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ORIGINAL PAPER

Recombinant production, characterization and industrial application testing of a novel acidic exo/endo-chitinase from *Rasamsonia emersonii*

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Abstract

An acid-active exo/endo-chitinase; comprising a GH18 catalytic domain and substrate insertion domain; originating from the thermophilic filamentous fungus *Rasamsonia emersonii,* was expressed in *Pichia pastoris*. In silico analysis including phylogenetic analysis, and recombinant production, purification, biochemical characterisation, and industrial application testing, was carried out. The expressed protein was identified by SDS-PAGE as a smear from 56.3 to 125.1 kDa, which sharpens into bands at 46.0 kDa, 48.4 kDa and a smear above 60 kDa when treated with PNGase F. The acid-active chitinase was primarily a chitobiosidase but displayed some endo-chitinase and acetyl-glucosamidase activity. The enzyme was optimally active at 50 °C, and markedly low pH of 2.8. As far as the authors are aware, this is the lowest pH optima reported for any fungal chitinase. The acid-active chitinase likely plays a role in chitin degradation for cell uptake in its native environment, perhaps in conjunction with a chitin deacetylase. Comparative studies with other *R. emersonii* chitinases indicate that they may play a synergistic role in this. The acid-active chitinase displayed some efficacy against non-treated substrates; fungal chitin and chitin from shrimp. Thus, it may be suited to industrial chitin hydrolysis reactions for extraction of glucosamine and chitobiose at low pH.

Keywords Acid active chitinase · Thermostable chitinase · *Rasamsonia emersonii* · Glucosamine · Chitooligosaccharides · Chitin-waste valorisation

Introduction

Chitin

After cellulose, chitin is the second most abundant polymer in nature. It is found in the shells of crustaceans, the exoskeletons of insects and the cell wall of fungi. The structure of chitin comprises repeating units of β-(1–4) glycosidic-linked N-acetyl-(D)-glucosamine in several hundreds to more than a thousand. The macro-structure is crystalline and highly resistant to degradation (Martínez et al. 2014). In natural sources, chitin is intimately associated with other structures (proteins, carbohydrates, minerals, etc.), making extraction of pure chitin and chitin derivatives difficult (Elieh-Ali-Komi and Hamblin 2016).

Chitin and chitin derivatives display properties such as biocompatibility, biodegradability, bioactivity, bioresorptivity and non-toxicity. This makes them attractive alternatives to synthetic polymers in healthcare, biomedical and pharmaceutical, waste and water treatment, textiles, food and beverage, cosmetics, agrochemical and edible/biodegradable packaging industries (Merzendorfer and Cohen 2019). Hence, large-scale and feasible extraction of pure chitin and chitin derivatives from their natural sources is desirable.

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The majority of industrial chitin is sourced from crustacean shells. Recently, some industrial chitin has been sourced from fungi (Chitosanlab Vegan 2021). No standard method for chitin isolation has been adopted, though typically, industrial chitin is extracted chemically. NaOH is the most common deproteinization reagent. It can be applied at concentrations ranging from 0.125 to 5.0 M, at varying temperatures (up to 160 °C). Demineralization of chitin is generally performed by acid treatment using HCl (Younes and Rinaudo 2015). However, chemical extraction methods have some limitations including environmental unfriendliness, products that are non-reproducible and of low molecular weight (Rinaudo 2006). Enzymatic extraction of chitin from natural sources overcomes many of these limitations in the industry as the processes employed are environmentally friendly, highly specific and therefore reproducible and can be engineered to yield products of specific molecular weight. Additionally, enzymatic processes offer intellectual property advantages over current chemical extraction methods.

Chitinases

Within the biopolymers of N-acetyl-glucosamine in chitin, chitinases catalyse the hydrolytic cleavage of the β(1, 4)-glycoside bonds. Chitinases are further classified as either exo-chitinases or endo-chitinases. Exo-chitinases (EC 3.2.1.96) catalyse the progressive release of N-acetyl-glucosamine or acetyl-chitobiose from the non-reducing end of chitin, and thus, are referred to as N-acetyl-glucosaminidase or chitobiosidase, respectively. Endo-chitinases (EC 3.2.1.14) randomly catalyse the cleavage at internal bonds in the chitin chain, yeilding chitooligosaccharides (CAZy 2021).

Development of industrial exo-chitinases is desirable in the production of glucosamine and chitobiose from natural chitinous material. Glucosamine is a supplement that promotes healthy joints and is used in arthritis treatments. It is also used in cosmetics to improve skin moisture and in sports food and drinks as a functional additive (Bissett 2006). Chitooligosaccharides have prebiotic properties (Lee et al. 2002) and can be added to foods and feed as a functional ingredient; chitobiose can be used in designed human milk oligosaccharides (Nyffenegger et al. 2015). Chitinases can be used directly as a biocontrol agent in agricultural crops (Binod et al. 2007; Veliz et al. 2017). Other potential industrial applications of chitinases include the valorisation of mushroom-based food waste (Dwyer 2019) and shrimp-shell waste, including one-step shrimp-chitin processing (Deng et al. 2020). Currently, chitinases are used in industry in alternative sustainable protein processing on mushroom substrates (Nagase (Europa) GmbH 2021). Industrial chitinases are somewhat limited, only two are available commercially; these are produced in *Streptomyces*.

Fungal chitinases

In fungi, chitinases play roles in morphogenetic, nutritional and parasitic functions (Duo-Chuan 2006; Merzendorfer and Zimoch 2003; Senol et al. 2014). Primarily, fungal chitinases belong to Glycosyl Hydrolase Family 18. Likely owing to the number of native roles which fungal chitinases carry out, the activity specificities they display are diverse (Hartl et al. 2012). In general, the molecular masses of fungal chitinases range from 30 to 200 kDa. The pH optima and temperature optima for most fungal chitinases varies from 4.0 to 7.0 and 20 to 40 °C, respectively (Khan et al. 2015). Thermophilic filamentous fungi, in particular, have gained attention in the literature as sources of chitinases for application in biotechnology owing to their ability to secrete enzymes to extracellular media displaying high-temperature optima and thermostability which are advantageous for industrial reactions (Karthik et al. 2014). Chitinases from thermophilic fungi have displayed pH optima from pH 3.5–8.0, with optima within 5.0–6.0 being the most common. Temperature activity optima have been seen in the ranges of 37–60 °C, with some chitinases from *Thermoascus aurantiacus var.levisporus*, *Chaetomium thermophilum* and *R. emersonii* displaying hyperthermostablity (Dwyer et al. 2021; Li et al. 2010).

R. emersonii

Rasamsonia emersonii (syn. *Talaromyces emersonii*) is a thermophilic filamentous fungus displaying optimum growth at 45 °C, and with a history of use in commercial food enzyme production. *R. emersonii* is known for producing industrially important enzymes which have been well-studied and described since the 1980s (Coughlan et al. 1984). However, only two individual chitinases from this source have been described in the literature recently. Chit1 and Chit2, were found to be markedly thermostable glyco-proteins that may have considerable biotechnological potential in shrimp chitin processing including one-step chitin hydrolysis, and alternative sustainable protein processing and the attractive emerging application of mushroom food-waste valorisation (Dwyer et al. 2021). The phylogeny and potential native roles of the chitinases were not discussed previously.

Materials and methods

Chemicals, strains and microorganisms

All chemicals were of the highest purity grade available and were purchased from Sigma-Aldrich Ireland unless otherwise stated. Herculase II Polymerase and protein ladders were from ThermoFisher Scientific Ireland Ltd.

MyTaq Polymerase was from Bioline Inc. (USA). Precast unstained SDS gels were purchased from Bio-Rad Laboratories (USA). *P. pastoris* EasySelect expression kit was obtained from Invitrogen (San Diego, CA); Restriction endonucleases and DNA-modifying enzymes were from NEB and used according to the manufacturer's recommendation. The acid-active chitinase (XM_013473152.1) nucleotide sequence was synthesized by Eurofins Genomics (Germany).

In silico analysis

The aim of our in silico analysis was to identify putative sequence(s) likely to display exo-chitinase activity. As such, the acid-active chitinase (XM_013473152.1) sequence was selected for recombinant production, purification and characterisation based on the in silico screening of the putative GH18 nucleotides from *R. emersonii* (CBS 393.64, taxid: 1,408,163).

