Carbohydrate Research 392 (2014) 16–20

Contents lists available at ScienceDirect

Carbohydrate Research

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

Note

Enzymatic sequencing of partially acetylated chitosan oligomers

Stefanie Nicole Hamer, Bruno Maria Moerschbacher, Stephan Kolkenbrock *

Institute of Plant Biology and Biotechnology, Westphalian Wilhelm's-University Münster, Schlossplatz 8, 48143 Münster, Germany

ARTICLE INFO

Article history:
Received 1 March 2014
Received in revised form 2 April 2014
Accepted 3 April 2014
Available online 13 April 2014

Keywords:
Chitosan oligomer
Enzymatic sequencing
Chitin deacetylase
Non-random pattern of acetylation
Thin-layer chromatography

ABSTRACT

Chitosan oligosaccharides have diverse biological activities with potentially valuable applications, for example, in the fields of medicine and agriculture. These functionalities are thought to depend on their degree of polymerization and acetylation, and possibly on specific patterns of acetylation. Chitosan oligomers with fully defined architecture are difficult to produce, and their complete analysis is demanding. Analysis is typically done using MS or NMR, requiring access to expensive infrastructure, and yielding unequivocal results only in the case of rather small oligomers. We here describe a simple and cost-efficient method for the sequencing of μg amounts of chitosan oligosaccharides which is based on the sequential action of two recombinant glycosidases, namely an *exo*-β-*N*-acetylhexosaminidase (GlcNAcase) from *Bacillus subtilis* 168 and an *exo*-β-D-glucosaminidase (GlcNase) from *Thermococcus kodakarensis* KOD1. Starting from the non-reducing end, GlcNAcase and GlcNase specifically remove *N*-acetyl glucosamine (A) and glucosamine (D) units, respectively. By the sequential addition and removal of these enzymes in an alternating way followed by analysis of the products using high-performance thin-layer chromatography, the sequence of chitosan oligosaccharides can be revealed. Importantly, both enzymes work under identical conditions so that no buffer exchange is required between steps, and the enzyme can be removed conveniently using simple ultra-filtration devices. As proof-of-principle, the method was used to sequence the product of enzymatic deacetylation of chitin pentamer using a recombinant chitin deacetylase from *Vibrio cholerae* which specifically removes the acetyl group from the second unit next to the non-reducing end of the substrate, yielding mono-deacetylated pentamer with the sequence ADAAA.

1. Introduction

The biopolymer chitin is widely distributed in nature, for example, in the exoskeleton of crustaceans and insects, and in the cell walls of fungi. Chitin is composed of *N*-acetyl-D-glucosamine (GlcNAc) units linked via β-(1,4)-glycosidic bonds. Chitin can be partially or completely deacetylated to yield chitosans which are composed of GlcNAc and D-glucosamine (GlcN) units. Chitosan polymers as well as chitin and chitosan oligomers have diverse biological activities and, therefore, have many potential applications in the fields of medicine, agriculture, and environmental protection.[1–3]

Today's commercially available chitosan polymers are mainly produced chemically from chitin that was isolated from shrimp or crab shell waste.[4] These vary in terms of their average degrees of polymerization (DP) and acetylation (DA), but their pattern of acetylation (PA) is invariably random.[5] All of these parameters have been shown or are thought to influence the biological activities.[6–8] Also, these chitosans are always mixtures of polymers, where the variability in terms of DP is described as the polydispersity index (Ip) while no measure exists for the variability in terms of DA. The same is also true for commercially available chitosan oligomers which are typically mixtures in terms of DP and DA, and also PA. More defined mixtures of chitosan oligomers can be produced using chitosan hydrolyzing enzymes such as chitinases or chitosanases with well defined cleavage specificities.[2] Single fully defined chitosan oligomers can be produced via chemical synthesis, but this is a demanding multi-step process with rather low yields.[9,10] Alternatively, well defined chitosan oligomers can be generated from chitin oligomers by the use of specific chitin deacetylases (EC 3.5.1.41) and chitin oligosaccharide deacetylases (EC 3.5.1.-).[11] A number of different chitin and chitin oligosaccharide deacetylases have been purified from different sources and analyzed in respect to their mode of action or products produced.[12–15] One of these chitin oligosaccharide deacetylases is COD from *Vibrio cholerae*, specifically deacetylating the second unit

* Corresponding author at present address: evocatal GmbH, Alfred-Nobel-Str. 10, 40789 Monheim am Rhein, Germany. Tel.: +49 2173 40994 20.

