Protein expression and purification

Biochemical characterization of chitinase A from *Bacillus licheniformis* DSM8785 expressed in *Pichia pastoris* KM71H

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ABSTRACT

Chitin is an abundant biopolymer found mainly in the exoskeleton of crustaceans and insects. The degradation of chitin using chitinases is one way to address the accumulation of chitin waste streams in the environment, and research has therefore focused on the identification, improvement and expression of suitable enzymes. Here we describe the production, purification and characterization of *Bacillus licheniformis* chitinase A in the *Pichia pastoris* expression system. Optimal enzyme activity occurred at pH 4.0–5.0 and within the temperature range 50–60°C. With colloidal chitin as the substrate, the K_m (2.307 mM) and V_{max} (0.024 mM·min^{-1}) of the enzyme were determined using a 3,5-dinitrosalicylic acid assay. The degradation products of colloidal chitin and hexa-*N*-acetylchitohexaose were compared by thin-layer chromatography. The activity of the glycosylated enzyme produced in *P. pastoris* was compared with the *in vitro* deglycosylated and aglycosylated version produced in

Escherichia coli. We showed that the glycosylated chitinase was more active than the deglycosylated and aglycosylated variants.

Keywords: molecular cloning, deglycosylation, enzymatic assay, TLC.

Abbreviations

BMGY, buffered glycerol-complex medium; BMMY, buffered methanol-complex medium; PCR, polymerase chain reaction; LB, lysogeny broth medium; MM, minimal medium; PBS, phosphate buffered saline; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; 4MUTC, 4-methylumbelliferyl β-D-*N,N,N*-triacetylchitotrioside; TLC, thin-layer chromatography; DNS, 3,5-dinitrosalicylic acid; NAG1, *N*-acetylglucosamine; NAG2, *N,N*-diacetylchitobiose; NAG3, *N,N,N*-triacetylchitotriose; NAG4, tetra-*N*-acetylchitotetraose; NAG5, penta-*N*-acetylchitopentaose; NAG6, hexa-*N*-acetylchitohexaoze.

INTRODUCTION

Chitin is the second most abundant natural biopolysaccharide on earth (after cellulose). It is composed of β(1⟶4)-linked *N*-acetyl-D-glucosamine residues. More than 10^9 tons of chitin is produced naturally every year, predominantly in the exoskeletons of marine crustaceans, mollusks, nematodes and insects, and in the cell walls of fungi [1-4]. Chitin waste streams accumulating in the environment are a significant hazard.

The β(1⟶4)glycoside linkages in chitin are cleaved by enzymes known as chitinases (EC 3.2.1.14) which are produced by many bacteria [5, 6], fungi [7-9], insects [10], plants [11] and mammals [12, 13]. Chitinases can be used to manage chitin waste streams, and are also used in medicine and agriculture, e.g. to kill fungal pathogens [14]. In nature, chitin is degraded very slowly by wild-type chitinases, which are synthesized in small amounts. The management of chitin waste streams therefore requires more active chitinases and a platform to produce large quantities of the superior enzymes. Chitinases can be used to convert chitin-containing biomass into useful derivatives such as chito-oligosaccharides, which are bioactive and potentially useful as pharmaceuticals[15].

The molecular weight of chitinase polypeptides is 20–90 kDa [16] but this is often increased by N-linked or O-linked glycosylation [17]. The native glycosylation of chitinases occurs in plants, insects and mammals, and in many cases is necessary for enzymatic activity [18-20]. Pichia pastoris is a popular expression system for the production of recombinant proteins, and chitinases expressed as recombinant enzymes in *P. pastoris* can be produced as either glycosylated or aglycosylated variants [21, 22].

Here we report the cloning and heterologous expression of a *Bacillus licheniformis* DSM8785 *chi*A gene encoding chitinase A (ChiA). The gene was expressed for the first time in *Pichia pastoris* KM71H and ChiA was secreted to the culture medium as a glycosylated recombinant enzyme. We compared the purified glycosylated ChiA with a deglycosylated variant produced *in vitro* and an aglycosylated form of the same enzyme produced in

Escherichia coli. We also developed an improved version of the chitin agar plate assay, a higher-resolution TLC method, and an alternative way to measure the kinetic properties of chitinase based on the concentration of product rather than the concentration of substrate. We consider this a better method to estimate the kinetic properties of hydrolytic enzymes because it overcomes inconsistencies in the substrate preparation methods. All substrates for hydrolytic enzymes are polymers, so the precise molar concentration is difficult to determine without extensive substrate purification, resulting in substantial inter-laboratory variation. Our novel approach provides the means to ensure uniform standards between experiments.

MATERIALS AND METHODS

Chemicals and enzymes

Chemicals were purchased form Carl Roth, Germany and Sigma-Aldrich, Germany unless otherwise stated. The enzymes used for cloning were acquired from Thermo Fisher Scientific, USA or New England Biolabs, USA. Kits for DNA purification, NucleoSpin® Plasmid Columns and PCR purification Columns were purchased from Macherey-Nagel, Germany.

Plasmids and genes

A synthetic *chi*A gene from *Bacillus licheniformis* DSM8785 (GenBank Accession Number FJ465148) was provided by GenScript (USA) and used as a template for molecular cloning in the yeast expression system. The *P. pastoris* expression vector pPICZαA (#V19520) was purchased from Invitrogen, USA.