Briefly, SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/) was used to predict the presence of a signal peptide within the amino acid sequences. The Basic Logic Alignment Search Tool Protein (BLASTP) program, hosted by NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi), was used to search protein databases for similar sequences. Hit sequences that had been characterised and published were downloaded and aligned with the query sequences, using the Custal Multiple Sequence Alignment tool (http://www.ebi.ac.uk/Tools/msa/). The isoelectric point and molecular weight of the putative amino acid sequences were predicted using the Compute pI/MW tool. The ELM Resource was used to indicate the presence of post-translational modification (PTMs) sites of the amino acid sequences. The InterPro Scan 5 programme, hosted by the European Bioinformatics Institute, was used for protein domain prediction (http://www.ebi.ac.uk/interpro/interproscan.html). SWISS model was used to source templates for protein structure homology-modelling (Waterhouse et al. 2018, accessed through https://swissmodel.expasy.org/). The files were downloaded in PDB format, and the 3D protein structures were visualised using the PyMol molecular visualisation software (https://pymol.org/2/). Phyre2, which uses a profile–profile matching algorithm, was used to visualise the secondary structures of the protein and to enhance the accuracy of the similarity search (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index). The 3D model was evaluated using the modules (ERRAT, VERIFY3D, PROVE, PROCHECK and WHAT_CHECK) of the SAVES server package (https://saves.mbi.ucla.edu/).

Comparative in silico analysis of the three *R. emersonii*-derived chitinases

Comparative in silico analysis was carried out on the acid-active chitinase and the other isolated and characterised chitinases from *R. emersonii* which were recently described in literature (chit1; XM_013472241.1 and chit2; XM_013474098.1). The amino acid sequences were aligned using the Clustal Multiple Sequence Alignment tool.

Phylogenetic analysis of chitinase proteins from 20 fungal genomes

Protein coding genes for the following Fungal species of interest were downloaded *Aspergillus chevalieri, Aspergillus nidulans, Aspergillus terreus, Aspergillus versicolor, Bjerkandera adusta, Cladosporium sphaerospermum, Myceliophthora heterothallica, Myceliophthora thermophila, Mycothermus thermophilus, Penicillium brevicompactum, Penicillium glabrum, Phialocephala scopiformis, Rasamsonia emersonii, Rhizomucor pusillus, Stereum hirsutum, Talaromyces cellulolyticus, Thermoascus stipitatus, Thermomyces dupontii, Thermomyces langinosus* and *Thielavia terrestris*. These fungi were of interest as they are available from the biobank at Monaghan Mushrooms Ireland, having been identified and isolated from the farm compost and mushroom substrates, with the exception of *R. emersonii* which was acquired commercially. The proteomes of these fungi are publicly available from JGI Fungal Resource, *Aspergillus* Genome Database, Genozymes Project and Ensemble Fungi (Table 1). All 20 genomes were concatenated to give a protein database of 210,780 protein-coding genes. The protein sequences of two chitinases, which were previously characterised from *Rasamsonia emersonii* (Dwyer et al. 2021), and the acid-active chitinase protein sequence were used as "bait' query sequences in a BLASTP (version 2.10.1 +) (PMID: 2,231,712) database search (*E* value 10^{-5}) against the protein database. All 227 non-redundant significant BLAST hits were extracted and the presence/absence of the PFAM Glycosyl Hydrolase family 18 (GH18) domain (PF00704) was determined using interproscan-5 (version 5.52–86.0) (PMID: 24,451,626). In total 219 proteins contained the GH18 domain and these were retained for phylogenetic analysis. The 219 protein sequences were aligned using muscle (PMID: 15,034,147) with the default settings and a phylogenetic tree was reconstructed using FastTree (version 2.1.1) (PMID: 20,224,823) with the lg protein substitution model (PMID: 18,367,465). Branch supports were determined using Shimodaira–Hasegawa local supports. The resultant phylogeny was curated using the iTOL webserver (Letunic and Bork 2019).

Table 1 Proteomes of Fungal species used in GH18 Phylogenetic Analysis

Species	Total ORFs	#GH18	Source
Aspergillus chevalieri	10,253	8	JGI
Aspergillus nidulans	10,776	18	Aspergillus genome database
Aspergillus terreus	10,406	20	Aspergillus genome database
Aspergillus versicolor	13,228	17	Aspergillus genome database
Bjerkandera adusta	15,473	11	JGI
Cladosporium sphaerospermum	8807	5	JGI
Myceliophthora heterothallica	10,061	5	JGI
Myceliophthora thermophila	9110	7	JGI
Mycothermus thermophilus	10,945	13	Genozymes project
Penicillium brevicompactum	11,536	13	JGI
Penicillium glabrum	12,328	15	JGI
Phialocephala scopiformis	18,573	12	JGI
Rasamsonia emersonii	9843	11	Ensemble fungi
Rhizomucor pusillus	10,979	10	Genozymes project
Stereum hirsutum	14,072	13	JGI
Talaromyces cellulolyticus	1618	2	Ensemble fungi
Thermoascus stipitatus	8798	14	JGI
Thermomyces dupontii	7920	6	Ensemble fungi
Thermomyces langinosus	6241	6	JGI
Thielavia terrestris	9813	13	JGI

Construction of acid-active chitinase expression vector for *P. pastoris*

The chitinase gene was ordered in a standard vector from Eurofins Genomics. It was amplified using the forward and reverse primers as follows: ggggctcgagAAAAGAGAG GCTGAAGCTGCTTTCATTCCTTACACTAATGATCGG TTT and aaaatctagagtGTTCGACGGGAATCCGGCCCT. The DNA sequence for acid-active chitinase is 1299 bp in length and is publically available from the National Centre for Biotechnology Information (NCBI) database under the reference XM_013473152.1.

The amplicons were digested with *Xho*I and *Xba*I and ligated into the equally treated vector pPICZα A. The procedures resulted in plasmids carrying genes encoding the acid-active chitinase, without the native signal sequences and stop codon, thus allowing the expressed protein to be C'-terminally histagged and secreted using the alpha-factor signal sequence via the methanol inducible AOX1 promoter included in the pICZalphaA sequence.

The aa-chit-pICZalphaA plasmids were electroporated into competent Top10F' E. coli and the resulting transformants were screened via PCR on colonies. Positive colonies were further analysed by DNA sequencing, carried out by Eurofins Genomics, Germany. *E. coli* cells harbouring aa-chit-pPICZaA were cultivated in LB low salt medium at 37 °C supplemented with 25 µg Zeocin/mL overnight. The aa-chit-pPICZaA plasmid was isolated and linearized with *Pme*I and transformed into electro-competent *P. pastoris* X-33 cells. Transformants were grown on YPD plates (10 g L-1 yeast extract, 20 g L-1 peptone, 10 g L-1 glucose and 15 g L-1 agar) containing 100 mg L-1, 500 mg L-1 and 1000 mg L-1 zeocin.

Expression screening, expression and purification of the acid-active chitinase enzyme

P. pastoris transformants were pre-cultured in 2 mL BMGY overnight, harvested and re-suspended in 2 mL of BMMY, to a final OD600 of 1, and incubated at 30 °C and 250 rpm. A negative control of empty-vector *P. pastoris* was used. The media was supplemented with methanol every 24 h to a final volume of 1% to maintain expression. At 48 h of induction, the secretome underwent SDS-PAGE analysis. The highest expressing *P. pastoris* cells were cultured and induced as before with some modifications; pre-cultures were scaled-up to 25 mL and expression cultures to 100 mL in 250 mL baffled flasks. Filter-top lids allowed aeration of the samples. Samples were harvested at 48 h to avoid proteolytic degradation of the recombinant proteins (Sinha et al. 2005).

Purification

The expression supernatant (approximately 95–100 mL) was equilibrated to final concentrations of 50 mM sodium phosphate, 300 mM sodium chloride, pH 7.4 and was loaded into a 1 × 6.0 cm econo-column (Bio-Rad) packed

with HisPur Cobalt with a bed volume of 3.0 mL. Using the Biologic LP purification system, equilibrium buffer and elution buffer (50 mM sodium phosphate, 300 mM sodium chloride, 150 mM imidazole; pH 7.4) was run through the column at a flow rate of 1 mL/min. Fractions of 3.0 mL were collected, assayed for chitinase activity, and protein concentration. Recombinant-protein-containing fractions were pooled, assayed for total activity and total protein content. The enzyme was dialysed (50 mM citrate buffer pH 4) using the 3 mL slide-A-lizer kit, as per manufacturer's manual. Sterilised glycerol was added to the dialysed, purified protein sample to a final concentration of 20%. The sample was stored in 1 mL aliquots at – 20 °C thereafter.

De-glycosylation reaction with PNGase F

De-glycosylation was carried on crude and pure acid-active chitinase using PNGase F enzyme from New England Biolabs, as per manufacturer's instructions for denaturing method, using H_2O, buffer and PNGase F as a negative control.

