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Improved fluorescent labeling of chitin oligomers: Chitinolytic properties of acidic mammalian chitinase under somatic tissue pH conditions

Satoshi Wakita[a,1], Masahiro Kimura[a,1], Naoki Kato[a], Akinori Kashimura[a], Shunsuke Kobayashi[a], Naoto Kanayama[a], Misa Ohno[a], Shotaro Honda[a], Masayoshi Sakaguchi[a], Yasusato Sugahara[a], Peter O. Bauer[b,c], Fumitaka Oyama[a,*]

[a] Department of Chemistry and Life Science, Kogakuin University, Hachioji, Tokyo, Japan
[b] Department of Neuroscience, Mayo Clinic, Jacksonville, FL, USA
[c] Bioinova Ltd., Prague 142 20, Czechia

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ABSTRACT

Acidic mammalian chitinase (AMCase) has been implicated in various pathophysiological conditions including asthma, allergic inflammation and food processing. AMCase is most active at pH 2.0, and its activity gradually decreases to up to pH 8. Here we analyzed chitin degradation by AMCase in weak acidic to neutral conditions by fluorophore-assisted carbohydrate electrophoresis established originally for oligosaccharides analysis. We found that specific fragments with slower-than-expected mobility as defined by chitin oligosaccharide markers were generated at pH 5.0 ~ 8.0 as by-products of the reaction. We established an improved method for chitin oligosaccharides suppressing this side reaction by pre-acidification of the fluorophore-labeling reaction mixture. Our improved method specifically detects chitin oligosaccharides and warrants quantification of up to 50 nmol of the material. Using this strategy, we found that AMCase produced dimer of *N*-acetyl-D-glucosamine (GlcNAc) at strong acidic to neutral condition. Moreover, we found that AMCase generates $(GlcNAc)_2$ as well as $(GlcNAc)_3$ under physiological conditions.

1. Introduction

Chitin is a β-1,4-linked polymer, insoluble in most solvents, composed primarily of *N*-acetyl-D-glucosamine (GlcNAc) residues. It is a major component of the exoskeletons of crustaceans and insects, the microfilarial sheaths of parasitic nematodes and fungal cell walls (Khoushab & Yamabhai, 2010; Koch, Stougaard, & Spaink, 2015). Thus, chitin is the second most abundant polysaccharide in nature.

Chitinases are glycosidases that break down glycosidic bonds in chitin. They are important enzymes responsible for chitin metabolism in a wide range of organisms, including bacteria, fungi, nematodes and arthropods (Bueter, Specht, & Levitz, 2013; Hamid et al., 2013; Khoushab & Yamabhai, 2010; Koch et al., 2015; Lee et al., 2011). Although mammals do not produce chitin, mice and humans express two active chitinases, chitotriosidase (Chit1) and acidic mammalian chitinase (AMCase) (Bussink, van Eijk, Renkema, Aerts, & Boot, 2006; Lee et al., 2011). Chit1 was the first mammalian chitinase to be purified and its gene was cloned (Boot, Renkema, Strijland, van Zonneveld, & Aerts, 1995; Renkema, Boot, Muijsers, Donker-Koopman, & Aerts, 1995). AMCase was the second mammalian chitinase discovered and was named for its acidic isoelectric point (Boot et al., 2001).

AMCase has attracted considerable attention due to its increased expression under certain pathological conditions related to immune response, for example in an induced asthma mouse model and antigen-induced mouse models of allergic lung inflammation (Reese et al., 2007; Zhu et al., 2004). Some polymorphisms and haplotypes in the AMCase gene are associated with bronchial asthma in humans (Bierbaum et al., 2005; Okawa et al., 2016; Seibold et al., 2009) and inhibition of its activity has been suggested as a therapeutic strategy against asthma (Sutherland et al., 2011; Yang et al., 2009). Furthermore, AMCase has been shown to be involved in eye (Bucolo, Musumeci, Maltese, Drago, & Musumeci, 2008; Bucolo,

* Corresponding author.
E-mail address: f-oyama@cc.kogakuin.ac.jp (F. Oyama).
[1] These authors contributed equally to this article.