Organism strains and growth conditions [23]

Pichia pastoris KM71H (#C18200) (Invitrogen) was cultured in BMGY and BMMY at 27°C. The basal components of both media, i.e. 1.34% (w/v) yeast nitrogen base, 1% (w/v) yeast extract and 2% (w/v) peptone, were autoclaved at 120°C for 30 min before supplementing with 100 mM potassium phosphate buffer pH 6.0 (autoclaved). To prepare the BMGY medium, glycerol solution was autoclaved separately and added at a final concentration of 2% (v/v). To prepare the BMMY medium, methanol was added at a final concentration of 0.5% (v/v). YPD medium, comprising 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (v/v) glucose and 0.125 mg/mL zeocin, with or without 2% (w/v) agar, was used to cultivate the yeast cells after transformation with the ChiA_pPICZαA and pPICZαA plasmids. Plasmids were amplified in *E. coli* XL10 Gold ultracompetent cells (#200314) from Agilent Technologies (USA). The bacteria were transformed with ChiA_pPICZαA and were grown in LB low-salt medium containing 0.5% (w/v) yeast extract, 1% (w/v) peptone and (w/v) 0.5% NaCl, with or without 2% agar (w/v). The pH was adjusted at 7.4 and the medium was autoclaved at 120°C for 30 min, cooled and supplemented with 0.025 mg/mL zeocin. Minimal medium containing 1.34% (w/v) yeast nitrogen base without amino acids and 1% (w/v) agar supplemented with 0.5% (w/v) colloidal chitin and was used to measure chitinase activity after

cultivation for 76 h at 27°C. In this case 98% methanol was sprayed on the cover of the agar plates to induce enzymatic expression.

Colloidal chitin preparation

Colloidal chitin was prepared as previously described, with modifications after Shimahara and Takiguchi Shimahara and Takiguchi [24] Concentrated HCl (140 mL) was slowly dripped onto 10 g of crab-shell chitin using a separation funnel. The viscous solution was mixed at 4°C overnight, then poured into 2 L ultrapure water and incubated for another 24 h at 4°C. The swollen chitin was centrifuged at 9000 ***g*** for 20 min at 20°C. The harvested chitin was washed several times with ultrapure water until the pH reached ~7. The resulting colloidal chitin, with a creamy texture, was sterilized and stored at room temperature.

Water-soluble chitin preparation

Water-soluble chitin was prepared as previously described, with modifications after Guo, Kikuchi, Matahira, Sakai and Ogawa [25]. We added 100 mL 48% NaOH slowly to 5 g crab-shell chitin and incubated the mixture for 48 h at 20°C before adding 215 g of ice and stirring for 10 days at 4°C. After 10 days of deacetylation, the pH was adjusted to 8.0 with 4 M HCl. The chitin mixture (turbid, with most of the material in an insoluble state) was boiled for 30 min and cooled overnight to room temperature. The chitin was collected by centrifugation at 9000 ***g*** for 20 min at 25°C and washed twice with ultrapure water to eliminate the salt. The precipitate containing the chitin was resuspended in 150 mL water and the pH was adjusted to 6.0 with 2 M HCl. The 150 mL chitin suspension (pH 6) was mixed with 300 mL 99% acetone and incubated overnight at room temperature before centrifuging at 9000 ***g*** for 20 min at 25°C and washing again with 300 mL 99% acetone. The white product was dried at 60°C for 2 h then crushed and sieved. The product was used to obtain 0.6% water-soluble chitin solution, after overnight shaking. The solution was filtered to remove the insoluble debris and stored at 4°C.

Subcloning the *B. licheniformis chi*A gene into the pPICZαA expression vector

The *chi*A gene was cloned by classical PCR/restriction method using forward primer FP ChiA Kpn I (5' ATC GGT ACC TTT GTC ATG TTG CTG AGC TTG TC-3') and reverse primer RP ChiA Xba I (5'-ATC TCT AGA TTC GCA GCC TCC GAT CAG C-3'). PCR amplification comprised an initial denaturation step at 95°C for 3 min followed by 25 cycles of 95°C for 30 s, 47°C for 30 s, and 72°C for 2 min, followed by a final extension step at 72°C for 10 min. The reaction products were treated with 1 µL Dpn I (10 U/µL) for 1 h at 37°C to eliminate the template, then purified on NucleoSpin Plasmid Columns.

The pPICZαA vector and *chiA* PCR product were each double digested with Kpn I and Xba I. The vector was treated with calf intestinal alkaline phosphatase and the components were ligated at room temperature for 3 h

using T4 DNA ligase. The ligated vector was introduced into E. coli XL10 Gold cells by heat-shock transformation [26]. The insert was verified by colony PCR and Sanger sequencing [27].

The verified *ChiA*_pPICZαA plasmid was linearized with Pme I and introduced into *P. pastoris* KM71H cells by electroporation [28].

Measuring chitinase activity using the chitin agar plate assay

Approximately 20 *P. pastoris* KM71H colonies containing *chiA*_pPICZαA were transferred to solid MM with 0.5% colloidal chitin to induce enzymatic expression and incubated at 30°C for 1 to 2 days. After this step, the yeast cells were removed by washing the plate surface with water and the plate was incubated for 1 h in 20 mL 10 x PBS buffer. After removing the buffer, we added 20 mL 0.2% (w/v) Congo red in water and the plate was incubated at room temperature for 1 h [29]. The plate was washed three times with 1 M NaCl for 15 min at room temperature. For positive controls, the plates were incubated with 5 µL commercial chitinase from *S. griseus* at three concentrations (0.1, 0.5 and 5 U/mL) overnight at 27°C.

