

Quantification of Chitinase Activity in *Fusarium oxysporum*

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[Abstract] Fungal morphogenetic development requires modification and plasticity of the cell wall, which implies synthesis and remodelling of its components, including chitin and glucan. Thus chitinase and glucanase activities are crucial for cell-wall biogenesis and cell division. Quantification of chitinase activity might be useful to identify structural defects that could negatively influence growth and morphogenesis of some filamentous fungi like *Fusarium oxysporum*, which produces both intracellular and secreted chitinases. The chitinolytic enzymes are categorized based on their enzymatic action on chitin substrates. Endochitinases are defined as the enzymes catalyzing the random cleavage at internal points in the chitin chain. Exochitinases catalyze the progressive release of acetylchitobiose or N-acetylglucosamine from the non-reducing end of chitin, and thus, are referred to as chitobiosidase and β -N-acetylglucosaminidase, respectively. Here we describe a simple method to easily purify chitinases in order to compare both endo- and exo-chitinase activity of different *F. oxysporum* strains. The protocol can be adapted to any fungal species.

Materials and Reagents

1. Monodur nylon filters 15 μ m diameter (Filtravibracion S.L., catalog number: Nylon-15)
2. Sterile plastic funnels (80 mm diameter) (Tecnylab, catalog number: 45000150)
3. Eppendorf tubes
4. Sterile spatula (Instrumentación Científica, S.L., catalog number: 2004NE8044)
5. Microtiter plates (Thermo Fisher Scientific, catalog number: 2205)
6. Dialysis membranes, cut-off 12 kDa (Sigma-Aldrich, catalog number: D9527-100FT)
7. *Fusarium oxysporum* f.sp. *lycopersici* microconida suspensions from wild type (J. Tello, Universidad de Almeria, Spain), the $\Delta con7-1$ and the $c\Delta con7-1$ mutant strains (Ruiz-Roldan *et al.*, 2015)
8. Glycerol (Merck, catalog number: 104092)
9. Potato dextrose broth medium (PDB) (Scharlau, catalog number: 01483)
10. Chitin (Sigma-Aldrich, catalog number: C6137)
11. Bio-Rad protein assay Dye reagent concentrate (Bio-Rad Laboratories, catalog number: 500-0006)
12. Bovine serum albumin (BSA) (Sigma-Aldrich, catalog number: 10 735 078 001)
13. Polyethyleneglycol 35,000 (Sigma-Aldrich, catalog number: 81310)

14. Na₂HPO₄ (Merck, catalog number: 1.06586.0500)
15. NaH₂PO₄ monohydrate (Merck, catalog number: 567549)
16. MgSO₄·7H₂O (Merck, catalog number: 1058865.5000)
17. KH₂PO₄ (Merck, catalog number: 104873.1000)
18. KCl (Merck, catalog number: 104933.0500)
19. NH₄NO₃ (Merck, catalog number: 1.01187.1000)
20. FeSO₄ (Merck, catalog number: 103965.0500)
21. ZnSO₄·7H₂O (Merck, catalog number: 108883)
22. MnSO₄ monohydrate (Merck, catalog number: 105941)
23. Glucose (Coger SAS, catalog number: 24379.363)
24. NaCl (Merck, catalog number: 1.06404.5000)
25. Fluorimetric Chitinase Assay Kit (Sigma-Aldrich, catalog number: CS1030). Contents:
 - a. Assay Buffer (25 ml) (Sigma-Aldrich, catalog number: A8730)
 - b. 4-Methylumbelliferyl N-acetyl-β-D-glucosaminide (5 mg) (Sigma-Aldrich, catalog number: M2133)
 - c. 4-Methylumbelliferyl β-D-N,N'-diacetylchitobioside hydrate (5 mg) (Sigma-Aldrich, catalog number: M9763)
 - d. 4-Methylumbelliferyl β-D-N,N',N''-triacetylchitotriose (5 mg) (Sigma-Aldrich, catalog number: M5639)
 - e. Chitinase from *Trichoderma viride* (1 mg) (Sigma-Aldrich, catalog number: C6242)
 - f. 4-Methylumbelliferone Standard Solution, 50 mg/ml (1 ml) (Sigma-Aldrich, catalog number: M3570)
 - g. Sodium carbonate (2 g) (Sigma-Aldrich, catalog number: S2127)
 - h. Dimethyl sulfoxide (DMSO) (1 ml) (Sigma-Aldrich, catalog number: D8418)
26. 0.05 M phosphate buffer (see Recipes)
27. Synthetic medium (SM) (see Recipes)
28. Phosphate-buffered saline (PBS) (see Recipes)
29. Strate stock solutions (20 mg/ml) (see Recipes)
30. Strate working solutions (see Recipes)
31. Chitinase control enzyme (see Recipes)
32. Stop solution (sodium carbonate solution) (see Recipes)
33. 4-Methylumbelliferone standard solution (see Recipes)