Identification of expressed protein

One-dimensional SDS-PAGE was carried out according to known methods (Laemmli 1970) using a acrylamide gels of various percentages and a vertical electrophoresis system. BioRad Stain Free pre-cast gel of 10%, 12% or 4–16% acrylamide was used as per the manufacturer's instructions. Western blotting was carried out to confirm the presence of the His6-tagged product according to standard methods as described in Sambrook and Russell (2001). Briefly, after electrophoresis, the proteins were transferred onto a PVDF membrane using the BioRad Turbo Blot transfer system and subsequently, using Bio-Rad Immun-Blot Goat Anti-Rabbit IgG (H + L)-HRP Assay Kit #1706463, the membrane was developed by initially blocking and washing the membrane with a milk-TTBS and TTBS solution respectively, then applying the antibody at 1–2 h at room temp or 4 °C overnight. The membrane was washed again in TTBS and the conjugate binding step occurred by incubating the membrane with Protein A-HRP in TTBS for 30 min to 2 h. The membrane was washed again with TTBS and once with TBS to remove residual Tween-20. The resulting signals were visualised via chemillumencessence settling on the BioRad Imaging System.

Chitinase assay

The assay used for the estimation of chitin degrading activity was based on methods in Sigma Chitinase Assay Kit (product code CS0980) with some modifications. The microtiter assay contained 0.2 mg of substrate, 4-nitrophenyl N,N-diacetyl-β-D-chitobioside in 100 mM glycine–HCl buffer pH 2.5, unless otherwise stated. The reaction proceeded for 15 min at the desired assay temperature and was stopped by the addition of 1 M sodium carbonate. All samples were measured in triplicate, against a substrate-enzyme blank. One unit of chitinase activity was defined as the amount of enzyme capable of releasing 1 µmol of 4NP/min under the defined assay conditions. Specific activity of the enzymes could be calculated using the absorbance at 280 nm in respect to a BSA standard curve from concentrations of 0.0–20.0 mg/mL. Specific activity of the enzymes was defined as the amount of enzyme capable of releasing 1 µmol of 4NP/min/mg under the defined assay conditions.

Biochemical characterisation of the acid-active chitinase

The effect of pH on enzyme activity was determined by measuring activity at pH 1.5–6.0 in 100 mM KCl–HCl (pH 1.5–2.2), *glycine–HCl (pH* 2.2–3.5), phosphate-citrate (pH 2.6–5.0) or citrate buffer (pH 5.0–6.0). The effect of temperature on enzyme activity was determined by measuring activity at 30–70 °C. The stability of the enzyme was determined by incubating the acid-active chitinase at 50 °C and pH 2.5, over a time course of 48 h. The relative activity remaining at each sample point was expressed as a percentage of the optimum activity observed. Inactivation data was determined as per the methods of Anthon and Barrett 2002. The substrate specificity of the chitinase was determined with respect to the substrates 4-nitrophenyl N-acetyl-β-D-glucosaminide, 4-nitrophenyl, N,N-diacetyl-β-D-chitobioside and 4-Nitrophenyl β-D-N,N,N-triacetylchitotriose. The first two sequences tested exo-chitinase activity; glucosaminidase activity and chitobiosidase activity, respectively. The 4-Nitrophenyl β-D-N,N,N-triacetylchitotriose substrate tested endo-chitinase activity. The kinetic parameters were determined with respect to the substrate 4NP- N,N′-diacetyl-β-D-chitobioside using methods available in literature (Biggs 1954; Khan et al. 2015) by monitoring the rate of substrate hydrolysis as a function of substrate concentration at pH 2.5 and 50 °C. Substrate concentrations ranged from 0.2 to 1.0 mg per mL. Plots of substrate concentration versus enzyme velocity were fit using non-linear regression in GraphPad Prism, which then estimated the kinetics constants Km and Vmax.

Industrial application testing of the acid-active chitinase

Hydrolysis reactions on complex industrial substrates were carried out to assess the industrial applicability of the acid-active chitinase. Chitin from shrimp (Megazyme, Ireland) was used as a substrate to reflect the majority of current industrial chitin bioprocessing. Additionally, chitinous substrates from commercial mushroom production were used, namely fungal chitin, glucan-chitin polymer (de-proteinated mushroom powder) and dried mushroom powder. The glucan-chitin polymer and dried mushroom powder were generated by Monaghan Mushrooms staff, and represent the emerging fungal chitin markets. In the reactions, 10 mg of the substrate was placed into 10 mL 20 mM sodium acetate buffer pH 4 and samples were incubated at 50 °C and 150 rpm overnight. Denazyme CBB from Nagase ChemteX Corporation was used as the market standard, and the negative control contained buffer only. The test sample contained 0.1 mg of each enzyme. In the case of the acid-active chitinase, to represent industrial enzyme processing, a crude 48 h expression sample was used, prepared as per expression methods. This corresponds to approx. 25 µmol/min of the acid-active chitinase (on 4-nitrophenyl N,N-diacetyl-b-D-chitobioside in optimum conditions). In the case of the control, the enzyme was resuspended in buffer, corresponding to approx. 175 µmol/min (as per product manual). After hydrolysis, the samples were filtered and analysed by HPAEC-PAD analysis using the Dionex ICS-5000 system, in conjunction with a 4 × 250 mm ThermoFisher CarboPac-PA10 anion exchange column (ThermoFisher Part # 035391) and Carbo Pac-PA10G guard column, 4 × 50 mm (ThermoFisher Part # 043096) and an Electrochemical Detector to detect and quantify sugars and oligosaccharides released from the hydrolysis reaction. The eluent system used 100 mM Sodium hydroxide/1 M Sodium Acetate (A), MilliQ water (B), and 100 mM Sodium hydroxide (C). Elution was performed in a linear gradient from 0:0:100 (% A:B:C) to 0:30:70 (% A:B:C) from 0 to 15 min, followed by isocratic elution at 0:100:0 (% A:B:C) for 3 min. The method was carried out at 25 °C and the flow rate was 1 ml/min. The peaks of interest eluted between 4 and 8 min. Standards for this method were made up in the water and included 50 µg/ml glucosamine, N-acetyl-glucosamine, N,N-diacetyl-chitobiose, N,N,N-triacetyl-chitotriose, N,N,N,N-tetraacetyl-chitotetraose and N,N,N,N,N,-pentaacetyl-chitopentaose. Comparison of the peak-retention time of the standards to the peak-retention times resulting from the test samples allowed the identification of the sugars and chitooligosaccharides release during hydrolysis. For quantification, standard curves which ranged from 1.62 to 103.88 µg/ml for glucose, n-acetyl-glucosamine or glucosamine were used for comparison. The quantification of these sugars was used to determine the efficacy of the chitinase to act on chitinous substrates. The size of the peaks in relation other peaks in the same hydrolysis sample allowed estimation of the amount of other chitooligosaccharides released during hydrolysis (i.e. N,N-diacetyl-chitobiose, N,N,N-triacetyl-chitotriose, N,N,N,N-tetraacetyl-chitotetraose and N,N,N,N,N,-pentaacetyl-chitopentaose), though these were not quantified.

Results and discussion

In silico analysis

R. emersonii (CBS 393.64, taxid: 1408163) was selected as a suitable source of chitin-degrading enzymes, due to its reputation as a producer of important industrial enzymes with high temperature optima (Coughlan et al. 1984; Tuohy et al. 2012), as well as previous literature describing the native chitinolytic cocktail (Hendy et al. 1990), the individual components of which would likely display properties desired in industry (such as thermoactivity and thermostability). Likelihood of exo-activity was considered a desirable feature during in silico analysis, as exo-chitinases yield chitobiose and/or N-acetyl-glucosamine from chitin, both of which have commercial value.

The genome of *R. emersonii* encodes 15 putative GH18s. The putative sequences were downloaded from the NCBI website and subjected to the in silico analysis as per the materials and methods section.

The putative sequence, subsequently termed "acid-active chitinase", showed similarity to other GH18 chitinases from *Aspergillus, Penicillium, Byssochlamys*. The sequence displayed 65% identity with 92% query cover to a chitinase gene from *Coccidioides immitis* (Hollis et al. 1998; Pishko et al. 1995; Yang et al. 1996), and 62% identity across 92% percent of the sequence cover with a chitinase from the fungal pathogen *Aspergillus fumigatus* (Hu et al. 2004), and 61% with 88% query cover to chitinase6 from *Coccidioides posadasii.* The predicted isoelectric point of the acid-active chitinase was pH 4.91. The predicted molecular weight was 45.81 kDa. The ELM Resource predicted that the acid-active chitinase contained 6 N-linked glycosylation sites, the backbone-locations of which can be seen in Table 2. The acid-active chitinase was predicted to include a signal sequence, GH18 catalytic region and an insertion domain. This chitinase insertion domain (CID) is known to be composed of five or six anti-parallel beta-strands and one alpha-helix and it inserts between the seventh alpha-helix and seventh beta-strand of the GH18 TIM barrel. The presence of the CID was considered an interesting feature as it is correlated to exo-chitinase activity. This type of activity is more likely to yield glucosamine or N-acetyl-glucosamine from chitin

than endo-chitinase activity. Hence, the acid-active chitinase was selected for further study.

