Bulletin of Entomological Research (2019) **109**, 741–751
doi:10.1017/S0007485319000191

Functional expression of the *Spodoptera exigua* chitinase to examine the virtually screened inhibitor candidates

L. Zhang[1]†, Z. Guan[1]†, Z. Pan[1], H. Ge[2], D. Zhou[3,4], J. Xu[5] and W. Zhang[1]*

[1]State Key Laboratory of Biocontrol and School of Life Sciences, Sun Yat-sen University, Guangzhou 510275, China: [2]Medical College, Qingdao University, No. 38 Dengzhou Road, Qingdao 266021, China: [3]Guangdong Provincial Key Laboratory of New Technology in Rice Breeding, Rice Research Institute, Guangdong Academy of Agricultural Sciences, Guangzhou 510640, China: [4]College of Life Sciences, South China Agricultural University, Guangzhou 510642, China: [5]Research Center for Drug Discovery, School of Pharmaceutical Sciences, Sun Yat-sen University, Guangzhou 510006, China

Abstract

Chitinase is responsible for insect chitin hydrolyzation, which is a key process in insect molting and pupation. However, little is known about the chitinase of *Spodoptera exigua* (*Se*Chi). In this study, based on the *Se*Chi gene (ADI24346) identified in our laboratory, we constructed the recombinant baculovirus P-Chi for the expression of recombinant *Se*Chi (r*Se*Chi) in Hi5 cells. The r*Se*Chi was purified by chelate affinity chromatography, and the purified protein showed activity comparable with that of a commercial *Sg*Chi, suggesting that we harvested active *Se*Chi for the first time. The purified protein was subsequently tested for enzymatic properties and revealed to exhibit its highest activity at pH 8 and 40 C. Using homology modeling and molecular docking techniques, the three-dimensional model of *Se*Chi was constructed and screened for inhibitors. In two rounds of screening, twenty compounds were selected. With the purified r*Se*Chi, we tested each of the twenty compounds for inhibitor activity against r*Se*Chi, and seven compounds showed obvious activity. This study provided new information for the chitinase of beet armyworm and for chitinase inhibitor development.

Keywords: *Spodoptera exigua*, chitinase, baculovirus expression system, homology modeling, inhibitor screening

(Accepted 15 January 2019; First published online 22 May 2019)

Introduction

Chitin is widely distributed in invertebrates and microorganisms (Rinaudo, 2007). In insects, chitin is distributed in the epidermis, exoskeleton, peritrophic membrane of the midgut, intestinal lining of the foregut and hindgut, and spiral band in the trachea (Lehane, 1997; Andersen, 2003; Merzendorfer & Zimoch, 2003). As the important role chitin plays in insects, chitinase is widely studied as an anti-pest method. Chitinase belongs to the glycoside hydrolase family and is widely distributed. Chitinase in baculovirus and Beauveria bassiana was found involved in the pathogenesis against insects (Fan *et al.*, 2007). Chitinase genes were cloned into transgenic cotton (Hao *et al.*, 2005) and Bacillus thuringiensis (Arora *et al.*, 2003; Lertcanawanichakul *et al.*, 2004;

*Author for correspondence
Phone: + 86 20 39332963
Fax: +86 20 39943515
E-mail: lsszwq@mail.sysu.edu.cn
†The first two authors contributed equally to this work

Hu *et al.*, 2009) for pest control. On the other hand, Chitinase-mediated chitin degradation plays an important role in the molting and pupation of insects (Schrempf, 2001; Carlini & Grossi-De-Sá 2002; Saville *et al.*, 2002). Insect chitinases are mainly present in the molting fluid as well as in the midgut. The function of the molting fluid is to digest the old epidermis before molting. In addition to digest the old peritrophic membrane, chitinase in the midgut may also be associated with food digestion. In the venom of certain insects, chitinase can be used to assist in the spread of toxic substances in the feeding object (Reynolds & Samuels, 1996).