Equipment

1. Hemocytometer (Thoma) (Marienfeld, catalog number: 06 407 10)
2. Mini-BeadBeater -16 homogenizer (BioSpec Products)
3. Mortar and pestle
4. Orbital incubator with shaking (Infors, model: Multitron Pro)

5. Microcentrifuge (Eppendorf MiniSpin plus, Fisher Scientific)
6. Fluorimeter (SpectraFluorPlus, TECAN, catalog number: F129005)
7. Water bath (Selecta, catalog number: 6000138)
8. Vortex (IKA, model: MS2 Minishaker)

Procedure

A. Sample preparation

1. *F. oxysporum* f.sp. *lycopersici* strains were stored at -80 °C with 30% glycerol as microconidial suspension.
2. For fresh microconidia production, aliquots of frozen microconidial stocks were inoculated into 100 ml of potato dextrose broth (PDB) and incubated for 3 days at 150 rpm and 28 °C in an orbital incubator. Cultures were then filtered through Monodur nylon filters placed on funnels to separate mycelia (Figure 1A), and centrifuged at 3,020 x g for 5 min to collect microconidia. Finally, pellets containing fresh microconidia were resuspended into 1 ml sterile dH₂O and counted using a hemocytometer.

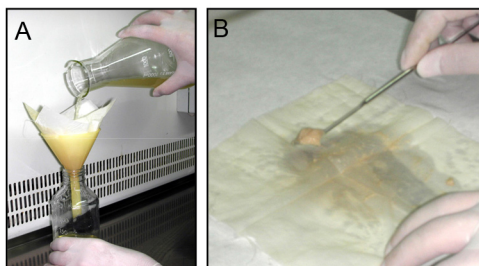


Figure 1. Filtration and mycelia harvesting procedure. A. Filtration of a fungal culture through a Monodur nylon membrane placed on a funnel. B. Subsequent harvesting of mycelia by scraping a nylon membrane using a spatula.

3. Aliquots containing 4×10^8 fresh microconidia from each strain were inoculated into 100 ml PDB and incubated in an orbital incubator at 28 °C with shaking at 170 rpm for 14 h (wild type and *cΔcon7-1* strains) or 24 h (*Δcon7-1* mutant).

Note: Conidial germination of Δcon7-1 mutant is significantly delayed compared to the wild type or the cΔcon7-1 strains. To normalize the growth delay of the mutant a longer incubation period was used for this strain.

4. Germlings were harvested by filtration through Monodur nylon membranes (Figure 1A), washed twice with sterile distilled water, scraped from the nylon filter using a spatula (Figure 1B) and directly transferred to 100 ml synthetic medium (SM) containing 0.5% chitin as sole carbon source.

5. After 24 h growth, culture supernatants were filtered (to separate mycelia) and centrifuged (to discard spores) (as described in step A2). Supernatants were used to determine secreted chitinase activity, while mycelia were used for intracellular chitinase activity measurement.
6. Culture supernatants were introduced into dialysis membranes that were sealed at both ends using plastic clamps, and subsequently submerged into 5 L distilled water for dialysis for 14 h at 4 °C.

Note: The dialysis step is necessary to eliminate remaining salts and nutrients from the culture medium.

7. After dialysis, membranes were transferred to a plastic tray, covered with PEG 35,000 powder and incubated at 4 °C until they were concentrated 100-fold (Figure 2). Samples were stored at -20 °C until use.



Figure 2. Concentration of culture supernatants using PEG 35,000

8. Mycelia from step A5 were washed twice with sterile distilled water, frozen at -80 °C and ground with a mortar and pestle under liquid nitrogen.
9. Samples were transferred to Eppendorf tubes containing 1ml phosphate buffer (0.05 M, pH 6.0) and homogenized by vortexing, followed by 2 min incubation at 4 °C.
10. The homogenates were centrifuged 30 min at 10,000 x g and 4 °C, and the supernatants were collected and stored at -20 °C.