To further confirm the presence of the chitin-insertion domain, visualisation of the putative acid-active chitinase was carried out. Template search with BLAST and HHblits performed against the SWISS-MODEL template library identified *Aspergillus fumigatus* chitinase B1 (PDB ID: 2a3c.1.A) as the suitable template for the structure prediction of the acid-active chitinase. The sequence and structure alignment shows 61.77% identity. The Procheck structural assessment tool showed 92.3% of residues were in the most favoured regions and 7.7% residues in the additional allowed regions of the Ramachandran plot. Verify3D showed that 96.72% of the residues have averaged 3D-1D score ≥ 0.2, indicating the reliability of the model prediction. Errat gave the overall quality factor of the model a score of 87.63%. During the homology modelling, the catalytic α/β barrel typical of GH18 enzymes could be observed using Pymol (Fig. 1). The model showed 14 α-helices and nine β-sheets. The chitin insertion domain was observed as comprising several anti-parallel β-sheets. To further support the visualisation of the acid-active chitinase, the amino acids involved in the secondary structure of the protein were predicted using Phyre2 and can be seen in Fig. 2.

Comparative in silico analysis of the acid-active chitinase and two characterised *R. emersonii*-derived chitinases

During the in silico analysis of the acid-active chitinase, the homology modelling showed the GH18 catalytic domain contains 8 strands of parallel β-sheets, forming a barrel laid down α-helices, which in turn from a ring to the outside. The CID domain forms a wall alongside the TIM barrel substrate-binding cleft of chitinase which increases the depth of the cleft, which can correlate to exo-chitinase activity. The mechanism of activity can be predicted by the class of chitinase. The active acid chitinase is a putative class II chitinase on the basis of amino acid sequence homology as analysed by Blast and Interpro scan, making it likely to hydrolyse β-glycosidic linkages through an inverting mechanism.

Other characterised GH18 chitinases derived from *R. emersonii* have either been seen to be comprised of a GH18 domain only or have contained a binding domain at the C-terminus. A number of plant and fungal proteins that bind N-acetylglucosamine contain this domain. The domain may occur in one or more copies and is thought to be involved in the recognition or binding of chitin subunits. The acid-active chitinase does not contain this chitin-binding domain nor does it share any notable sequence identity with other chitinases characterised from *R. emersonii* (Fig. 3). It is not unusual for fungal chitinases of the same GH family and source to differ in sequence as fungal chitinases carry out a number of native functions and activities. Additionally, the GH18 family includes the broad activity range of endo-chitinase, chitobiosidase and glucosamindase (Oyeleye and Normi 2018). The similarity between the sequences which does occur occurs within the predicted GH18 catalytic regions.

Phylogenetic analysis of GH18 from 20 fungal species

Twenty Fungal proteomes of interest were concatenated to build a local BLAST database of 210,780 proteins. BLASTP homology database searches of the acid-active chitinase as well as the Chit1 and Chit2 proteins from *R. emersonii* followed by filtering for the presence of the GH18 domain (PF00704) resulted in a dataset of 219 proteins. *T. cellulolyticus* was found to encode only two chitinases while *A. terreus* encodes 20. Including Chit1, 2 and the acid-active chitinase *R. emersonii* was found to encode 11 (Table 1). Our phylogenetic analysis shows that Chit 1, 2 and the acid-active chitinase are not species-specific duplications as they

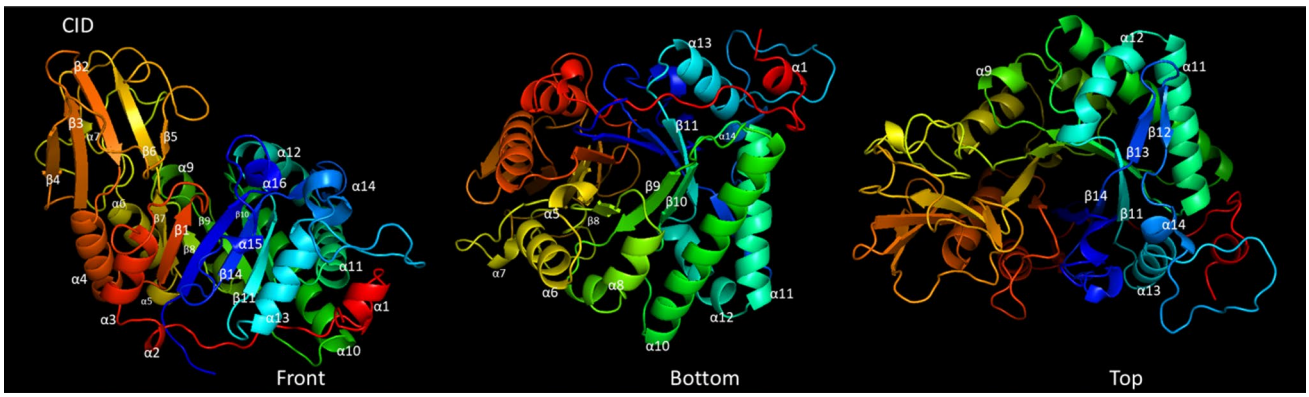

Fig. 1 Homology modelling with predicted protein domains within the acid-active chitinase as generated by SWISS model and visualised using PyMol. GH18 family domain is detected as well as a chitin insertion domain. The α-helices, β-sheets, α/β barrel and CID are labelled

Fig. 2 Protein secondary structure analysis of the acid-active chitinase generated by Phyre2

are not grouped beside one another, furthermore, orthologs are found in many different species (Fig. 4). In terms of phylogenetic relatedness *R. emersonii* acid-active chitinase shares a close relationship to *Penicillium* species, *R. emersonii* Chit1 is most similar to *T. cellulolyticus* and *R. emersonii* Chit2 is most similar to *Thermomyces* species (Fig. 4). It is interesting to note that the difference in the number of GH18 proteins between different species is predominantly down to species-specific duplications, a select number of these are highlighted for *C. sphaerospermum*, *Thielavia terrestris* and *Penicillium* species (Fig. 4). Interproscan analysis showed that 40 proteins including *R. emersonii* Chit2 contain a Chitin binding PFAM domain (PF00187) while in total 34 contained a LysM PFAM domain (PF01476). The LysM domain aids in anchoring to extracellular polysaccharides such as chitin (Fig. 4).

Cloning

Amplicons resulting from the Polymerase Chain Reactions were seen to occur in the expected region of 1299 bp. Successful ligation of the digested amplicons and the pICZα

Table 2 Shows predicted PTMs for the translated acid-active chitinase sequences

Predicted PTM	Matched sequence Chit3	Amino acid positions	Pattern
Generic motif for N-glycosylation	SNSSST	33–38	
	QNLTTE	59–64	
	NNATEA	177–182	(N)[^P][ST]
	DNYSAK	197–202	
	ANGTHL	204–209	
	GNESLI	391–396	

PTMs were predicted by the ELM Resource under "*Rasamsonia emersonii*" and "extracellular" conditions

expression vectors was confirmed by the plasmid-insert analysis. Purified pICZα vectors containing inserts were sent to Eurofins Genomics for sequencing analysis. The resulting sequences were aligned with the original acid-active chitinase sequence yielding a homology score of 100%. The growth of single colonies indicated that the transformation of linearized aa-chit-pICZαA in *P. pastoris* X-33 had been successful. The rising concentration of zeocin encouraged the separation of high-copy integrals, as visualised by a decrease in the numbers of transformants with rising zeocin levels on the selection plates.