Previous studies have discovered that insect chitinases are composed of a family with eighteen chitnases (Henrissat & Bairoch, 1993) and are present in all studied insect orders (Müller, 1992). They are mostly internal cleavage enzymes that can hydrolyze chitins containing three or more N-acetamide glucose residues. Several insect chitinases were previously cloned and expressed in different systems such as *E. coli* (Shinoda *et al.*, 2001; Paek *et al.*, 2012; Fan *et al.*, 2015), *Pichia pastoris* (Fitches *et al.*, 2004; Wu *et al.*, 2013; Liu *et al.*, 2017) and insect cells (Shen & Jacobs-Lorena, 1998; Shinoda *et al.*, 2001; Yan *et al.*, 2002; Zheng *et al.*, 2002; Fan *et al.*, 2015; Fan *et al.*, 2018). The successful expression of chitinases from different species, including *Spodoptera littoralis* (Shinoda *et al.*, 2001), *Glossina morsitans* (Yan *et al.*, 2002), *Choristoneura fumiferana* (Zheng *et al.*, 2002), *Lacanobia oleracea* (Fitches *et al.*, 2004), *Mamestra brassicae* (Paek *et al.*, 2012), *Ostrinia furnacalis* (Wu *et al.*, 2013; Liu *et al.*, 2017), *Lithocolletis ringoniella* (Fan *et al.*, 2015), and *Lymantria dispar* (Fan *et al.*, 2018) has been reported previously. In some studies, insect chitinases expressed in *E. coli* exhibited lower enzymatic activity (Shinoda *et al.*, 2001; Fan *et al.*, 2015) than expressed in insect cells. Therefore, we tend to express the *Se*Chi in insect cells via baculovirus expression system in this study.

Due to the importance of chitinase in insect molting and pupation, several inhibitors of insect chitinase have been discovered. Allosamidin was firstly isolated and reported in 1987, showed strong inhibitory activity against silkworm chitinase (Sakuda *et al.*, 1987). (GlcN)2–7 not only inhibits *Of*ChtI *in vitro* but also arrests *Ostrinia furnacalis* larval stage *in vivo* (Chen *et al.*, 2014). *Of*Chi-h was found inhibited by TMG-(GlcNAc)4 (Liu *et al.*, 2017). Phlegmacin B1 showed inhibitory activities against insect GH18 enzymes such as *Of*Chi-h and *Of*Hex1 (Chen *et al.*, 2017). The traditional screening methods usually directly detect whether a random mixture inhibits the target chitinase and then screen for a single component with inhibitory activity. For instance, the chitinase inhibitor A82516 was screened from the fermentation product of *Streptomyces* (Somers *et al.*, 1987). A neutral protease with chitinase inhibitory activity was screened from the soil by the plate-clear circle method (Xie *et al.*, 2006). ChI-06 bacteria, which are capable of expressing chitinase inhibitors, were screened from marine microorganisms (lv, 2007). Traditional methods have massive randomness and require large amounts of the substrate, enzyme, and candidate inhibitor. Since the 1990s, drug development has entered a new stage of 'rational drug design', in which the development of computer-aided drug design methods has resulted in a significant breakthrough. For instance, an azide-bearing inhibitor against *Serratia marcescens* chitinase B (*Sm*ChiB) was generated by *in situ* click chemistry (Hirose *et al.*, 2013). TKUPSP017, a small synthetic boron-containing molecule with a BF3K side-chain, was confirmed to have inhibition activity against the chitinase purified from *Bacillus cereus* (Liang *et al.*, 2014). *Ostrinia furnacalis Of*Chi-h (a GH18 chitinase) and *Of*Hex1 (a GH20 Hex) were used together to screen a library of microbial secondary metabolites (Chen *et al.*, 2017). A novel inhibitor of *T. viride* chitinase was designed and synthesized by an *in silico* target fishing protocol (Maccari *et al.*, 2017).

The chitinase of *Spodoptera exigua* was discovered in this lab and reported in 2012. Two chitinse genes, namely *Se*Chi and *Se*Chi-h, were identified and silenced through specific double-stranded RNA (dsRNA) injection relatively (Zhang *et al.*, 2012). According to the classification method by Arakane and Muthukrishnan (Arakane & Muthukrishnan, 2010), *Se*Chi contains a signal peptide, a catalytic domain, a PEST-rich linker region, and a chitin-binding domain, thus belongs to the group I Chitinase (Zhang *et al.*, 2012). The reduction of *Se*Chi mRNA led to a death rate around 60% and different phenotypic defects. In the pupal stage of some individuals, the cuticle of the head split open. In the adult stage, some individuals could not shed their pupal shell completely (Zhang *et al.*, 2012). In the meantime, the detailed information of *Se*Chi was up load to gene bank by this lab (ADI24346). Although several insect chitinases have been cloned and expressed in recent years, no further researches on *Se*Chi were reported ever since. In this study, the *Se*Chi gene was cloned and expressed in Hi5 cells by an insect cell expression system, and recombinant *Se*chi (r*Se*Chi) was obtained by isolation and purification and showed high concentration and high activity. Subsequently, the purified r*Se*Chi was analyzed for enzymatic properties and screened for optimum conditions with the highest chitinase activity. In addition, virtual screening technology was used to obtain *Se*Chi candidate inhibitors. Twenty compounds were tested against the purified r*Se*Chi, and seven showed inhibitory activity. These results provided information for the *Se*Chi and are useful for the development of chitinase inhibitors.