Heterologous expression and protein purification

One *P. pastoris* KM71H colony containing *chi*A_pPICZαA was used to initiate an overnight culture in BMGY medium and 5 mL of this pre-culture was transferred to 250 mL BMGY medium and incubated at 27°C, overnight. The cells were collected by centrifugation at 3000 ***g*** for 20 min at 20°C, resuspended in 50 mL BMMY expression medium and incubated for 7 days at 27°C shaking at 160 rpm. We added 0.5% methanol every day and withdrew samples to check for the presence of ChiA protein (SDS-PAGE) and chitinase activity (4MUTC assay).

For larger-scale expression, 5 mL of an overnight pre-culture was inoculated into 4 L of BMGY growth medium (initial OD_{600} = 0.081) and incubated at 27°C shaking at 160 rpm for ~19 h (OD_{600} ~1.8). The cells were collected by centrifugation at 9000 ***g*** for 15 min at 20°C, resuspended in 1 L BMMY expression medium and incubated for 7 days at 27°C, shaking at 160 rpm. The cells were then recovered by centrifugation at 12000 ***g*** for 30 min at 4°C. The supernatant containing recombinant chitinase was pumped through a 0.2-µm rapid flow filter (Nalgene, USA) and an ultrafiltration membrane with a cutoff of 30 kDa using a Heidolph, Model PD 5201 peristaltic pump system at 120 rpm (1.8 mL/min).

The concentrated enzyme solution (~25 mL) was dialyzed against 4 L 10 mM Tris-HCl buffer (pH 7.2) at 4°C for 48 h with one change of buffer after 24 h. The dialysate (44 mL) was then injected to a 20-mL HiPrep DEAE FF 16/10 anion-exchange Sepharose column (GE Healthcare, UK) on an AKTA chromatography system following equilibration with three volumes of 10 mM Tris-HCl (pH 7.2). Bound proteins were eluted in a salt gradient of 0–1 M NaCl in 10 mM Tris-HCl (pH 7.2) over 20 column volumes (2 mL collected fractions). Except for sample loading, when a flow rate of 0.5 mL/min was used, the nominal flow rate was 1 mL/min.

Deglycosylation of purified chitinase

The purified chitinase was deglycosylated by mixing of 20 µL purified chitinase (0.75 mg/mL protein concentration) with 1 µL of 10 x G5 reaction buffer and 1 µL Endo H enzyme (1 million U/mL) in a total volume of 50 µL, adjusted with ultrapure water.

SDS-PAGE and zymogram analysis

Samples containing recombinant chitinase before and after purification and deglycosylation were analyzed by SDS-PAGE (8% polyacrylamide) under reducing conditions [30].The samples were prepared by mixing 15 µL glycosylated/deglycosylated chitinase (0.3 mg/mL) with of 5 µL reducing buffer and incubating at 95°C for 10 min. Each gel lane was loaded with 18 µL of the mixture and the samples were separated at 120 V for 90 minutes.

For zymogram analysis, we prepared 8% polyacrylamide separating gels containing 0.2% water-soluble chitin. The samples were prepared under non-reducing conditions (62.5 mM Tris-HCl, pH 6.8, 1% bromophenol blue, 25% glycerol) and 10 µL aliquots were mixed with 5 µL non-reducing sample buffer. Each gel lane was loaded with 10 µL of the mixture and the samples were separated as above. The gel was immersed in 50 mL 100 mM sodium acetate buffer (pH 6) containing 1% Triton X-100 and incubated overnight at 37°C, with gentle agitation, before staining with Coomassie brilliant blue R250, followed by destaining [31]. A SDS-PAGE under reducing conditions was run using glycosylated chitinase. The gel was overlapped with an 8% polyacrylamide gel containing 0.1% water soluble chitin, and incubated overnight, at room temperature, in 5 mL of 100 mM sodium acetate buffer (pH 6) containing 1% Triton X-100, without mixing and stained using Coomassie brilliant blue R250.

Analysis of chitinase activity using 4MUTC as a fluorogenic substrate

Chitinase activity was quantified using the fluorogenic substrate 4MUTC by mixing 2 µL of chitinase (0.3 mg/mL) with 30 µL 0.1 M sodium acetate buffer (pH 6) and 18 µL 0.5 mg/mL 4MUTC. The fluorescent product was immediately measured at 355 nm excitation and 460 nm emission using a Berthold Tech Mithras LB 940 fluorescence spectrophotometer. One unit of enzyme was defined as the amount of enzyme that liberates 1 µmol 4-methylumbelliferone per minute under the experimental conditions.

Analysis of chitinase activity on colloidal chitin

The activity of ChiA on colloidal chitin was measured using a DNS assay [32] comprising 500 µL 84 mg/mL colloidal chitin in 100 mM sodium acetate buffer (pH 6) and 50 µL 0.9 mg/mL pure enzyme. The mixture was incubated for 2 h at 37°C with slow agitation, then 60 µL was combined with 120 µl DNS and incubated for 15 min at 100°C. The mixture was then centrifuged for 1 min to pellet the non-hydrolyzed chitin, and 80 µL of the supernatant was transferred to a cuvette and the absorbance at 540 nm was measured to determine the concentration of reducing sugars. One unit of enzyme was defined as the amount of enzyme that liberates reducing sugar ends equivalent to 1 µmol *N*-acetyl-glucosamine per minute under the experimental conditions.

Determination of optimal pH and temperature ranges

Chitinase activity was tested within the pH range 2.0–11.0 using 100 mM acid citric–trisodium phosphate buffer. The reaction comprised 500 µL 84 mg/mL colloidal chitin and 50 µL 0.9 mg/mL pure enzyme, and was incubated at 37°C for 2 h, shaking at 900 rpm. The optimal temperature was determined using the same reaction components, but the pH was held at 6.0 and the following temperatures were applied: 28, 40, 50, 60, 70, 80 and 90°C.