Fig. 3 Sequence alignment of the three *R.emersonii*-derived chitinases generated by Custal Omega

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Chit1      MKTSLLAFASFTGLLASSHAAP-----------LRSNNATTKLSG--VAAWWGQGSGASL 47
Chit2      -----MLRLLLLGLLA---------------------AHAAALR---YVMYIDQYHKDNL 31
Chit3      MQ---LKLALLLGLSWLANAAFIPYTNDRFRLHSRSNSSSTGFRSVAYFVDWAIYARNFN 57
                 :    : **                          :: :

Chit1      DEVCGEKSFDIVILSFLDTFFGAGGYP--G--MNMQGLYSPSPAQRAAGATDLLDGTPLV 103
Chit2      PEPDQTQGITHAIMAFAKSDLFNSD----------------------SP-PQFTPFEPI 67
Chit3      PQNLTTETLTHVLYAFANVDQNTGEVSLADTYADIQKHYPTD-SWNEQGN-NVYGCVKQL 115
            :    : :  .: :* .     .                          .         :

Chit1      PAIKKCQSAGKKVFLSLGGADASSKLDSDEQGAQLADTVWNLFLGGTKDAALRPFQNVTL 163
Chit2      STMRGRFSKDTKVMIAIGGWGDTSGFSAGAKDNTTRA--------RFAKNVAAMLDSLGA 119
Chit3      -FLLKKKQRNLKTLLSIGGYTYSPKFSTFTTTESGRQ--------TFASTAVQMVQNLGF 166
             :    . . *.::::**   :  :.:                    . .   .:.:

Chit1      DGIDLDNENHDPTGY-----------------LGLVNQLRANMKSDSS---------KQY 197
Chit2      DGVDIDWEYPGGNGQDYKQIPNSAKVDEIETYPLFLQAIRDAISKDKLLSIAVPGLQRDM 179
Chit3      DGIDIDWEYPNNATEADNMVA------------LLKDVREALDNYS---AKYANGTHLL 210
           **:*:* *  .                      ::: :*  :.. .          :

Chit1      YISAAPQCPRPDASLSDDVLQKTDMVWVQFYNNQQANCCNGQDG---FL----------- 243
Chit2      IAFTKEQGPKIFAP------------VDFVNVMTYDLMNRRDNVTKHHTSVQ------- 219
Chit3      LSVASPAGPSNYQPMHLAE----MDQYLDFWNLMAYDYTGSWSTVAGHMANVFPSTSNPA 266
              :    *                 ::* *    :  .  .    .

Chit1      ----NSLQA----WSKAVSPAQVWIGAPAAASAVG--DGGYISASTMQAEIAQAKKLGLS 293
Chit2      ----GSLETINNYLDIGMDPAKANLGFAFYAKYFTTDPSSDCATEPLGCKVVPLENPDGS 275
Chit3      STPFNTDQAVSYYTSNGVAPSKINLGIPLYGRSFLQTDGPGTAYNGVG---------PGE 317
               .: ::     . .: *::  :*    .  .    .   : . :           .

Chit1      NFGGVALWDAELAIQNGNFQDA----------------------------------- 315
Chit2      DNGKSGAYTFEPIHMAPPPSNLKTSTDGTCGIDKGKCPEGLCCSQYGNCGTTDEFCQVGC 335
Chit3      WEGGPGVWDYKALPQAGA------------------------------------------ 335
             *  . :  :

Chit1      ------------------------------------------------------------ 315
Chit2      LSDYGTCKGVSVTDSWRKAQSDGKTDEEAGGQYYWDADNQLFWSWDTPDLMAKKFTDIVS 395
Chit3      --------------------TVYELDQPIASYSYDPNQKIMISYDTPNVVAWK-GDYIQ 373


Chit1      ----------------------VASAL-----------------------------AS- 322
Chit2      AKKLGGVMAWSLGEDSYDWS-HV-------------------------KALRDGVNSL 427
Chit3      QKGIGGGMYWESSGDKSGNESLIATLVNRVGGTAVLDQSPNLLSYPASIYDNLRAGFPSN 433
                                 :                                    *

Chit1      ---- 322
Chit2      TTST 431
Chit3      ---- 433
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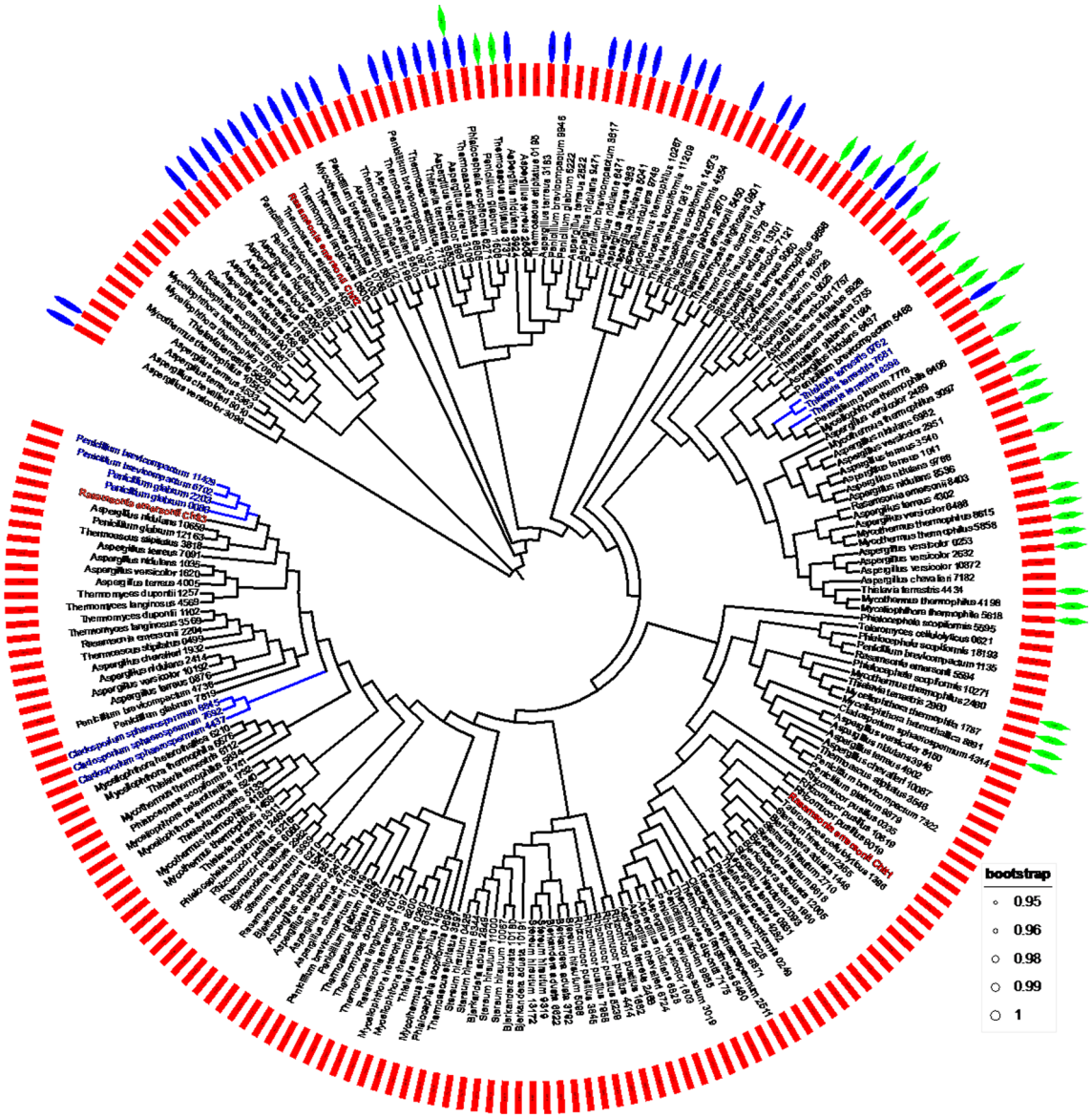

Fig. 4 Maximum likelihood phylogeny of 219 chitinases (GH18) from 20 fungal species. *R. emersonii* Chit1, Chit2 & the acid-active chitinase are highlighted in red text. Branch supports are indicated, only inferences with a score of 0.95 are shown. A select number of species-specific duplications are highlighted with blue branches/text. The presence of select PFAM domains is shown. Red rectangles indicate the presence of a GH18 domain (PF00704), green hexagons indicate the presence of the LysM domain (PF01476) and blue ellipses indicate the presence of a chitin-binding domain (PF00187)

Expression and purification

The acid-active chitinase enzyme was effectively secreted into the expression media with crude activity levels of 239.71 µmol/mL on chitobiose substrates after 48 h of methanol induction. The chitinase was purified to apparent homogeny in a one-step IMAC purification protocol, recording a yield of 64% with a purification factor of 106.47.