B. Quantification of protein content

The protein content of culture supernatants and mycelia was quantified according to the method of Bradford (Bradford, 1976) using the Bio-Rad protein assay kit with bovine serum albumin as standard.

1. Standard curve
 - a. Dilute Bradford protein assay reagent 1:4 into distilled water.
 - b. Aliquot 200 µl of diluted Bradford protein assay reagent per well into a microtiter plate.
 - c. Prepare solutions containing serial dilutions of standard BSA ranging from 0 to 1 µg/ml.
 - d. Start reaction by adding 5 µl of each standard BSA solution. Make at least two replicates of each standard dilution.
 - e. Determine Absorbance at 595 nm using a Fluorimeter.
 - f. Generate a standard curve for a series of BSA concentrations.

2. Sample analysis

- a. Follow the directions given in Standard Curve, substituting BSA standard solutions for 5 μ l fungal experimental samples. Please note that the total volume of the experimental sample assays should be equal to that of the BSA standard assays.
- b. Calculate the amount of protein in the experimental samples from the standard curve.

C. Enzyme activity determination

Endo- and exochitinase activities were individually quantified by using the Fluorimetric Chitinase Assay Kit following the manufacturer's instructions. The kit provides three different substrates for the detection of the various types of the chitinolytic activity:

4-Methylumbelliferyl β -D-N,N'-diacetyl-chitobioside, substrate suitable for exochitinase activity detection (chitobiosidase activity)

4-Methylumbelliferyl N-acetyl- β -D-glucosaminide, substrate suitable for exochitinase activity detection (-N-acetylglucosaminidase activity)

4-Methylumbelliferyl β -D-N,N',N''-triacetylchitotriose - substrate suitable for endochitinase activity detection

Chitinase activity is expressed as μ g of 4-methylumbelliferone released from the substrate per μ g of protein. Assays were performed in two independent experiments, each including two replicates.

1. Preparation of solutions (see Recipes 4-8)
2. Assay

The chitinase hydrolysis is performed in an acidic environment (pH ~5.0) at 37 °C. The enzymatic hydrolysis liberates 4-methylumbelliferone (4MU). The fluorescence of liberated 4MU is measured in alkaline pH using a fluorimeter with excitation at 360 nm and emission at 450 nm. In order to quantitate the total chitinolytic activity, separate reactions should be run with the three substrates supplied in the kit. Note that in crude preparations there may be additive/synergist activity of different chitinases (*i.e.*, 4-Methylumbelliferyl N,N'-diacetylchitobioside can be cleaved by β -N-acetylglucosaminidase and also chitobiosidase). It is recommended to perform the assays in duplicates.

For each substrate, perform a separate activity assay according to the following instructions of the manufacturer:

- a. Equilibrate the substrate working solution(s) and the standard solution(s) to 37 °C by incubating for several minutes in a water bath.
- b. Set the fluorimeter at an excitation wavelength of 360 nm and an emission wavelength of 450 nm.
- c. Add the reaction components to 96 well plates according to Table 1 and mix by pipetting. Prepare the standard samples first (samples No. 4-8). Then, prepare the enzyme samples: add the substrate solution to the appropriate wells first and then add the enzyme (positive control or test sample).

Table 1. Reaction scheme

No.	Assay	Substrate working solution	Sample or standard solution	Assay buffer
1	Blank*	100 µl	-	-
2	Positive control	90 µl	10 µl of chitinase control enzyme	-
3	Fungal test sample	90 µl	10 µl of fungal test sample	-
4	Standard blank	-	-	100 µl
5	10 ng of assay standard	-	2 µl of 5 µg/ml 4MU	98 µl
6	100 ng of assay standard	-	2 µl of 50 µg/ml 4MU	98 µl
7	500 ng of assay standard	-	10 µl of 50 µg/ml 4MU	90 µl
8	1,000 ng of assay standard	-	2 µl of 500 µg/ml 4MU	98 µl

*A Blank reaction (substrate solution without enzyme) should be run to account for the spontaneous hydrolysis of the substrate during the incubation time.

- d. Incubate the plate for 30 min at 37 °C. If required, the incubation time for highly active samples can be reduced to 15 min. On the other hand, in order to detect low levels of enzyme activity, the incubation time can be extended to 1 h.
 - e. Stop the reactions by adding 200 µl of stop solution to each well.
 - f. Measure the fluorescence at an excitation wavelength of 360 nm and an emission wavelength of 450 nm no later than 30 min after ending the reaction.
3. Calculation

Unit definition: One unit of chitinase activity will release 1 mM of 4-methylumbelliferone from the appropriate substrate per minute at pH 5.0 at 37 °C.