Kinetic analysis

The kinetic characteristics of ChiA were determined under the optimal reaction conditions defined above, using 500 µL of colloidal chitin substrate ranging from 0 to 1.53 mM *N*-acetylglucosamine (calculated based on the initial concentration of reducing sugars measured by the DNS assay and determined according to the calibration curve obtained using *N*-acetylglucosamine). The initial velocities (V0) versus substrate concentration ([S]) were plotted as Lineweaver–Burk and Michaelis-Menten curves using Minitab software (Minitab 17 Inc., USA).

TLC

The hydrolytic activity of the recombinant chitinase on colloidal chitin and hexa-*N*-acetyl chitohexaoze was determined by TLC analysis as previously described, with modifications [33]. Two reaction mixtures containing 100 µL 0.5% colloidal chitin in 0.1 M sodium acetate buffer (pH 6) or 25 µL 100 µM NAG 6 were incubated at 37°C for 48 h with 10 µL of purified enzyme (0.75 mg/mL). The reactions were stopped by boiling at 98°C for 10 min and centrifuged at 11000 ***g*** for 5 min at room temperature. We then loaded 3 µL of the supernatant from each reaction in six 0.5 µL aliquots onto a TLC Aluminum Silica Gel 60 F254 plate (Merck, Germany) with a drying step between each aliquot. We used 1.5 µL NAG1–NAG6 mix (50 mM NAG1, 25 mM NAG 2, 16.5 mM NAG 3, 12.5 mM NAG 4, 10 mM NAG 5, 7.5 mM NAG 6) as standards. The samples were separated using a 2:2:1 (v/v/v) mix of 1-butanol, acetic acid and water as the mobile phase. The plate was dried using a hair dryer then wiped with a BGCM00010 Biopore PTFE cloth (Merck KGaA, Germany) dipped in aniline-diphenylamine reagent (400 µL aniline, 0.4 g diphenylamine, 20 mL acetone, 3 mL 85% ortho-phosphoric acid). The TLC plate was gradually heated to 150°C on a heating plate to develop the signal.

RESULTS AND DISCUSSION

Molecular cloning of the *B. licheniformis chi*A gene in the pPICZαA expression vector

The *Bacillus licheniformis* DSM8785 *chi*A gene has an open reading frame (ORF) of 1743 bp and encodes a protein of 694 amino acids with a molecular weight of 76.20 kDa predicted by the ExPASy Bioinformatic Resource program, and an isoelectric point of 4.94, an acidic range specific for bacterial chitinases [6]. The *chi*A sequence was amplified using primers to add restriction sites compatible with the pPICZαA vector and inserted by standard restriction/ligation. The recombinant plasmid was then introduced into *P. pastoris* KM71H competent cells, and

colonies expressing ChiA were identified using a rapid screening assay in which chitin agar plates were stained with Congo red [34]. The traditional Congo red staining procedure could not be used because the dye precipitates in acidic media, an unavoidable situation under our experimental conditions because the colloidal chitin preparation method and culture media for yeast reduces the pH of the agar medium to 5.0. We therefore modified the assay by including a pre-incubation step in 10 x PBS to neutralize the pH. This restored the contrast between the bright halos (with diameters proportional to the level of chitinase activity) and the surrounding darker medium, as shown in Figure 1 A.

The negative control in Figure 1 B appears to have a small halo, reflects the low-level intrinsic chitinase activity of the yeast cells, which is required for the remodeling of the cell wall during cell division and growth [35]. We used this modified agar plate assay to screen hundreds of P. pastoris transformants and to select those with the largest and brightest halos, probably representing clones with multiple integrated copies of the chitinase gene and the highest chitinase expression levels.

Protein expression and purification

The recombinant *B. licheniformis chi*A gene was controlled by the *AOX* promoter in *P. pastoris* allowing the induction of gene expression using 0.5% methanol. The coding sequence was also fused to the α-factor pro-peptide to ensure the secretion of the enzyme to the extracellular medium. The expression of ChiA in *P. pastoris* strain KM71H was first optimized by growing the strain transformed with *chi*A_pPICZαA in a small volume of BMMY expression medium, and the supernatant and cell pellet were tested for the presence of ChiA over the next 7 days.

The supernatant was assessed by SDS-PAGE, starting at the onset of methanol induction (lane 0) followed by one sample per day over the next 7 days. The yield of recombinant ChiA increased throughout the fermentation (Figure 2). The estimated molecular mass of the expressed enzyme ranged from 70 to 130 kDa, with the variation in size being attributed to the presence of different glycosylation levels of chitinase recombinantly expressed in *P. pastoris* [36]. A negligible amount of the enzyme was detected in the cell pellet (data not shown).

Based on the optimized small-scale fermentation conditions, the recombinant *P. pastoris* strain was cultivated in 4 L of BMGY medium for 19 h at 27°C. The cells were centrifuged and resuspended in 1 L of BMMY expression medium and incubated for 7 days at 27°C. Having established that negligible amounts of ChiA separate with the cell pellet, we discarded the pellet and have further used only the protein from the supernatant. The supernatant was processed by serial microfiltration and ultrafiltration, followed by dialysis and DEAE anion-exchange chromatography. The activities and protein concentrations obtained after each purification step are presented in Table 1. Samples from all purification steps were analyzed by SDS-PAGE (Figure 3).

