A high-throughput matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry-based assay of chitinase activity

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Abstract

A high-throughput matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry (MALDI–TOF MS) assay is described for determination of chitolytic enzyme activity. The assay uses unmodified chitin oligosaccharide substrates and is readily achievable on a microliter scale (2 µl of total volume containing 2 µg of substrate and 1 ng of protein). The speed and sensitivity of the assay make it potentially well suited for the high-throughput screening of chitinase inhibitors. The mass spectrum is acquired in approximately 2 min, as opposed to typically 30–40 min for a single run with a high-performance liquid chromatography (HPLC)-based assay. By using the multiple-place MALDI MS targets, we estimate that 100 assays could be run in approximately 2–3 h without needing to remove the target from the instrument. In addition, because the substrate and product chitomers are visualized simultaneously in the TOF spectrum, this gives immediate information about the cleavage site and mechanism of the enzyme under study. The assay was used to monitor the purification and transgenic expression of plant class IV chitinases. By performing the assay with chitomer substrates and C-glycoside chitomer analogs, the enzyme mechanism of the class IV chitinases is described for the first time.

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Materials and methods

Protein purification

ChitA and ChitB were purified from kernels of maize inbred LH8 by a method described previously [14]. Recombinant ChitA (rChit-A) was expressed and secreted by a heterologous strain of Pichia pastoris [15]. Purification by method 1 was described previously [15]. In method 2, the ammonium sulfate precipitation was replaced by an acetone precipitation (60% acetone final). All of the chemicals used were purchased from Sigma–Aldrich (St. Louis, MO, USA).

Synthesis of chitooligosaccharide C-glycoside ketones

The chitooligosaccharide C-glycoside ketones dp C4, dp C5, and dp C6 were synthesized from chitotetrose, chitopentaose, and chitohexaose, respectively, using methods described previously [16]. The chitooligosaccharide starting materials were obtained from Seikagaku (Tokyo, Japan).

Chitinase assays

All assays were performed in 10 mM sodium acetate (pH 5.2) containing 1 mM substrate. Reactions were initiated by the addition of chitinase (0.01 mg protein/ml). Reactions were stopped by adding an equal volume of matrix (saturated 2,5-dihydroxybenzoic acid [2,5-DHB] in acetonitrile) and immediate spotting onto the matrix-assisted laser desorption/ionization (MALDI) MS target.

MS analysis

MALDI–TOF mass spectra were recorded on a Bruker Daltonics OmniFlex instrument (Billerica, MA, USA) operating in reflector mode. Assay mixtures (typically 2 μl) were mixed with 2.5-DHB matrix (2 μl, saturated in acetonitrile) and cocrystallized on a conventional 49-place stainless steel target. The MS spectra were recorded in triplicate with matrix suppression up to m/z 250 or without suppression. Ion source 1 was set to 19.0 kV and source 2 was set to 14.0 kV, with lens and reflector voltages of 9.20 and 20.00 kV, respectively. A 200-ns pulsed ion extraction was used. Excitation was set at 337.1 nm, typically at 75% of 150 μJ maximum output, and 60 shots were accumulated.

Results

MALDI–TOF MS-based assay for chitolytic enzymes

The aim was to develop a high-throughput enzyme assay that would be generally applicable to chitinases and require minimal amounts of expensive substrates, buffer, and enzyme. Hence, the reactions were conveniently undertaken on a 2-μl scale, and the activity was stopped by the addition of an equal volume of saturated 2,5-DHB in acetonitrile. The latter also acts as the matrix for the MALDI–TOF MS. From this total volume (4 μl), aliquots (0.5 μl) were transferred to the MALDI–TOF MS target using standard sequencing pipette tips. The 49-place stainless steel targets supplied by Bruker Daltonics for use with the OmniFlex mass spectrometers was suitable for 16 reactions (or time points) run in triplicate, and 100-place and larger targets are also available commercially. The chitin oligosaccharide fragments released by the enzyme activity were observed as [M+Na]+–sodium adduct ions as listed in Table 1.

For the maize ChitB, these optimized conditions were shown to give complete hydrolysis of chitohexaose (dp 6) to chitotriose (dp 3) and chitobiase (dp 2) within 30 min (Fig. 1). The intermediate chitotetrose (dp 4) was observed at the 0- and 15-min time points but was completely hydrolyzed by 30 min. Similar results were observed with ChitA, although slower kinetics was evident from the residual dp 6 peak (m/z 1259.4) at 15 min and the dp 4 peak (m/z 953.2) at the 1-h time point (see Suppl. Fig. S1 in supplementary material). It is noteworthy that the ChitA and ChitB activities on the chitohexaose did not give rise to chitotetrose (1033.41 Da, calculated [M+Na]+ = 1056.40) or the monomer sugar GlcNAc (221.09 Da, calculated [M+Na]+ = 244.08). This suggested that these chitinases are endoglucosidases that hydrolyze chitobiase units from larger chitin oligosaccharide substrate.