E-mail addresses: Stefanie.Hamer@uni-muenster.de (S.N. Hamer), Bruno.Moerschbacher@uni-muenster.de (B.M. Moerschbacher), S.Kolkenbrock@evocatal.com (S. Kolkenbrock).

http://dx.doi.org/10.1016/j.carres.2014.04.006
0008-6215/

from the non-reducing end of chitin oligomers, resulting in a mono-deacetylated chitosan oligomer.[14]

The PA of the products of a specific chitin oligosaccharide deacetylase such as COD can be analyzed using either mass spectrometry (MS) or nuclear magnetic resonance spectroscopy (NMR), both requiring access to expensive infrastructure.[14,16] Alternatively, specific glycosidases, namely *exo*-β-*N*-acetylhexosaminidase (GlcNAcase, EC 3.2.1.52) and *exo*-β-D-glucosaminidase (GlcNase, EC 3.2.1.165) removing GlcNAc and GlcN residues from the non-reducing end of chitosan oligomers, respectively, may be used to reveal the sequence of pure chitosan oligomers.[17] We have now developed this approach into a full enzymatic sequencing method for partially acetylated chitosan oligomers without the need of using MS or NMR. Our method is based on recombinant enzymes, namely the GlcNAcase NagZ from *Bacillus subtilis* 168[18] and the GlcNase $GlmA_{TK}$ from *Thermococcus kodakaraensis* KOD1.[19]

By adding both enzymes in an alternating manner and removing the previous one, the PA of chitosan oligomers with known DP and a single PA can easily be determined and analyzed using high-performance thin-layer chromatography (HPTLC). Importantly, these two enzymes have similar buffer requirements so that the buffer does not need to be exchanged between steps.

2. Results and discussion

2.1. Production of enzymes in *Escherichia coli* and determination of activity

The *cod* gene from *V. cholerae* coding for a chitin oligosaccharide deacetylase, the *glm*A_{TK} gene from *T. kodakaraensis* KOD1 coding for a GlcNase, and the *nagZ* gene from *B. subtilis* 168 coding for a GlcNAcase, all including a C-terminal Strep-tagII, were expressed in *E. coli* Rosetta 2 (DE3) [pLysSRARE2]. The recombinant enzymes were purified by strep-tag affinity chromatography. SDS–PAGE and Western blot analysis (Fig. 1) revealed a single band for each enzyme, with an apparent molecular mass of 45.5 kDa for COD, 90.6 kDa for $GlmA_{TK}$, and 72.2 kDa for *Bs*NagZ.

The activity of COD was tested in vitro against chitin pentamer. HPTLC and matrix assisted laser desorption ionization—time of flight mass spectrometry (MALDI-TOF-MS) revealed that the hydrolysis product was a mono-deacetylated chitosan oligomer. Enzyme activities and specificities of $GlmA_{TK}$ and *Bs*NagZ were tested with GlcNAc and GlcN pentamers. HPTLC revealed that $GlmA_{TK}$ shows GlcNase activity, but no GlcNAcase activity, whereas *Bs*NagZ exhibited GlcNAcase activity, but no GlcNase activity (Fig. 2).

2.2. Enzymatic sequencing

$GlmA_{TK}$ and *Bs*NagZ were applied to determine the specific PA of a chitosan oligomer generated by the deacetylation of a fully acetylated GlcNAc pentamer using COD. COD is described as deacetylating the second unit from the non-reducing end exclusively[14] and will, thus, generate chitosan oligomers exhibiting a PA of ADA_n, that is, the sequence ADAAA when acting on the chitin pentamer in this proof-of-principle study. This defined chitosan oligomer was used to validate the enzymatic sequencing method which makes use of alternate actions of the GlcNAcase *Bs*NagZ and the GlcNase $GlmA_{TK}$. After each step, the products were analyzed using HPTLC (Fig. 3), and their identification was verified using MALDI-TOF-MS (Table 1).