The yield of pure recombinant glycosylated chitinase was 44 mg protein/L with a specific activity on colloidal chitin of 0.026 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ (U/mg) protein (*N*-acetylglucosamine reducing sugars). SDS-PAGE analysis

revealed that the starting material (fermentation broth) contained small amounts of contaminating proteins in addition to the glycosylated ChiA (represented by a heterogeneous band of 70 to 130 kDa under reducing conditions, situation described also by other authors [37]) but these were removed during purification. Under non-denaturing conditions, the mobility of the ChiA band was between 60 and 90 kDa, probably due to different interactions of reduced and non-reduced chitinase with SDS. In the corresponding zymogram, the white area showing the activity of chitinase is brighter at the migration front (60-80 kDa) but it is present also in other areas of the migration line (Figure 3, C) confirming the activity of the purified glycosylated enzyme [38]. To determine if the activity on the zymogram corresponds to the major chitinase band (60 and 90 kDa) or if there are other contaminants in the purified sample that show chitinase activity, an overlap zymogram was performed. In this case two distinct polyacrylamide gels (one with migrated chitinase and another with water soluble chitin substrate) were used and the result showed that chitinase band migrated at around 60 kDa (Figure 3D, lane 1) corresponding to the same band of enzyme activity in the overlay gel (Figure 3D, lane 2).

Comparison of glycosylated/deglycosylated chitinase activities

The native *B. licheniformis* ChiA was previously purified and identified as an aglycosylated enzyme [6] and when expressed in *E. coli*, the recombinant ChiA was also aglycosylated [39]. We found that the enzyme was ectopically glycosylated in *P. pastoris*, and the NetNGlyc 1.0 server[40] predicted that the ChiA amino acid sequence contains seven Asn-Xaa-Ser/Thr sequence (where Xaa denotes any amino acid except proline) that are potential *N*-linked glycosylation targets.

We therefore deglycosylated the recombinant *P. pastoris* ChiA protein *in vitro* using the enzyme Endo H, which cleaves intact *N*-linked glycans between two *N*-acetylglucosamine residues, leaving one *N*-acetylglucosamine residue attached to the asparagine residue on the polypeptide backbone.

Figure 4 A compares by SDS-PAGE analysis the recombinant ChiA after deglycosylation for 2 days (lane 2) to the untreated glycosylated protein as a control (lane 3) and the aglycosilated enzyme expressed in *E. coli* BL21 (DE3) strain (#C600003) (Invitrogen) (lane 1). After 2 days, ChiA was almost entirely deglycosylated, confirming that the enzyme is sensitive to Endo H. We then compared the activities of the glycosylated, deglycosylated and aglycosilated variants of recombinant ChiA using the fluorogenic substrate 4MUTC (Figure 4 B). Interestingly, although only an aglycosylated version of the enzyme is produced by nature, the glycosylated recombinant ChiA produced by *P. pastoris* clearly demonstrated approximately 10% greater activity than the deglycosylated variant. This observation is in concordance with the published results of other types of glycosylasted and deglycosylatyed chitinases [37]. The loss of some of the activity after deglycosylation indicates a possible role of glycans in structural integrity of recombinant chitinase.

Because *in vitro* deglycosylation leaves a residual *N*-acetylglucosamine unit attached to asparagine in the polypeptide background, a true comparison between the native ChiA and the recombinant glycosylated version produced in *P. pastoris* would require access to an aglycosylated variant. We therefore performed the 4MUTC-based activity assay comparing the glycosylated recombinant enzyme produced in *P. pastoris* to the same enzyme produced in *E. coli*, which lacks the machinery for *N*-linked glycosylation and therefore produces a true aglycosylated form of ChiA presumably identical to the native enzyme. As shown in Figure 4 B, the *P. pastoris* glycosylated ChiA is almost seven times more active than the *E. coli* aglycosylated form, confirming that the ectopically glycosylated recombinant enzyme produced in *P. pastoris* is superior in activity, at least under the reaction conditions we tested. There are published results showing that recombinant chitinase expressed in E. coli has a lower activity compared with the expression of the same gene in P. pastoris [41]. Although we cannot explain, based on the results presented in this work, the differences in activities we may hypothesize that glycosylated form is more stable (to proteases attacks and temperature promoted unfolding) than aglycosylated form. As the reaction assay is performed over 2 hour period or longer, the stability of the enzyme can be an important feature that may explain the observed differences in enzymatic activities. Many investigators are trying to elucidate the mechanisms involved in this process [42], however there is currently no understood general pattern as glycosylation has been observed to have both stimulating and inhibiting effects on activity.

The pH and temperature optima of glycosylated recombinant ChiA

The optimal pH and temperature of the glycosylated recombinant ChiA were determined using the DNS assay with colloidal chitin as the substrate. The optimum pH at 37°C was between 4.0 and 5.0, but the enzyme retained at least 50% of its maximum activity over a wide range of values between pH 3.0 and 10.0 (Figure 5 A). The optimum temperature at pH 6.0 was 50–60°C, but the enzyme retained at least 50% of the maximal activity throughout the temperature range we tested, with extreme values of 28 and 70°C (Figure 5 B). These results concur with the reported pH and temperature optima of other authors [39, 43, 44].

Enzyme activity and kinetic analysis

The effect of substrate concentration on the activity of recombinant glycosylated ChiA was determined using the DNS assay with wet colloidal chitin as a substrate. The values of the initial substrate concentration were expressed as mM of reducing ends, determined from calibration curves based on known *N*-acetylglucosamine concentrations.