The predicted molecular weight of the acid-active chitinase was 45.81 kDa. The expressed protein was a glycoprotein was identified as a smear from 56.3 to 125.1 kDa, which sharpens into two bands at 46.0 kDa and 48.4 kDa, and a smear above 60 kDa when treated with PNGase F. The smear above 60 kDa is the acid-active chitinase which has not been fully de-glycosylated as it shows a positive result for histag presence (Fig. 5). Despite efforts to deglycosylate the acid-active chitinase, the smear remained, possibly due to the 6 predicted N-glycosylation sites within the sequence, plus the chitin insertion domain may limit the accessibility of the chitinase backbone to the PNGase F enzyme, though the glycosylation sites do not occur within that region.

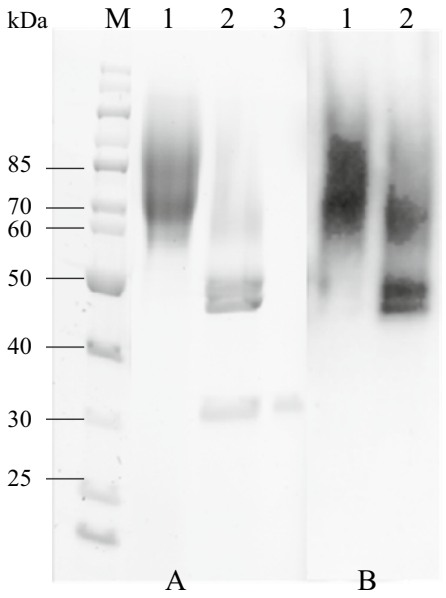

Fig. 5 SDS-PAGE (**a**), and western blot (**b**) of deglycosylation carried out purified acid-active chitinase. (M) PageRuler Unstained Protein Ladder, (1) acid-active chitinase, (2) deglycosylated acid-active chitinase, (3) PNGase F control

Enzymes which are natively secreted from eukaryotic organisms are likely to undergo PTMs. The observed smears are due to glyco-sidechains which migrate unpredictably in SDS gels (Bonifacino 2004; Hames 1998). The multiple banding after de-glycosylation is likely reflective of proteolytic processing.

pH and temperature profile

The acid-active chitinase shows optimum activity at pH 2.8, as seen in Fig. 6. As far as we are aware, a fungal chitinase

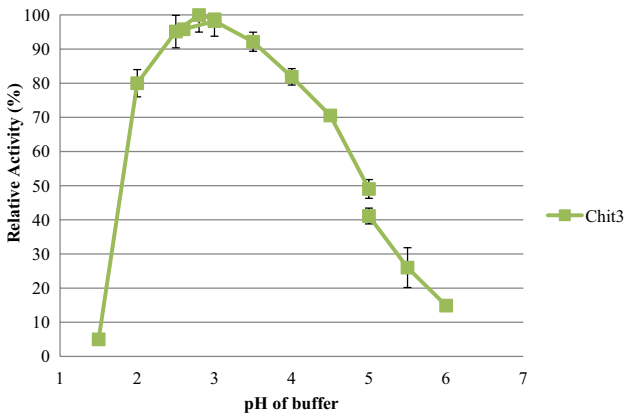

Fig. 6 pH profiles of the purified acid-active chitinase. Values for 100% relative activity on this substrate (4-nitrophenyl N,N-diacetyl-b-D-chitobioside) were 268.07 µmol/mg (mean values + _ DS, $n = 3$)

displaying such acidic activity optima, as well as high relative activity displayed at pH 2, has not been described in literature previously. The temperature curve of the acid-active chitinase can been seen in Fig. 7, it shows the optimum temperature of the enzyme is 50 °C. Previously, the enzymatic secretome of *R. emersonii* demonstrated the ability to degrade chitin and chitin derivatives at temperature optima of 65 °C (Hendy et al. 1990; McCormack et al. 1991). However, to date, the individual chitinases display a temperature optimum of 50–55 °C; with Chit1 and Chit2 also having fallen into this range.

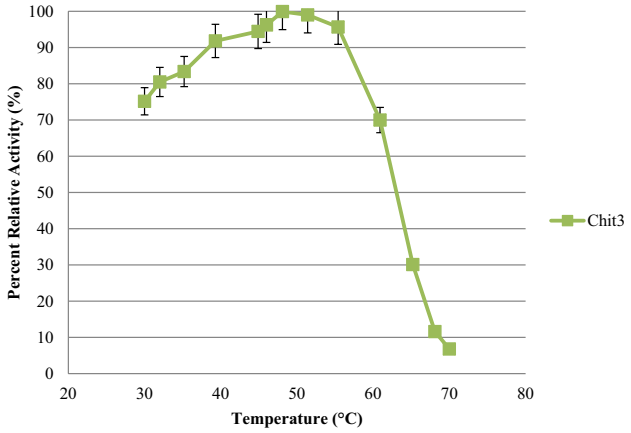

Fig. 7 Temperature profile of purified acid-active chitinase. Values for 100% relative activity on this substrate (4-nitrophenyl N,N-diacetyl-b-D-chitobioside) were 268.07 µmol/mg (mean values + _ DS, $n = 3$)

The stability profile of the acid-active chitinase can be seen Fig. 8. The acid-active chitinase retains 63.3% and 28.9% relative activity remaining after 24 h and 48 h, respectively, of incubation at 50 °C and pH 2.5. The inactivation data revealed that the *D* value, the time required to reduce the enzyme activity to 10% of its original value at 50 °C, was 3.7 days. The enzyme can be classed as a thermostable chitinase.

Substrate specificity

The acid-active chitinase is predominately an exo-chitinase, with preference shown for 4-nitrophenyl N,N-diacetyl-b-D-chitobioside. It has 24.88% relative activity as an endo-chitinase. It is the only characterised chitinase from *R. emersonii* to display any N-acetyl-glucosaminidase activity, but this is minimal with 1.16% relative activity shown. The exo-chitinase activity of this enzyme is likely due to the presence of the chitin insertion domain, which plays a role in deepening the substrate binding cleft of the enzyme. This has been seen to result in increased exo-chitinase activity (Fig. 9).

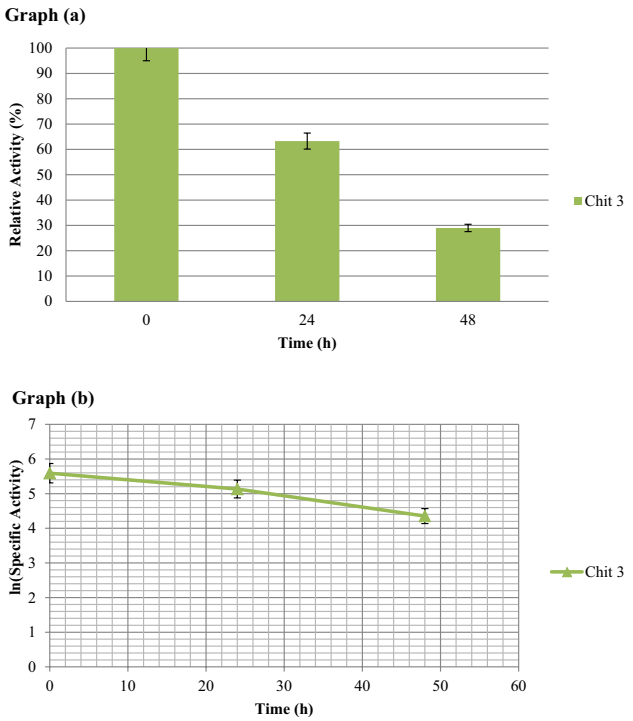

Fig. 8 Stability profile of the acid-active chitinase (graph (**a**)) at 50 °C, pH 2.8 over a 48 h time course. Values for 100% relative activity on this substrate (4-nitrophenyl N,N-diacetyl-b-D-chitobioside) was 268.07 µmol/mg (mean values + _ SD, $n = 3$). Thermal inactivation (graph (**b**)) data was generated using the equation ln(specific activity) vs time to obtain an estimate of the kD; kD is the time required to reduce enzyme activity to 10% of its original value

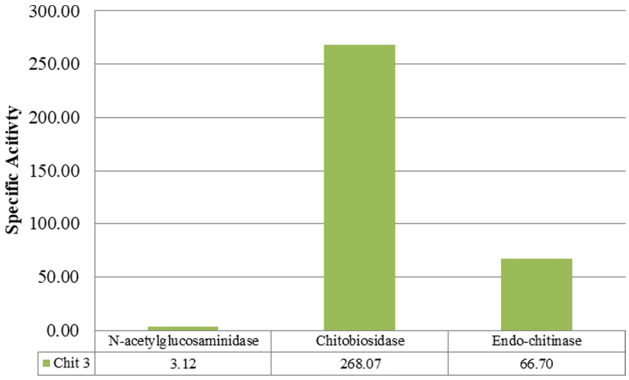

Fig. 9 The specific activity (µmol/mg) of the acid-active chitinase displayed towards various substrates (mean values + _ DS, $n = 3$). The reaction was carried out at 50 °C, pH 2.5. The substrates used were 4-nitrophenyl N-acetyl-β-D-glucosaminide, 4-nitrophenyl, N,N-diacetyl-β-D-chitobioside and 4-Nitrophenyl β-D-N,N,N-triacetylchitotriose. The first two sequences tested exo-chitinase activity; glucosaminidase activity and chitobiosidase activity, respectively. The 4-Nitrophenyl β-D-N,N,N-triacetylchitotriose substrate tested endo-chitinase activity

Kinetic properties of the chitinases

The Vmax of the acid-active chitinase was calculated by GraphPrisim Pad to be 5034 µmol/mg/min, thus making the Km estimate 1.15 mg/mL. Previously, of the *R. emersonii*-derived chitinases, Chit2 had the highest enzyme velocity of 7351 µmol/mg/min (Dwyer 2019), possibly due to the presence of the chitin-binding domain which may allow the faster location of the substrate backbone in solution. Its velocity was nearly twice that of the acid-active chitinase which contains the substrate insertion domain. Chit1 is estimated to have the highest Km value, indicating that it would not become as quickly saturated by the substrate as other *R. emersonii* chitinases in a hydrolysis reaction.