Materials and methods

Cells

The *Spodoptera frugiperda* cell line Sf9 and the *Trichoplusia ni* cell strain Hi5 were donated by Professor Weihua Xu. Sf9 cells were cultured in Grace's medium supplemented with 10% fetal bovine serum at 27°C. Hi5 cells were cultured at 27°C in Grace's medium supplemented with 10% fetal bovine serum or Express Five Medium.

Construction of a recombinant baculovirus containing the Spodoptera exigua *chitinase gene*

In this study, the bacmid bMON14272 was used to generate the r*Se*Chi virus in the Bac-to-Bac system. First, the r*Se*Chi fragment with a 6xHis tag at the 3′ end of the *Se*Chi ORF was generated by PCR with the 5′-RACE-Ready cDNA as the template and with the primers *Se*Chi-F/*Se*Chi-R (the primer sequences used in this study are listed in table 1). The PCR product was subsequently digested with *Bam*H I/*Xho* I and subcloned into *Bam*H I/*Xho* I-digested pFastBac1 (donated by Professor Weihua Xu) to generate the donor plasmid pFastBac1-Chi.

Because a mini-attTn7 site has been integrated into the AcMNPV *polyhedron* (*polh*) locus in bMON14272, foreign genes can be easily inserted therein via site-specific transposition (Luckow *et al.*, 1993). Thus, the donor plasmid pFastBac1-Chi was transformed into competent DH10Bac

https://doi.org/10.1017/S0007485319000191 Published online by Cambridge University Press

Table 1. List of primers used in this paper.

Primers	Sequences[1]	Restriction enzyme sites
*Se*Chi-F	5′- TACGGATCC**ATG**AGAGCGATACTGGCG	*Bam*H I
*Se*Chi-R	5′-TCTCTCGAG**CTA**ATGATGATGATGATGAT GGGGCTCGCAGTCTTGACGGTC	*Xho* I
*Se*Chi-MF	5′- CGCTACGGCATTGAAGATA	
*Se*Chi-MR	5′- GTTACCAGCTGACAGAGTGAA	

[1]Restriction enzyme sites are underlined. Translation starting and termination codons are in bold.

cells (containing bMON14272 and the helper plasmid pMON7124 as previously described (Wu *et al.*, 2006), donated by Professor Weihua Xu). The resulting bacmid was named Bacmid-Chi, with r*Se*Chi under control of the *poly* promotor.

As mentioned above, Sf9 cells were previously cultured in Grace's medium supplemented with 10% fetal bovine serum. For transfection, 9×10^5 Sf9 cells were washed twice with Grace's medium without fetal bovine serum and transfected with 1 μg of Bacmid-Chi using Cellfectin II reagent (Invitrogen Life Technologies). After the culture was incubated for 5 h, the transfection buffer was replaced with TMM-FH medium, and the time point was designated 0 h post transfection (h p.t.). The supernatant was collected and filtered through 22 nm filter membrane at 72 h p.t., and the BV titer was determined by a 50% tissue culture infective dose (TCID50) endpoint dilution assay as previously described (Kitts & Possee, 1993). The harvested supernatant with recombinant BV was named P1-Chi. For virus propagation, Sf9 cells were infected with P1-Chi at a multiplicity of infection (MOI) of 0.01–0.1 (pfu/cell), and the supernatant was collected and filtrated at 72 h post infection (h p.i.), followed by BV titer determination. The resulting virus solution was named P2-Chi. Similarly, P3-Chi and P4-Chi were harvested and preserved at −20°C.

*Expression and purification of the r*SeChi

To obtain the highest protein expression level, we explored the best expression conditions for r*Se*Chi. First, 6×10^5 Hi5 cells were seeded in each well of a 12-well plate and infected with P4-Chi of different MOIs (0, 2, 5, 10, 20, and 30). The supernatants and cells were harvested at 96 h p.i. and tested for protein expression level by SDS-PAGE and western blot, in which anti-His or anti-*Se*Chi (a polyclonal antibody prepared by injecting rabbit with the full-length purified r*Se*Chi) was used as the primary antibody. Subsequently, we tested the most suitable time point to harvest the supernatants. Since infection with MOIs of 20 and 30 did not show higher expression levels than infection with an MOI of 10 (fig. 1c), Hi5 cells were infected with P4-Chi with an MOI of 10 in the following experiments. The supernatants were harvested and tested as described above at different time points (0, 24, 48, 72, 96, 120, 144 h) p.i. The results showed that 72 h is the most suitable expression time course (fig. 1d).