The concentration (initial moment) of colloidal chitin varied between 0 and 1.53 mM. The initial velocities (V_0) versus substrate concentration ([S]) were plotted as Lineweaver–Burk and Michaelis-Menten curves using Minitab software (Figure 6). The Lineweaver–Burk plot shows a linear distribution with a correlation coefficient of 0.991.

The Michaelis-Menten constant (Km) and the maximum velocity (Vmax) values of the recombinant glycosylated ChiA, calculated by non-linear regression (R^2=0.989) were 1.981 mM and 0.022 mM min^{-1}, respectively (Figure 6). When the kinetic constants were calculated using a linear Lineweaver-Burk equation (R^2=0.991) based on an *N*-acetylglucosamine standard curve, the values were 2.307 mM and 0.024 mM min^{-1}, respectively. In many articles, kinetic data for chitinases are obtained using various synthetic substrates such as *p*-nitrophenylchitobiose [39] or 4MUTC [45]. Although chitin is the natural substrate, it is rarely used to determine the kinetic properties of chitinases because the precise molecular mass is difficult to calculate and it is insoluble in water. Partially soluble colloidal chitin is obtained by the acid hydrolysis of chitin, but this method is difficult to reproduce and the kinetic data vary widely [6, 44].Therefore, the comparison of different chitinases using different chitin substrates provides largely subjective data. By instead basing the kinetic data calculations on the concentration of *N*-acetylglucosamine reducing ends, using wet colloidal chitin as the substrate, we have provided a new approach that allows the precise calculation of kinetic values.

Thin layer chromatography analysis

The hydrolytic products of recombinant glycosylated chitinase were analyzed by TLC using colloidal chitin and NAG6 as substrates (Figure 7). In both cases, the most abundant reaction products were *N*,*N*-diacetylchitobiose (NAG2), followed by *N*-acetylglucosamine (NAG1). The presence of NAG2 as the most abundant hydrolysis product of both substrates confirms that ChiA is an endochitinase. Furthermore, the data suggest that short-chain substrates such as NAG6 are degraded more effectively than colloidal chitin, resulting in large amounts of NAG2 and NAG1 but only small amounts of NAG3, NAG4 and NAG5, broadly in agreement with the activity of ChiA expressed in *E.* coli [39] TLC analysis of recombinant glycosylated ChiA on a chitosan substrate revealed that chitobiose was the major product, followed by glucosamine (data not shown).

One major challenge faced by researchers analyzing chito-oligosaccharides by TLC is the low resolution of the TLC plates, which can make the results difficult to interpret and reproduce. Many parameters interfere with the optimal separation of products by TLC, including the choice of solvent, the degree of saturation with solvent vapors, the accuracy of sample pipetting, the sample volume and concentration, the sample migration time, the type of dye used for staining and the coloring method [46] To improve the resolution of our TLC separation, we used small aliquot volumes (0.5 µL) and transferred several aliquots to the same spot with drying time between transfers. After migration, the TLC plate was dried with a hair dryer and the reaction products were detected by wiping the plate with a filter cloth dipped in aniline-diphenylamine reagent. Alternatives such as spraying the TLC plate with reagent [47], using cotton balls [39] or dipping of the TLC plate in the reagent [48] may cause reagent leakage or damage to the silica layer, reducing the quality of the results. The TLC plate was gradually heated to 150°C on a heating plate for a few minutes, to avoid the cracking of the silica layer.

CONCLUSIONS

The *B. licheniformis* chiA gene encoding chitinase A (ChiA) has been expressed for the first time in the *P. pastoris* KM71H expression platform. Unlike the native enzyme, the recombinant ChiA produced in *P. pastoris* was glycosylated. Comparative analysis revealed that the glycosylated enzyme was at least twice as active as a deglycosylated variant produced in vitro, with a pH optimum of 4.0–5.0 and a temperature optimum of 50–60°C. The glycosylated variant was also more active than an aglycosylated version produced in *E. coli*. We conclude that glycosylation enhances the activity of the enzyme, potentially by increasing its stability. Glycans can have different roles in protein activity and have been shown to modify turnover rates, substrate recognition, specificity and binding affinity.

In this paper, we have also developed an improved version of the chitin agar plate assay compatible with acidic media, an alternative method to calculate the kinetic properties of chitinase using colloidal chitin as a substrate and a strategy to improve the resolution of TLC for the analysis of chito-oligosaccharides.

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Declaration of Interest: The authors declare that they have no conflict of interest.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

Author contributions: R.O. conceived, lead the study, analyzed data and revised the manuscript, G.M. performed the experiments, analyzed data and wrote the manuscript, V.O., R.P., R.F. have analyzed data, supervised and performed critically revising of the manuscript.

FIGURE LEGENDS

Figure 1. Chitin agar plate assay. (A) Commercial chitinase produced in *S. griseus* chitinase (positive control) at concentrations of (1) 0.1 U/mL, (2) 0.5 U/mL, and (3) 5 U/mL. (B) Chitinase from *P. pastoris* KM71H *chi*A_pPICZαA transformants: (N) negative control (base vector only), (1) *P. pastoris* KM71H *chi*A_pPICZαA transformant.

Figure 2. SDS-PAGE analysis of supernatant samples containing recombinant glycosylated ChiA expressed by *P. pastoris* KM71H. M = protein molecular marker, lane 0 = negative control, corresponding to the beginning of the methanol induction phase, lanes 1-7 = days after induction. The recombinant glycosylated chitinase has a molecular mass ranging between 70~130 kDa.