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Musumeci, Musumeci, & Drago, 2011; Musumeci et al., 2009) and stomach diseases (Cozzarini et al., 2009; Nookaew et al., 2013).

We have reported that AMCase mRNA is synthesized in the mouse stomach at exceptionally high levels. These levels are comparable to pepsinogen, the precursor of the major digestive enzyme in gastric fluid, pepsin, suggesting a digestive role of AMCase (Ohno et al., 2013; Ohno, Tsuda, Sakaguchi, Sugahara, & Oyama, 2012). Moreover, we recently showed that AMCase is a proteases-resistant glycosidase in mouse digestive system, further supporting the hypothesis of AMCase functioning as a digestive enzyme (Ohno et al., 2016). We have also shown that beside stomach, AMCase mRNA is highly expressed in submaxillary gland and lung (Ohno et al., 2012). In addition, recombinant AMCase and its catalytic domain had the highest activity at around pH 2.0, when it produces primarily $(GlcNAc)_2$, and lower activities at more neutral pH (pH 3.0 ~ 7.0) (Boot et al., 2001; Kashimura et al., 2015; Kashimura et al., 2013). The AMCase activity under somatic tissue conditions at pH 5 ~ 8 remains to be elucidated.

Chitin and chitosan oligosaccharides (*N*-acetyl-chitooligosaccharides) prepared either chemically or enzymatically, have been shown to have anti-cancer and anti-inflammatory properties (Azuma, Osaki, Minami, & Okamoto, 2015; Masuda et al., 2014) and have various biological activities in mammalian cells (Aam, Heggset, Norberg, Sorlie, Varum, & Eijsink, 2010; Khoushab & Yamabhai, 2010). We hypothesized that upregulated AMCase under certain pathological conditions can generate specific degradation products associated with those pathologies.

Here we analyzed the chitinase activity of AMCase by incubating the enzyme with chitin substrates at pH 2.0 ~ 8.0 followed by fluorophore-assisted carbohydrate electrophoresis (FACE), a method based on labeling the reducing ends of oligosaccharides with a fluorophore (Jackson, 1990). FACE is very sensitive (pmol amounts) as compared to high-performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR) spectrometry, and is often used for detection of very low oligosaccharide quantities (Boot et al., 2001; Jackson, 1990).

During our research, we found a pH-dependent generation of an unexpected by-product with a slower mobility than $(GlcNAc)_2$, the main fragment resulting from chitin substrates digestion by AMCase. This by-product was observed at pH 5.0 ~ 8.0. To optimize the digestion reaction, we established an improved method for a specific detection of chitin oligosaccharides. Using this procedure, we found that AMCase generates $(GlcNAc)_2$ at broad pH range of 2.0 ~ 8.0.

2. Materials and methods

2.1. Recombinant AMCase expressed in Escherichia coli and enzymatic activity assays

We expressed and purified Protein A-AMCase-V5-His from the periplasmic fraction of the *E. coli* as described previously (Kashimura et al., 2015; Kashimura et al., 2013). The protein-containing fractions were desalted using PD MidiTrap G-25 (GE Healthcare, Milwaukee, WI, USA) equilibrated with TS buffer [20 mM Tris-HCl (pH 7.6), 150 mM NaCl and protein inhibitor (Complete Mini; Roche Diagnostics, Basel, Switzerland)].

Chitinolytic activity was determined using a synthetic chromogenic substrate, 4-nitrophenyl *N,N′*-diacetyl-β-D-chitobioside (Sigma-Aldrich, St. Louis, MO, USA) as described previously (Kashimura et al., 2015; Kashimura et al., 2013). AMCase unit definition was also reported previously (Kashimura et al., 2013).