Based on the experiments above, Hi5 cells were cultured and proliferated in 75 cm^2 cell culture bottles until the logarithmic growth phase and infected with P4-Chi with an MOI of 10. At 72 h post infection, the cell medium was harvested and centrifuged (4 °C, 10,000 *g*) for 10 min; the supernatant after centrifugation was separated from the precipitate and storage at −20°C or applied for purification.

Since the C-terminus of r*Se*Chi has a 6xHis tag, the protein was purified by Ni^{2+} chelate affinity chromatography in this study. Ni Sepharose™ 6 Fast Flow (GE Healthcare) was used, and the experimental procedure was performed according to the kit instructions. The purified protein was analyzed by SDS-PAGE electrophoresis and western blot analysis, and the protein concentration was measured by the BCA Protein Concentration Assay Kit produced by Mizuno Biology. The procedures for standard curve preparation and sample determination were performed according to the instructions.

*Enzyme activity assay of r*SeChi

To detect the chitinase activity of r*Se*Chi, CM-Chitin-RBV (Loewe Biochemica GmbH, Germany) was used as a substrate. A total reaction volume of 0.4 ml (250 mg l^{-1} of purified r*Se*Chi, 0.5 mol l^{-1} CM-Chitin-RBV, and 50 mmol l^{-1} sodium acetate solution) at pH 5.5 was added to a 1.5 ml centrifuge tube and placed at 40°C for 2 h. Afterwards, 0.1 ml of 1 mol l^{-1} HCl was added to the reaction system to terminate the reaction, followed by an ice bath for 10 min and centrifugation (14,900 *g*, 4°C) for 5 min. The absorbance at 550 nm was measured using a spectrophotometer. The control group was prepared and treated as above except that the reaction time was 0 instead of 2 h.

*Enzymatic property assays of r*SeChi

The optimum reaction temperature and reaction pH of r*Se*Chi were analyzed. Except for the following differences, the experimental procedure was consistent with the above. To determine the optimum reaction temperature, nine different reaction temperatures (10, 20, 30, 40, 50, 60, 70, 80, 90 °C) were tested. To determine the optimum pH, Britton-Robinson buffer was prepared with 10 different pH values between 1.5 and 12 (2.87, 3.78, 4.78, 5.72, 7.0, 7.96, 8.95, 9.91, 10.88, and 11.92). The experiments under each condition were repeated at least three times.

*Detection of the enzyme kinetics of r*SeChi

The enzyme activity of r*Se*Chi at different substrate concentrations was measured. Except for the following differences, the experimental procedure was consistent with the above. The final concentrations of the substrate CM-Chitin-RBV were 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1.0 mM. In addition, the reaction time was 1 h instead of 2 h.

Finally, the K_m and V_{max} of r*Se*Chi were calculated using the Lineweaver-Burk equation.

Homology construction of the 3D model of SeChi

The amino acid sequence of *Se*Chi (ADI24346) obtained from this laboratory was submitted to the SMART website (http://smart.embl-heidelberg.de/) for domain analysis. The

https://doi.org/10.1017/S0007485319000191 Published online by Cambridge University Press