Figure 3. SDS-PAGE analysis of recombinant glycosylated chitinase before and after purification. (A) Reducing conditions. (B) Non-reducing conditions. (C) Non-reducing conditions followed by zymography on a gel containing water-soluble chitin. The lanes in each panel contain the same samples. M = protein molecular markers. Lane 1 = sample after ultrafiltration; lane 2 = sample after dialysis; lane 3 = pure enzyme following anion-exchange chromatography. (D) Non-reducing conditions for glycosylated chitinase after purification (lane 1) and zymography on a gel containing water-soluble chitin (lane 2).

Figure 4. Comparison of glycosylated and deglycosylated ChiA produced in *P. pastoris* and aglycosilated ChiA produced in *E.coli*. The enzyme was deglycosylated with Endo H at 37°C and samples were analyzed after 2 days. (A) SDS-PAGE analysis under reducing conditions. M = protein molecular markers; lane 1, aglycosylated chitinase produced in *E.coli*; lane 2, deglycosylated chitinase after 2 days of incubation produced in *P. pastoris*; lane 3, glycosylated chitinase isolated from *P. pastoris*. (B) Effect of deglycosylation on chitinase activity measured using the fluorogenic substrate 4MUTC. Data represent means of two replicate experiments with standard deviations.

Figure 5. Optimum activity of recombinant glycosylated ChiA produced in *P. pastoris* KM71H. Activity was determined using a DNS assay with colloidal chitin as the substrate. (A) Activity within the pH range 2–11 at 37°C. (B) Effect of different temperatures (28–90°C) at pH 6.0. Data represent means of three replicate experiments with standard deviations.

Figure 6. Michaelis-Menten fitted line plot of recombinant glycosylated ChiA activity (Minitab software). The inset Lineweaver-Burk plot relates *P. pastoris* KM71H ChiA reaction velocity to N-acetylglucosamine associated with the concentration (initial moment) of wet colloidal chitin (0–1.53 mM).

Figure 7. TLC analysis of recombinant glycosylated ChiA reaction products on colloidal chitin and hexa-N-acetylchitohexaose (NAG6). M = N-acetylchito-oligosaccharide molecular markers (NAG1-NAG6); lane 1 = ChiA products with the colloidal chitin substrate after 48 h; lane 2 = ChiA products with the hexa-N-acetylchitohexaose substrate after 48 h.

Table 1. Summary of purification of recombinant chitinase

Fraction	Volume (mL)	Total Protein (mg)	Activity (mu)	Total Activity (mu)	Specific Activity (mu/mg)	Fold Purification	Yield (%)
Fermentation media	1000	506	1.7	1700.0	3.4	1.0	100.00
Ultrafiltration concentrate	25	452	65.2	1630.0	3.6	1.07	95.88
Dialysate	44	437	36.2	1592.8	3.6	1.08	93.69
Chromatography DEAE (pooled fractions)	35	46	34.1	1193.5	25.9	7.72	72.35
After dialysis	25	46	47.1	1177.5	25.6	7.62	69.26

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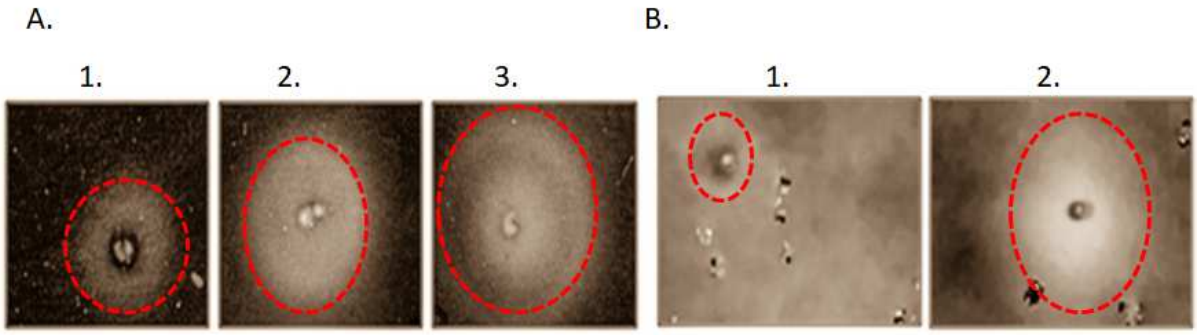