2.2. Degradation of colloidal chitin and $(GlcNAc)_6$ by E. coli-expressed mouse AMCase

Colloidal chitin was prepared from shrimp shell chitin (Sigma-Aldrich), as described previously (Kashimura et al., 2013), and used as a substrate to determine the chitinase activity of AMCase. All enzymatic reactions using colloidal chitin (at a final concentration of 1 mg/mL) or *N*-acetyl-chitohexaose $(GlcNAc)_6$ (0.2 μmol/mL) (Seikagaku Corporation, Tokyo, Japan) were carried out in a volume of 50 μL containing 0.8 mU or 0.1 mU *E. coli*-expressed mouse AMCase in McIlvaine's buffer (mixture of 0.1 M citric acid and 0.2 M Na_2HPO_4; pH 2.0 to pH 8.0). The reaction mixtures were incubated for 1 h at 37 °C.

2.3. Fluorophore labeling by the method of Jackson

Generated chitin fragments or chitin mono- and oligomers of $(GlcNAc)_{1\sim6}$ (Seikagaku Corporation) as molecular weight markers were labeled covalently at their reducing end groups with the fluorophore 8-aminonaphthalene-1,3,6-trisulphonic acid (ANTS, Invitrogen, Carlsbad, CA, USA), and separated by 40% polyacrylamide gel electrophoresis (PAGE), as described by Jackson (Jackson, 1990). Briefly, the enzymatic reaction samples were lyophilized and 5 μL of 0.2 M ANTS in acetic acid/water (3:17, v/v) and 5 μL of 1.0 M $NaCNBH_3$ in dimethyl sulfoxide (DMSO) were added. The mixture was incubated at 37 °C for 16 h. The reaction was neutralized by 10 μL of 1 M NaOH, followed by addition of 10 μL Laemmli sample buffer (Laemmli, 1970) without SDS, 2-mercaptoethanol and bromophenol blue. The samples were separated by PAGE and quantified using the Luminescent Image Analyzer (ImageQuant LAS 4000, GE Healthcare), according to the manufacturer's instructions. Exposure condition was fixed as follows: exposure type, precision; sensitivity, high resolution; exposure time, 1 s.

2.4. Pre-acidification method for labeling chitin oligomers

Enzymatic reactions were lyophilized and 5 μL of 0.2 M ANTS in acetic acid/water (3:17, v/v), 5 μL of 1.0 M $NaCNBH_3$ in DMSO and 5 μL of 17.5 M acetic acid were added for reaction acidification followed by incubation at 37 °C for 16 h. The reaction was neutralized by 15 μL of 1 M NaOH, followed by loading buffer addition. The samples were analyzed by PAGE as described above.

2.5. Separation of degradation products from $(GlcNAc)_6$ by AMCase using HPLC

Enzymatic reactions using $(GlcNAc)_6$ (0.6 μmol/mL) were performed in a volume of 300 μL containing 4.2 mU of *E. coli*-expressed mouse AMCase in McIlvaine's buffer (pH 2.0 or pH 7.0), 30 mM Gly-HCl (pH 2.0) or 30 mM Tris-HCl (pH 7.0). The reaction mixtures were incubated at 37 °C for 1 h. Generated GlcNAc oligomers were separated by gel permeation chromatography (GPC) essentially as described previously (Kazami et al., 2015).

3. Results

3.1. Detection of pH dependent fluorophore-labeled products at pH 5.0 ~ 8.0

Previously, we have shown that the *E. coli*-produced AMCase has the highest chitinolytic activity at around pH 2.0 which is decreasing in less acidic environment (pH 3.0 ~ 7.0) against the synthetic chromogenic substrate, 4-nitrophenyl *N,N′*-diacetyl-β-D-chitobioside [4NP-$(GlcNAc)_2$] (Kashimura et al., 2015; Kashimura et al., 2013). To determine whether AMCase can generate distinct