by running in solution C for 10 min before sample injection (step 5). Separations were performed while running isocratically in solution C. Generally, 25 μl of (GlcNAc)1–6 standards or samples were injected using the autosampler equipped with a 50 μl sample loop. Standards and samples contained an internal standard (IS) of 10 μM l-fucose. Following separation, 225 mM sodium hydroxide was delivered at 2 ml.min⁻¹ and mixed in the mixing coil, with a 3 s residence time. Detection of sugars was performed during 16 min after injection using a standard quadruple potential

---

Table 3

<table>
<thead>
<tr>
<th>Analyte</th>
<th>LODμmol/20 μl</th>
<th>LODppm (pmol)</th>
<th>LOQμmol/20 μl</th>
<th>LOQppm (pmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(GlcNAc)1</td>
<td>3.0±0.3</td>
<td>0.8</td>
<td>7.5±0.8</td>
<td>2.7</td>
</tr>
<tr>
<td>(GlcNAc)2</td>
<td>3.1±1.8</td>
<td>10.0</td>
<td>7.8±3.0</td>
<td>3.3</td>
</tr>
<tr>
<td>(GlcNAc)3</td>
<td>0.9±1.0</td>
<td>0.7</td>
<td>2.3±2.3</td>
<td>2.2</td>
</tr>
<tr>
<td>(GlcNAc)4</td>
<td>2.4±1.8</td>
<td>0.6</td>
<td>5.8±4.2</td>
<td>2.2</td>
</tr>
<tr>
<td>(GlcNAc)5</td>
<td>1.5±0.9</td>
<td>0.5</td>
<td>3.4±2.1</td>
<td>1.7</td>
</tr>
<tr>
<td>(GlcNAc)6</td>
<td>1.1±0.2</td>
<td>1.0</td>
<td>2.5±0.6</td>
<td>3.3</td>
</tr>
</tbody>
</table>

---

Table 4

<table>
<thead>
<tr>
<th>GlcNAc</th>
<th>Km (μM)</th>
<th>kcat (s⁻¹)</th>
<th>kcat/Km (μM⁻¹ s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(GlcNAc)1</td>
<td>49.5±6.9</td>
<td>15.9±0.9</td>
<td>0.32±0.05</td>
</tr>
<tr>
<td>(GlcNAc)2</td>
<td>19.3±2.4</td>
<td>12.4±0.5</td>
<td>0.65±0.09</td>
</tr>
<tr>
<td>(GlcNAc)3</td>
<td>15.0±0.5</td>
<td>15.1±0.5</td>
<td>1.05±0.14</td>
</tr>
<tr>
<td>(GlcNAc)4</td>
<td>23.3±2.2</td>
<td>156.2±2.2</td>
<td>8.4±0.12</td>
</tr>
</tbody>
</table>

a n.d.: not determined.

b Statistically significant differences between CfcI and CfcISYN.
waveform.\textsuperscript{31} Integration was performed using a Chromelone 6.8 chromatography data system. The concentration of chitin oligosaccharides in each sample was calculated using a calibration curve giving the relation between the analyte amount and the ratios of the peak areas of standards prepared with known amounts of GlcNAc oligosaccharides divided by IS peak areas.

To investigate whether epimerization reactions were likely to cause the presence of extra peaks in the chromatogram, COS were incubated for 0.2 and 7 h in 25 mM NaOH, neutralized and desalted using a Carbograph Ultra-Clean solid phase extraction column (Grace, Deerfield, USA). Subsequently, these samples were dissolved in ultrapure water and injected on the HPAEC-PAD.

4.3. Method validation

Specificity and selectivity was assessed through determination of chromatography retention times of 20 μM of the carbohydrates N-acetyl-glucosamine (with degree of polymerization (DP) 1–6), glucosamine (DP1–6), N-acetyl-galactosamine, galactosamine, glucose, galactose, mannose and 4-nitrophenyl labeled GlcNAc-pNP, (GlcNAc)\(_2\)-pNP and (GlcNAc)\(_3\)-pNP.

The accuracy of the method was determined by measuring known amounts of analytes under conditions resembling those of actual samples. Sample-like backgrounds (10 mM sodium acetate buffer, 1 μg ml\(^{-1}\) BSA) were spiked with 2 μM, 10 μM or 25 μM COS and 25 μl was injected in duplicate. Five determinations were performed under intermediate precision conditions as detailed below.