The chitinase activity can be calculated using a standard curve prepared from the fluorescence readings of the 5 standard solutions (see Table 1).

Alternatively, the chitinase activity can be calculated using only a single standard concentration. It is recommended to measure the fluorescence of 100 ng (1.9 nmole/ml) Standard and then use the following equation:

$$\text{Units/mL} = \frac{(\text{F}_{\text{sample}} - \text{F}_{\text{blank}}) \times 1.9 \times 0.3 \times \text{DF}}{\text{F}_{\text{standard}} \times \text{Time} \times \text{V}_{\text{enz}}}$$

Where:

F_{sample} = fluorescence of the test sample

F_{blank} = fluorescence of the Blank (containing only substrate working solution)

0.3 = final reaction volume in milliliters after addition of the stop solution

DF = enzyme dilution factor

$\text{F}_{\text{standard}}$ = fluorescence of the standard solution minus the fluorescence of the Standard Blank

Time = min

V_{enz} = volume of the sample in milliliter

Finally refer the resulting activity to the amount of protein calculated for each sample in previous steps.

Representative data

Figure 3 shows a representative example of data obtained following this protocol.

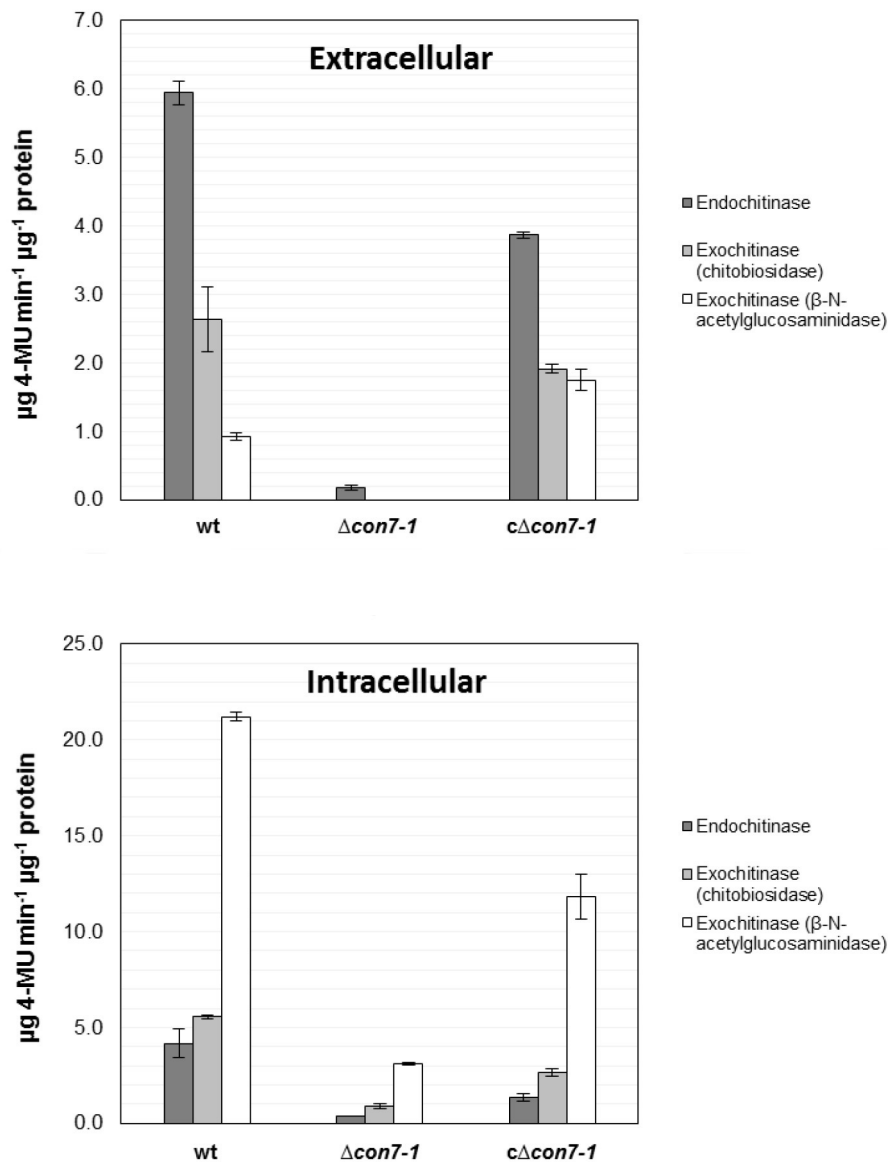


Figure 3. Quantification of intra- and extracellular chitinase activity. Intra- and extracellular protein extracts were obtained from germlings of the wild type strain (wt), $\Delta con7-1$ mutant and the $c\Delta con7-1$ complemented strains after growth in SM supplemented with 0.5% chitin. Endo- and exochitinase (chitobiosidase and β -N-acetylglucosaminidase) activities were measured using the Fluorimetric Chitinase Assay Kit. Specific chitinase activity is expressed as μ g of 4-methylumbelliferone (4MU) released from the appropriate substrate per μ g of protein. Bars represent standard errors calculated from two independent experiments with two replicates each (Taken from Ruiz-Roldan *et al.*, 2015).

Recipes

1. 0.05 M phosphate buffer (pH 6.0)

Prepare the following solutions:

- 1M Na₂HPO₄ (14.2 g in 100 ml distilled water)
- 1M NaH₂PO₄·H₂O (13.8 g in 100 ml distilled water)

Mix 12 ml solution A and 88 ml solution B

Dilute to 0.05 M final concentration

2. Synthetic medium (SM)

0.2 g/L MgSO₄·7H₂O

0.2 g/L KH₂PO₄

0.2 g/L KCl

1 g/L NH₄NO₃

0.01 g/L FeSO₄

0.01 g/L ZnSO₄·7H₂O

0.01 g/L MnSO₄·H₂O

10 g/L glucose

Dissolve in deionized water and sterilize by autoclaving

3. Phosphate-buffered saline (PBS)

8 g/L NaCl

0.2 g/L KCl

1.44 g/L Na₂HPO₄