Industrial application testing of the acid-active chitinase

The commercial chitinase was the most effective in degrading the chitinous substrates under the defined assay conditions (Table 3). It released 1.27 mg/mL, 1.39 mg/mL, 0.92 mg/mL and 1.29 mg/mL of N-acetyl-glucosaminide from shrimp chitin, fungal chitin, fungal chitin-glucan polymer and dried mushroom powder, respectively. The commercial enzyme yielded many peaks between 8 and 18 min which were not identified over longer retention times, particularly in the shrimp chitin and fungal chitin hydrolysis samples (Fig. 10). These are likely to be chitooligosaccharides of various chain lengths as a result of random cleavage of the chitin backbone. The acid-active chitinase displayed efficacy in degrading the pure chitinous substrates, i.e. shrimp chitin and fungal chitin under assay conditions, yielding 0.55 mg/mL and 1.06 mg/mL N-acetyl-glucosaminide from shrimp chitin and fungal chitin, respectively. The enzyme displayed less activity on the more complex substrates of fungal chitin-glucan polymer and dried mushroom powder. The acid-active chitinase gave the highest relative peak activity for diacetyl-chitobiose, with 47.46% and 30.50% for shrimp chitin and fungal chitin, respectively, with second highest peaks for N-acetyl-glucosaminide, indicating a preference for exo-chitinase activity, which is expected given the enzyme's preference towards exo-chitinase activity during the substrate specificity tests, as well as the presence of the insertion domain which would indicate a processive mode of action.

Table 3 N-acetyl-glucosaminide and Glucosaminide in mg/ml produced by approximately 0.1 mg of each crude enzyme over 24 h hydrolysis reactions at 150 rpm, 50 °C on industrial chitinous substrates

Sample name	NAG (mg/ml)	GA (mg/ml)
Shrimp chitin		
Commercial enzyme	1.27 ± 0.09	0.21 ± 0.01
Acid-active chitinase	0.55 ± 0.03	0.14 ± 0.01
Fungal chitin		
Commercial enzyme	1.39 ± 0.10	0.00 ± 0.00
Acid-active chitinase	1.06 ± 0.09	0.06 ± 0.01
Fungal chitin-glucan polymer		
Commercial enzyme	0.92 ± 0.10	0.72 ± 0.05
Acid-active chitinase	0.00 ± 0.00	0.22 ± 0.03
Dried mushroom powder		
Commercial enzyme	1.29 ± 0.08	0.13 ± 0.01
Acid-active chitinase	0.00 ± 0.01	0.07 ± 0.01

Discussion

Physiochemical properties

The acid-active chitinase displays pH optima of pH 2.8. Of the characterised fungal chitinases to date, most display optimal pH ranges and pH stability within the ranges of pH 4 to pH 8 (Karthik et al. 2014). There are some fungal chitinases that show pH stability at pH 2; Chit1 from *R. emersonii* was considered a particularly acid-active enzyme with over 60% relative activity remaining at pH 2, a chitinase isolated from *Aspergillus fumigatus* and one from *Penicillium janthinellum* also retained notable activity at this pH value (Di Giambattista et al. 2001). Another chitinase from *Trichoderma viride* displayed pH optima of 3.5, but lost activity below pH 3, and a chitinase from *Anaeromyces mucronatus* displayed a pH activity range as low as pH 2.5 but optimum pH activity occurred at pH 6.5 (Karthik et al. 2014; Ihrmark et al. 2010), none, however, display such low pH optimum activity as this acid-active chitinase.

The temperature optimum of the acid-active chitinase is 50 °C, which is similar to the optima displayed by the other two chitinases isolated from *R. emersonii*. Additionally, the chitinases isolated from *R. emersonii* display over 50% relative activity occurring within the ranges of 30–60 °C. The acid-active chitinase retains more relative activity at lower temperatures. Other research has shown that most thermophilic fungal chitinases display temperature optimum within 40–60 °C, with activity range displayed across the temperatures of 30–70 °C (Karthik et al. 2014). *Thermomyces lanuginosus, Gliocladium catenulatum, Rhizopus oryzae* have been shown to produce chitinase enzymes that exhibit temperature optima at 55–60 °C (Chen et al. 2013; Gui-Zhen Ma 2012; Guo et al. 2008; Prasad and Palanivelu 2012). Thus, the temperature profile of the acid-active chitinase is in line with temperature optima displayed by thermophilic filamental fungal sourced chitinases, including the other characterised *R. emersonii* derived enzymes chitinases.

The thermostability displayed by acid-active chitinase at 50 °C is expected given the thermophilic source of the enzyme. Research describing chitinases from thermophilic fungi such as *Gliocladium catenulatum, Rhizopus oryzae,*

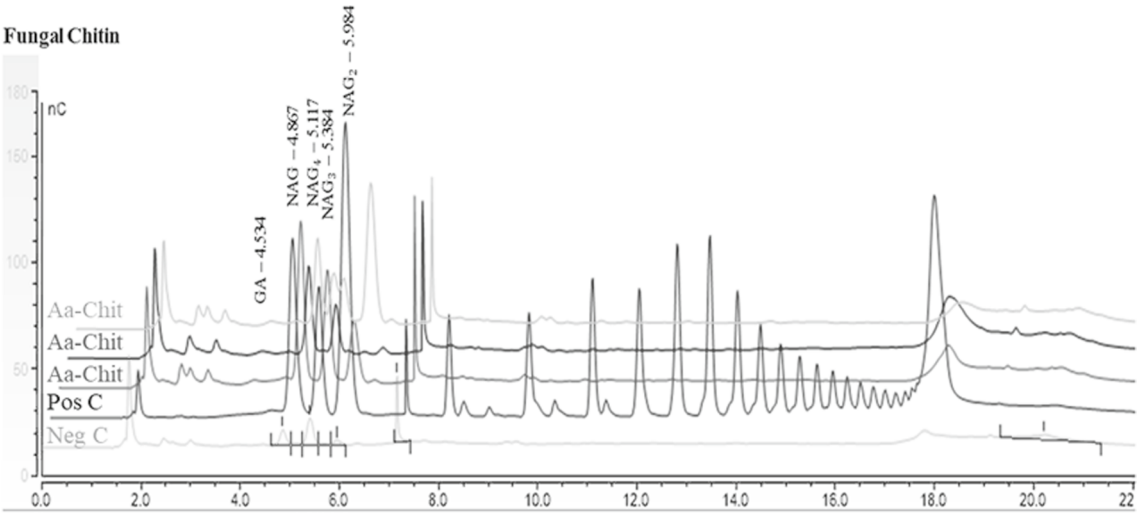

Fig. 10 Chromatogram resulting in industrial applicability testing. GA, NAG, NAG_2,NAG_3, NAG_4 refer to glucosamine, N-acetyl-glucosamine, N,N-diacetyl-chitobiose, N,N,N-triacetyl-chitotriose and N,N,N,N-tetraacetyl-chitotetraose, respectively. The chromatogram shows results generated from the chitinase which was tested in the triplicate, positive control (Denazyme CBB) and negative control (buffer)

Thermomyces lanuginosus and strains of *Trichoderma* exhibit thermostability up to 50 °C (Chen et al. 2013; Gui-Zhen Ma 2012; Guo et al. 2008; Prasad and Palanivelu 2012). For Chit1 and Chit2 previously, the *D* value was 19.2 and 2.3 days, respectively, and Chit1 remains the only hyperthermostable chitinase from *R. emersonii*.