The first step of the enzymatic sequencing of the mono-deacetylated chitosan pentamer was performed using the GlcNAcase *Bs*NagZ, cleaving the terminal GlcNAc residue from the non-reducing end. As seen in the HPTLC, this yielded GlcNAc monomer (A_1) and a partially deacetylated chitosan tetramer which was identified as the mono-deacetylated D_1A_3, by MALDI-TOF-MS. In the next step, the *Bs*NagZ enzyme was removed and replaced by the GlcNase $GlmA_{TK}$, which cleaved the terminal GlcN residue from the non-reducing end of the tetramer, yielding GlcN monomer (D_1) and GlcNAc trimer (A_3), as well as the GlcNAc monomer (A_1) from the previous step. A further incubation of this sample with again the first enzyme, *Bs*NagZ, resulted in the production of GlcNAc and GlcN monomers only (A_1 and D_1), with no oligomers left. Consequently, there must be a single GlcNAc unit at the non-reducing end, followed by a single GlcN unit, followed by three GlcNAc units.

This is a proof of principle study to show that the mode of action of a chitin oligosaccharide deacetylase (COD), here the COD from *V. cholerae*, can be determined by sequencing the product of the enzymatic reaction via the sequential use of a

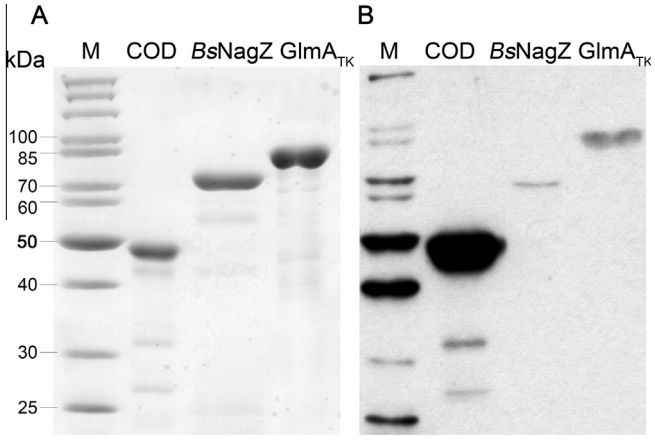

Figure 1. SDS–PAGE and corresponding Western blot of recombinant COD, *Bs*NagZ and $GlmA_{TK}$ purified from the crude extract from *E. coli* Rosetta 2 (DE3) [pLysSRARE2] harboring either pET-22b(+)::*cod*_StrepIIC, pET-22b(+)::*nagZ*_StrepIIN+C, or pET-22b(+)::*glm*ATK_StrepIIC, respectively. The enzymes were purified by Strep-Tactin affinity chromatography and 3 μg of each enzyme was applied to the gel. Enzymes were visualized either by staining with ethyl violet/zincon (A) or by enhanced chemiluminescence using HRP-coupled StrepII affinity protein after Western blotting (B). M: peqGOLD Protein-Marker II (Peqlab, Erlangen, Germany).

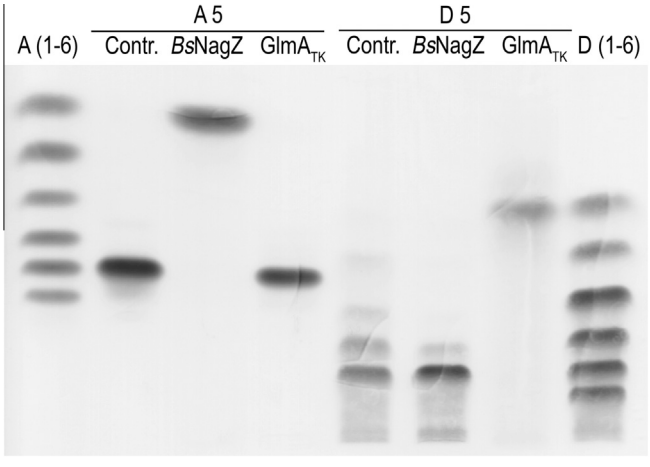

Figure 2. HPTLC analysis of $GlmA_{TK}$ and *Bs*NagZ activity as well as specificity toward GlcNAc (A5) and GlcN (D5) pentamers. The control (contr.) contains water instead of enzyme. $GlmA_{TK}$ shows GlcNase activity, but no GlcNAcase activity, whereas *Bs*NagZ exhibited GlcNAcase activity, but no GlcNase activity. For HPTLC analysis, 20 μg of each sample was applied after an incubation time over night at 80 °C for $GlmA_{TK}$ and at 37 °C for *Bs*NagZ. Fully acetylated GlcNAc monomers and oligomers (A_{1-6}) as well as fully deacetylated GlcN monomers and oligomers (D_{1-6}) were used as standards (4 μg of each monomer/oligomer).