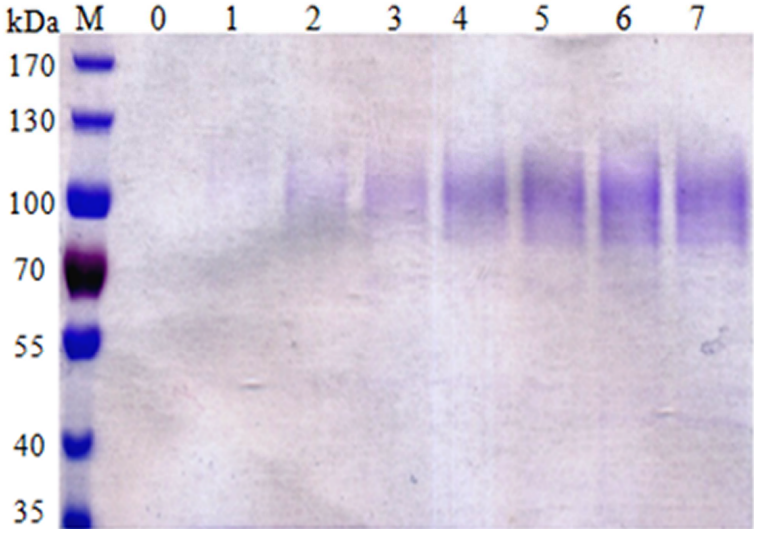

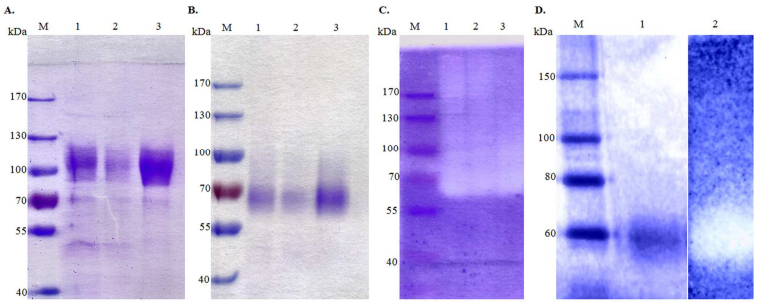

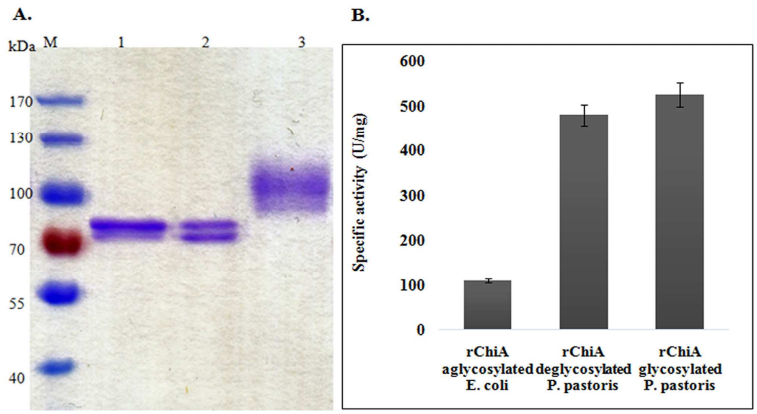

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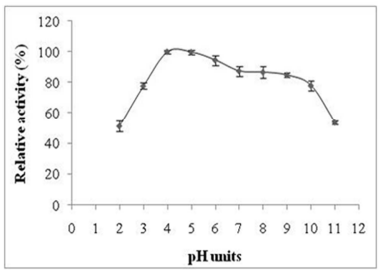

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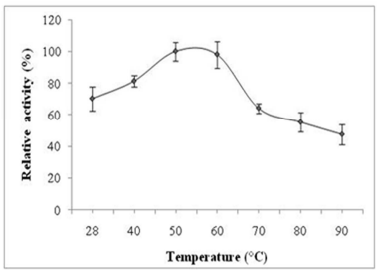

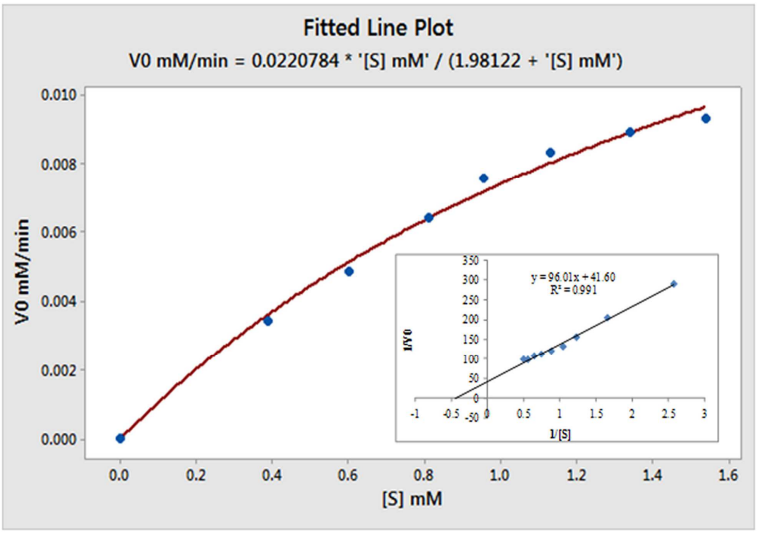

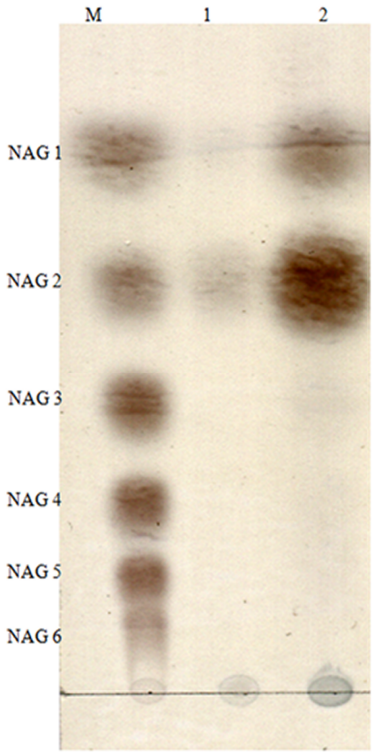

Protein expression and purification

Highlights

- Chitinase A gene from *Bacillus licheniformis* expressed in *Pichia pastoris* as a glycosilated enzyme
- Glycosylated chitinase produced by *P. pastoris* is more active than the deglycosylated and aglycosylated variant (produced by *E. coli*)
- An improved version of the chitin agar plate assay compatible with acidic media was developed.
- An alternative method to calculate the kinetic properties of chitinase using colloidal chitin as a substrate was designed.
- A strategy to improve the resolution of TLC for the analysis of chito-oligosaccharides was detailed.