Potential role in nature of the individual *R. emersonii* GH18 chitinases

Most chitinolytic fungi have been shown to produce different types of chitinases, likely owing to the number of roles that fungal chitinases play natively as well as the complexity of natural chitin substrates. *Trichoderma* species have been well characterised in terms of chitinase production and have been seen to produce several types of individual chitinase with activities including N-acetyl-glucosamidases, chitobiosidases and endo-chitinases (Katarina Ihrmark et al. 2010; Nogawa et al. 1998; Ulhoa and Peberdy 1991). The entomopathogenic fungus *Metarhizium anisoplae* also produces at least six different chitinases (da Silva et al. 2005; Staats et al. 2013). A number of chitinases were described in the secretome of *Stachybotrys elegans* under different growth conditions (Tweddell et al. 1994).

A potential native role of the chitinases is chitin-degradation for nutritional uptake. Multiple chitinases from the same fungal source are thought to play a synergistic role in chitin degradation and the demonstrated differences in the activity profile of Chit1, Chit2 and the acid-active chitinase could be representative of this as *R. emersonii* has already been shown to be capable of complete chitin hydrolysis (Hendy et al. 1990).

It's also possible that the demonstrated differences in the activity profile of the *R. emeronii* chitinases as well as the different predicted domains and relative un-relatedness of the enzymes indicates that they carry out different native roles. The three chitinases contain native signal sequences and are destined for extracellular application according to Interpro Scan, hence it is unlikely that the enzymes play a role in cell wall remodelling, as the fungal chitin layer faces the intracellular area of the cell. The exception is perhaps Chit2 which may play a role in mitosis, as indicated by the amphipathic motif which was previously detected in its amino acid sequence by the ELM Resource. This motif binds to yeast Cdc20 and acts as an APC/C degron enabling cyclin Clb5 degradation during mitosis. Chit1 displays homology to include a group of chitinases from *Trichoderma* which are expressed during mycoparasitism-related conditions, and chitinases from *Metarhizium anisopliae* which is an entomopathogenic fungus used in the biological control of some agricultural insect pests. Hence, Chit1 may play a native role in competition or defence against other microbes.

In hypothesizing the native role of the acid-active chitinase, it's possible this enzyme plays a role in chitin degradation for cell uptake. The *R. emersonii* genome encodes at least one chitin deacetylase, which is also a secretory enzyme. As the chitin deacetylase carries out the deacetylation reaction, this would release the acetyl groups, causing the environment surrounding the chitin to become acidic. With a pH optima of pH 2.8, the acid-active chitinase would likely remain active in the acidic environment immediately surrounding the chitin/chitosan, catalysing the hydrolytic cleavage of the chitin/chitosan backbone, likely yielding chitobiose, a disaccharide small enough to be absorbed by the *R. emersonii* cell.

Potential industrial applications of the acid-active chitinase

The hydrolysis of mushrooms for sustainable alternative protein processing represents the only current use of chitinases in industrial chitin hydrolysis reactions (Nagase (Europa) GmbH 2021). This occurs at 45–60 °C for 24 h. The use of chitinases in crustacean chitin hydrolysis has been long-since proposed, but it is yet to be realised in the industry in a significant way. The acid-active chitinase has shown some efficacy in acting against both fungal and crustacean chitin. During our industrial testing the acid-active chitinase predominately produced peaks of N-acetyl-glucosaminide, N,N-diacetyl-chitobiose, and N,N,N-triacetyl-chitotriose from chitin. Indicated a preference for exo-activity on these substrates. In comparison, the commercial chitinase primarily yielded peaks that were likely larger chitooligosaccharides. This indicates that the commercial chitinase was better able to locate and act within the chitin polymer, apparently displaying higher endo-chitinase activity than the acid-active chitinase. The commercial chitinase was produced by submerged fermentation of either classical or self-cloned *Streptomyces*, and is likely either not purified or only partly purified. Indeed, SDS-PAGE analysis revealed the presence of at least 7 protein bands (data not shown), thus it's likely this sample contains more than one chitin-degrading enzyme. The synergy of multiple chitin-degrading enzymes would explain the increased ability of the commercial chitinase in accessing the backbone of the complex substrates.

With regards to chitinases previously isolated from *R. emersonii*, the three enzymes could potentially work in synergy in chitin hydrolysis. The same synergistic principal applies to the theoretical addition of a chitin de-acetylase in producing glucosamine from chitin. Typically, hydrolysis reactions occur at pH 4–4.5, where Chit2 would be most active. As the chitin deacetylase converts the chitin to chitosan, an acetyl group would be released as a by-product of the reaction. This would lower the pH value of the reaction where Chit1 (pH 3.5–4) and then the acid-active chitinase

(pH 2.5–3.5) would reach their optimum activities. Previously, the native chitinolytic secretome of *R. emersonii* was seen to be effective in hydrolysing chitin (Hendy et al. 1990; McCormack et al. 1991). However, the efficacy of the other individual chitin-degrading components on shrimp and fungal chitin, and efficacy of acid-active chitinase in combination with other chitin-degrading enzymes represents an area of further study.

The commercial chitinase and the acid-active chitinase both showed the highest activity on the fungal chitin substrate, followed by chitin from shrimp. Increased activity against these substrates is not unexpected. The glucan, which is intrinsically linked to the chitin in the fungal chitin-glucan polymer, will restrict the accessibility of the chitin backbone to the chitinases thereby reducing their efficacy in releasing N-acetyl-glucosaminide. Similarly, the dried mushroom powder contains both glucan and protein which will have the same restricting effect. The increased activity towards fungal chitin over shrimp chitin is also not unexpected. Shrimp chitin is composed of α-chitin; comprised of alternating antiparallel polysaccharide strands, stabilized by a high number of distinct hydrogen bonds formed within and between the molecules; it is the most stable of the three allomorphs of chitin. Hence it is also the most resistant to enzymatic degradation. Fungal chitin is comprised of γ-chitin, which is formed by two parallel chains alternating with an antiparallel strand. Although γ-chitin is also highly stable and non-reactive, the packing tightness and numbers of inter-chain hydrogen bonds are reduced in comparison to α-chitin, resulting in an increased number of hydrogen bonds with water (Merzendorfer and Zimoch 2003). The higher degree of hydration and reduced packaging tightness result in more flexible and soft chitinous structures. In addition, non-crystalline, transient states of chitin exist in fungi (Martínez et al. 2014). All of which make γ-chitin more susceptible to enzymatic degradation. Further-to-this, the *R. emersonii* fungus, the genomic source of the acid active chitinase studied, was originally isolated from compost samples. In that native environment, the most abundant natural substrate of the chitinases expressed is, presumably, fungal chitin (followed by insect chitin which is comprised of the three chitin allomorphs). Thus, regardless of the role of the chitinase in defence against competition, breakdown of chitin for nutritional uptake or cell wall remodelling, etc., it's not unexpected that the acid-active chitinase displayed higher efficacy in degrading fungal chitin over shrimp chitin.

In terms of potential application, the acid-active chitinase is potentially suited to industrial chitin hydrolysis reactions for extraction of glucosamine and chitobiose at low pH. The increased activity of acid-active chitinase on fungal chitin indicates suitability in hydrolysis of mushrooms for sustainable alternative protein processing and release of fungal-derived glucosamine, N-acetyl-glucosamine and chitobiose. In a broader sense, it may find application in valorisation of the mushroom stalk waste stream of industrial mushroom production. The physiochemical characteristics of acid-active chitinase, namely; the thermostability, exo-chitinase activity and low pH-optima; indicate it may be effective in producing commercially valuable chitobiose and glucosamine from natural crustacean chitin, particularly as it has shown some efficacy against these non-treated substrates. An acid-active chitinase may be suited to the hydrolysis of treated crustacean chitin as the pre-treatment/demineralisation of shrimp chitin occurs at low pH. Theoretically, it can be added to the treated shrimp chitin with minimal or no pH adjustment.

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Author contributions KD, GW, DG and ISB conceived and designed the research. KD conducted experiments and analysed data. KD, GW, ISB and DAF wrote the manuscript. DAF carried out a phylogenetic analysis. KD and AS carried out the bioinformatics analysis. EMG and ET contributed new reagents and/or analytical tools. All authors read and approved the manuscript.

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Availability of data and materials The authors confirm that the data supporting the findings of this study are available within the article or its supplementary materials.

Declarations

Conflict of interest KD, DG, ISB, EMG, and ET declare they were employed by Monaghan Mushrooms Ireland and received a salary during the time this study was conducted.

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