This latter hypothesis was tested by observing the activity of the ChitA and ChitB on various chitin oligosaccharide substrates of different sizes (dp 6, dp 5, dp 4, dp 3, and dp 2). The MS data were recorded for the start of these reactions (Fig. 2), at which time dp 6 was observed to give rise to dp 4 (m/z 853.2), dp 3 (m/z 650.0), and dp 2 (m/z 446.8). Starting dp 5 similarly gave rise to a small amount of dp 2, whereas no hydrolysis was observed for starting dp 4, dp 3, or dp 2 (Fig. 2). By 15 min of reaction time, the dp 6 and dp 5 substrates were completely hydrolyzed to mixtures of dp 3 and dp 2, whereas dp 4 was hydrolyzed only to dp 2 (see Suppl. Fig. S5 in supplementary material). The starting dp 3 and dp 2 substrates remained unhydrolyzed even after 1 h of reaction (see Suppl. Fig. S3). Similar data were acquired for ChitB (not shown). These results were consistent with those observed during the time course experiment (Fig. 1) and confirmed that chitin oligosaccharides larger than dp 4 are ultimately hydrolyzed to dp 3 and dp 2 but do not undergo a simple loss of GlcNAc monomer. Hence, neither [M+Na–GlcNAc]+ ions nor released [GlcNAc+Nac]+ or [GlcNAc+H]+ ions (m/z 244.08 or 222.08, respectively) were observed for any of the substrate or enzyme combinations tested. Moreover, chitotriose and chitobiase were unaffected by treatment with the maize chitinases, indicating that dp 4 is the minimal substrate size for these enzymes.

Application to chitin oligosaccharide analogs: chitooligosaccharide C-glycoside ketones

The above data indicated that chitin oligosaccharides larger than dp 3 bind to the active site of the maize chitinases and are hydrolyzed by the release of chitobiase (dp 2) products but not by the loss of monomeric products (GlcNAc, dp 1). To test whether the chitobiase is released from the reducing or nonreducing terminus of the starting substrates, we synthesized a variety of chitin oligosaccharide analogs in which the reducing terminus has been converted to a C-glycoside ketone (see Fig. 3). The effect of this reducing end modification on the enzyme kinetics was also of interest. The chitooligosaccharide C-glycoside ketones dp 4, dp 5, and dp 6 were tested for catalytic hydrolysis by purified maize ChitA using the described MALDI–TOF MS chitinase assay (Fig. 3)
The C-glycoside ketone modification results in substrates and products that are 40 Da larger than the parent chitin-oligosaccharides [16]. The chitohexaose-C-glycoside ketone (dp 6, m/z 1299.7) was hydrolyzed to smaller C-glycoside ketones (dp 3, m/z 690.1, and dp 2, m/z 486.9) and to chitotriose (dp 3, m/z 650.0) and chitobiose (dp 2, m/z 446.8).
During the same time course, the chitotetraose-C-glycoside ketone (dp 4, m/z 893.1) was hydrolyzed to dp 2 and dp 2, with no evidence for the formation of either dp C3 or dp 3 (see Suppl. Fig. S4). The ChitA activity on the chitopenctaose-C-glycoside was noticeably slower, with the starting substrate (dp C5, m/z 1096.2) still present even after 1 h of reaction time (see Suppl. Fig. S5). The products arising from dp C5 were dp 3 and dp C2, indicating that hydrolysis occurs by the release of a chitobiose unit from the reducing end of the substrate.

**Application to heterologously expressed maize chitinase in the Pichia yeast expression system**

As a further example of the versatility of the described methods, the MALDI–TOF MS-based assay was used to monitor the activity of rChitA after purification from *P. pastoris* expression cultures. rChitA was purified from cultures by two different methods. Protein purified by method 1 was shown to have chitinase activity on chitohexaose using the MALDI–TOF MS assay (Fig. 4). In the presence of rChitA, dp 6 was rapidly hydrolyzed to dp 4, dp 3, and dp 2. As with the plant-derived enzymes, there was no evidence for dp 5 via a simple loss of monomer. After 5 min, the dp 6 oligosaccharide was entirely hydrolyzed to dp 3 and dp C2 (Fig. 4). Comparable results were obtained with protein purified by method 2 (acetone precipitation), albeit with slower kinetics (see Suppl. Fig. S6 in supplementary material). However, no observable hydrolysis of dp 6 (m/z 1259.5) was apparent at the 5-min time point, and dp 4 (m/z 853.3) was still in evidence after 5 min. It is noteworthy that in protein-free control experiments, the chitohexaose starting substrate was unaffected (see Suppl. Fig. S7).