0.24 g/L KH₂PO₄

Dissolve the reagents listed above in 800 ml of H₂O. Adjust the pH to 7.4 with HCl, and then add H₂O to 1 L

4. Strate stock solutions (20 mg/ml)

- Add 0.25 ml of DMSO to the contents (5 mg) of each of the appropriate substrate bottle:

4-Methylumbelliferyl N-acetyl-β-D-glucosaminide, or

4-Methylumbelliferyl β-D-N,N'-diacetylchitobioside hydrate, or

4-Methylumbelliferyl β-D-N,N',N''-triacetylchitotriose

- Vortex until dissolved

Note: 4-Methylumbelliferyl N-acetyl-β-D-glucosaminide 4-Methylumbelliferyl β-D-N,N',N''-triacetylchitotriose do not dissolve easily in DMSO. Mix by 15 min vortexing at room temperature and then incubating up to two h at 37 °C with shaking at 170 rpm until they completely dissolve. Aliquot the substrate solutions and keep at -20 °C.

5. Strate working solutions

- Just before the assay dilute an aliquot of the substrate stock solution (20 mg/ml) 40-fold with Assay buffer to a concentration of 0.5 mg/ml.

- b. Mix by vortexing. Approximately 100 µl of substrate working solution are required for each test (each well).

Note: If required, the substrate concentration in the substrate working solution can be reduced to a concentration of 0.2 mg/ml.

6. Chitinase control enzyme

- a. Add 5 ml of PBS to the contents of the chitinase bottle to give a final chitinase concentration of 0.2 mg/ml.
- b. Vortex until dissolved. The chitinase dissolves immediately to give a slightly hazy solution. For long term storage, aliquots are stored at -20 °C (stable for at least 3 months at -20 °C).
- c. Just before use, dilute an aliquot of the chitinase 200-fold with PBS and use 10 µl of the diluted enzyme per assay.

7. Stop solution (sodium carbonate solution)

- a. Add 47.2 ml of ultrapure water to the contents of the sodium carbonate bottle and mix well with a magnetic stirrer until completely dissolved.
- b. Store the stop solution at room temperature.

8. 4-Methylumbelliferone standard solution

Before performing the assay, dilute an aliquot of the 4-Methylumbelliferone (4MU) standard solution in stop solution. Prepare 100-, 1,000-, and 10,000-fold dilutions to final concentrations of 500, 50 and 5 µg/ml, respectively. The final volume of the diluted standard solution in the assay should not exceed 10 µl.

Acknowledgments

This research was supported by the Spanish Ministerio de Economía y Competitividad (BIO2013-47870, and Ramon y Cajal Program), and Junta de Andalucía (P11-CVI-7319).

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