Precision was determined by measuring known amounts of analyte as repeatability (intraday) and as intermediate (interday) precision. Sample-like backgrounds were spiked with 2 μM, 10 μM or 25 μM COS. Repeatability was determined from six replicate injections for each concentration performed in one HPAEC run. Intermediate precision was estimated from five determinations with the same equipment, performed by two individuals on five separate days using five separately prepared batches of buffer.

To assess the linearity, calibration curves were produced in duplicate as described in experimental section 4.2, using analyte standards (\(n=12\), injected at least in four-fold) in the range of 0.25 nmol–1 nmol (corresponding to 0.01–40 μM in 25 μl injections). The linear range of the curve was assessed by the deviation from the mean of the relative response of analytes, the value of the linear correlation coefficient and the residuals.

The limit of detection (LOD), defined as the ‘signal level, which may be a priori expected to lead to detection’\textsuperscript{28} was estimated from the standard deviation of the calibration curve as LOD=\(l+2(d/s)\) (with \(t\)-student \(t\)-value for \(\alpha=0.05\), \(d=n-2\), 1-sided test, \(l=\)intercept, \(s=\)standard deviation of intercept) and converted to the concentration domain.\textsuperscript{34} The quantification limit (LOQ), defined as the smallest amount of analyte that can be quantified with a limit of error 10% RSD, was estimated from the calibration curve as LOQ=\(l+10s\) (with \(l=\)intercept, \(s=\)standard deviation). Values were calculated as mean±SD of 2 independent sets of measurements with \(n=6\) each. The LOD and LOQ were experimentally verified by determining the value for 3s and 10s from a six fold injection of 5 pmol COS, respectively.

4.4. Enzyme production

Vector pET-15b-25 (encoding A. niger CfcI, GenBank: XM_001400452\textsuperscript{37}) served as template for the production of pET-15b-69, which encodes CfcI\(_{YNF}\) harboring mutations S322G, Y324G, N326C and F333G. Plasmid pET-15b-69 was produced in two rounds of PCR, in a protocol based on the QuikChange kit (Stratagene). Mutations S322G, Y324G and N326G were introduced with primer F64QC (5’- GGAAGTTGTCGGTGATCAGGGC- GAGGGTGCGTACAAACGC-3’, mutations underlined) and its identical, reverse complement, primer R65QC. A second PCR with primers F69QC (5’-GGTACAACCGACGGTCGACAAACG-3’) and its identical, reverse complement, primer R69QC, introduced mutation F333G. PCR was performed for 20 cycles with annealing at 62°C and elongation for 14 min at 72°C, using pfu DNA polymerase. Introduction of the desired mutations was confirmed by nucleotide sequencing. CfcI and CfcI\(_{YNF}\) were expressed in E. coli after induction with 0.1 mM IPTG at 18°C and purified using the N-terminal 6xHis-tag as described before.\textsuperscript{13}

4.5. Enzyme assays

Enzyme activity with (GlcNAc)\(_3\)-pNP was determined as described\textsuperscript{11} to quantify specific activity. One unit of enzyme activity (U) was defined as the amount of activity needed to release 1 μmol pNP min\(^{-1}\) from 1 mM (GlcNAc)\(_3\)-pNP at 45°C, pH 5.0.

Kinetic constants were determined at 45°C in 450 μl incubations containing 10 mM sodium acetate buffer pH 5.0 and 25–600 μM (GlcNAc)\(_3\) or 5–100 μM (GlcNAc)\(_4\)-s. Reactions were started by the addition of 0.11 mM CfcI or CfcI\(_{YNF}\) protein. At 1, 2, 3 and 4 min, 100 μl sample was inactivated in 100 μl ultrapure water containing 20 μM fucose, preheated at 95°C. Product formation was quantified by HPAEC-PAD as described above. The initial rate of GlcNAc release was calculated from at least three data points. Product formation was linear in time under assay conditions used with at maximum 15% of the available substrate consumed. At least three measurements were performed at six substrate concentrations. No indications of transglycosylation or product inhibition were detected in any of the experiments and thus the rates obtained from two (CfcI) or three (CfcI\(_{YNF}\)) independent experiments were each fitted to the Michaelis–Menten equation using OriginPro 8.0. Resulting values are given as mean±SE. Statistical significance of differences between CfcI and CfcI\(_{YNF}\) were tested using the Student’s t test.

Acknowledgments

We acknowledge SenterNovem for funding of IOP Genomics project IGE07008 and we thank Prof. J.P. Kamerling for helpful discussions.

References