**Discussion**

The chitinase family of glycolytic enzymes are generally classified into type GH 18 or GH 19 [17], which lack structural and sequence similarities and also use different catalytic mechanisms. GH family 18 chitinases are found in a wide diversity of organisms. GH family 19 chitinases are found mainly in plants but were also recently discovered in bacteria [17]. Plant chitinases also use two different hydrolytic mechanisms and are potent inhibitors of fungal growth [4,18]. GH family 19 plant chitinases are *endo*-chitinases, and the catalytic mechanism involves neighboring group participation by the 2-N-acetyl group on the GlcNAc residue in the -1 site, with hydrolysis of the C1 anomeric bond to the GlcNAc residue in the adjacent +1 site. Nucleophilic attack by water on C1 of the oxazolinium ion gives rise to the new reducing end GlcNAc in the -1 binding site [3]. This leads to the formation of a stable cyclic oxazolinium intermediate in the -1 site, with hydrolysis of the C1 anomeric bond to the GlcNAc residue in the adjacent +1 site. Nucleophilic attack on C1 of the oxazolinium ion gives rise to the new reducing end GlcNAc in the -1 binding site; hence, GH family 18 chitinases proceed with anomeric retention. By contrast, GH family 19 chitinase catalysis occurs by direct nucleophilic attack on the anomeric carbon in the active site, resulting in inversion of configuration as the leaving group is expelled.

This mechanism also explains the powerful inhibitory effect of two chitinase inhibitors, allosamidin and chitomer thiazolines, that mimic the structure of the cyclic oxazolinium ion in the -1 site [19,20]. These differ from native chitin oligosaccharides by structural differences at the reducing end, although allosamidin also contains an allo-configuration N-acetyl amino sugar at the non-reducing terminus that protects it from degradation by exo-N-acetylhexosaminidases without compromising the binding to chitinases [21]. The described chito oligosaccharide C-glycosides are also selectively modified at the reducing terminus, and these

**(dp 2, m/z 446.8) (Fig. 3).** During the same time course, the chitotetraose-C-glycoside ketone (dp 4, m/z 893.1) was hydrolyzed to dp C2 and dp 2, with no evidence for the formation of either dp C3 of dp 3 (see Suppl. Fig. S4). The ChitA activity on the chitopenctaose-C-glycoside was noticeably slower, with the starting substrate (dp C5, m/z 1096.2) still present even after 1 h of reaction time (see Suppl. Fig. S5). The products arising from dp C5 were dp 3 and dp C2, indicating that hydrolysis occurs by the release of a chitobiose unit from the reducing end of the substrate.

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also display a distinctly slower rate of hydrolysis than the native substrates. It is noteworthy that a similar pseudotetrasaccharide, TMG-chitotriomycin (which is modified only at the nonreducing end), is specific for β-N-acetylhexosaminidases [22]. Similar reasoning also explains the potent inhibition of exo-N-acetylhexosaminidase by NAG-thiazoline [23], XylNAc-isofagomine [24], and certain GlcNAc-inspired iminocyclitiols [25]. There are also several reports stating that unmodified chitooligosaccharides act as chitinase inhibitors [21,26], and other noncarbohydrate inhibitors, such as arginfin and argadin, also act as induced substrate analogs [21].

In addition to its potential as a high-throughput screen for the identification of chitinase inhibitors, MALDI MS analysis of chitinase reaction products can also be used to study chitinase mechanisms. The results reported here demonstrate, to our knowledge for the first time, a fundamental mechanistic difference between the two types of catalytic domains in plant GH family 19 chitinases. Plant class I and II chitinases, which have been studied structurally [27–29] and enzymatically [30–33], have longer catalytic domains, whereas plant class IV chitinases have evolved to be shorter through a series of three deletions (Fig. 5A) [3]. The longer chitinases are thought to contain six sugar binding sites, labeled −3 (nonreducing end), −2, −1, +1, +2, and +3 (reducing end), to accommodate binding of dp 6 with cleavage occurring between −1 and +1. Unlike the class IV enzymes ChitA and ChitB, class I and II chitinases have minimal activity with dp 4. Efficient conversion of dp 4 to dp 2 supports a model in which the deletions in the catalytic domain resulted in loss of the two outer sugar binding sites. The observed cleavage of dp C6 by ChitA at three different bonds further supports this model (Fig. 5B). These results are consistent with the recent publication of structures of the catalytic domain of a class IV chitinase, Chia4-Pa, which showed that its catalytic cleft is shorter and wider than that of class I and II chitinases [29]. The use of molecular biology techniques to produce mutant rChitA proteins combined with MALDI MS analysis of resulting changes in activity will allow further study of this mechanism.

The MALDI–TOF MS-based assay described here requires 2 μl of total volume containing only 1 ng of enzyme and 2 μg of oligosaccharide substrate. The 60 laser shots used to acquire a mass spectrum take approximately 2 min, as opposed to typically 30–40 min
for a single HPLC run. Moreover, by using the standard multiple-place MALDI MS targets, we estimate that up to 100 assays could be run in approximately 2–3 h without needing to remove the target from the instrument. We have shown that this assay is readily applicable to different-sized chitin substrates (up to dp 6), or structural analogs of chitomers, and to time course estimates. In addition, because the substrate and product chitomers are visualized simultaneously in the TOF spectrum, this gives immediate information about the cleavage site and mechanism of the enzyme under study. However, because of the intrinsic semiquantitative response of MALDI–TOF MS, the assay is not recommended for classical enzyme kinetic studies in its current form. Several other MS-based enzyme assays have been described [34,35], and the MALDI–TOF MS technique described here may be generally applicable to screen other glycosylases using the native oligosaccharide as substrate.

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Appendix A. Supplementary data


References


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