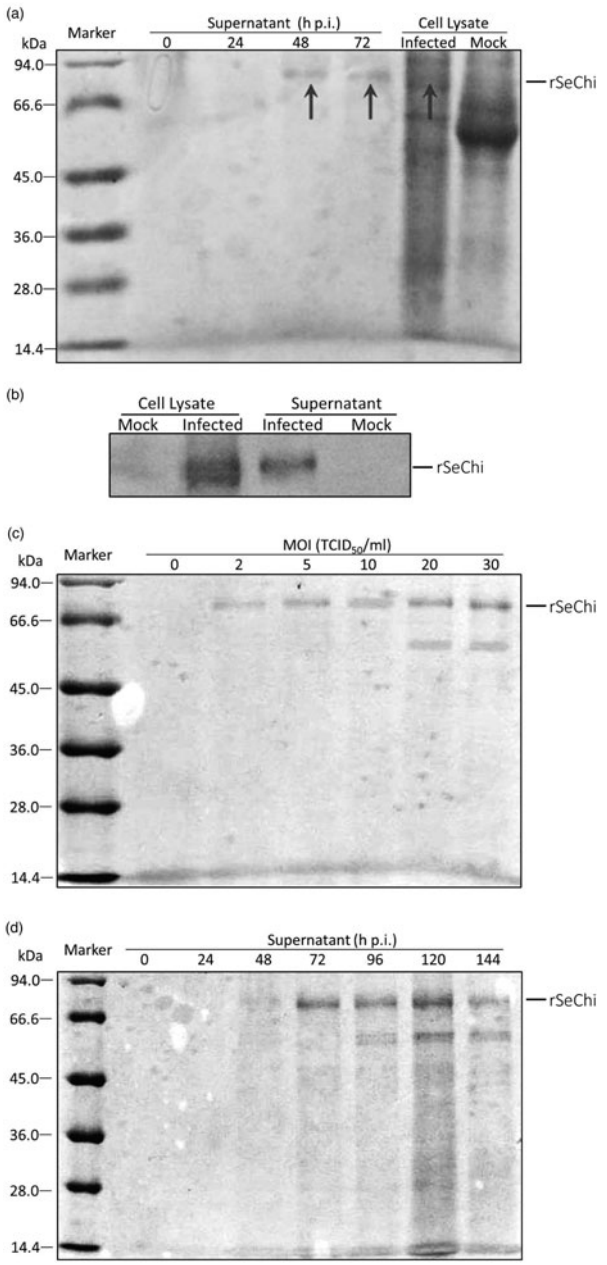

Fig. 1. Expression levels of r*SeChi* detected by SDS-PAGE or western blotting. (a) SDS-PAGE showing the proteins in the supernatants and cell lysate of Hi5 cells infected with P4-Chi recombinant virions. (b) Western blot analysis with anti-His showing a 74 kDa protein in the supernatant and cell lysate of Hi5 cells infected with P4-Chi recombinant virions. (c,d) SDS-PAGE showing proteins in the supernatants of Hi5 cells infected with P4-Chi recombinant virions with MOIs of 2, 5, 10, 20, and 30 (pfu/cell) and harvested at 96 h p.i. (c), or with an MOI of 10 and harvested from 0–144 h p.i. (d). Infected, Hi5 cells infected with P4-Chi; mock, mock-infected Hi5 cells.

software 'Discovery Studio v2.5' was used for homology modeling. The 'Sequence Analysis' function was used for a homology search in the PDB database. Three homologous protein structures were downloaded from the RCSB website (http://www.rcsb.org/). Using *Se*Chi as the 'Input Model Sequence' and the three templates as 'Input Template Structures', homology modeling was performed using the 'Protein Modeling' module. The results were evaluated subsequently by the probability density function energy (PDF Energy), DOPE Score, Ramachandran Plot, and Profile-3D; one optimal model was selected. Finally, the unreasonable amino acid residue conformations were manually adjusted according to the Ramachandran plot, and the final model was optimized with the 'Side-chain Refinement' function.

Virtual screening for SeChi *inhibitors*

Based on the results of a series of prescreening benchmark evaluations (data not shown), the modeling packages MOE and Surflex were chosen for inhibitor screening in this study. MOE was used for the initial screening. The *Se*Chi model file was further refined in MOE using the 'Protonate 3D' module. Subsequently, a small molecule library, the Guangdong Small Molecule Tangible Library (GSMTL) (Gu *et al.*, 2010), was screened. According to the calculated binding energy (ΔG) with *Se*Chi, the top 1000 compounds were selected. These compounds were further evaluated with the Surflex module in Sybyl. The eighty compounds with the highest scores were finally selected, twenty of which had available pure samples.

Measurement of the SeChi *inhibitor activity of the selected compounds*

Each of the twenty obtained compounds was reacted with r*Se*Chi. The reactions were carried out with r*Se*Chi, CM-Chitin-RBV, and each candidate inhibitor. A total reaction volume of 0.4 ml (250 mg l^{-1} of purified rSeChi, 0.5 mol l^{-1} CM-Chitin-RBV, and 10^{-3} mol l^{-1} candidate inhibitor) at pH 9 was added to a 1.5 ml centrifuge tube and placed at 40°C for 2 h. Afterwards, 0.1 ml of 1 mol l^{-1} HCl was added to the reaction system to terminate the reaction, followed by an ice bath for 10 min and centrifugation (14,900 *g*, 4°C) for 5 min. The absorbance values at 550 nm of the inhibition group AX0, the control group A0, the reaction group AX, and the reaction control group AS were detected (where X refers to the inhibitor name).

$$\text{Inhibition}\% = [(\text{AS} - \text{A0}) - (\text{AX} - \text{AX0})]/(\text{AS} - \text{A0})$$

Finally, the three compounds with the highest activity were selected to determine the correlation between inhibitor concentration and inhibitory rate. The reaction procedure was consistent with the above except that each candidate inhibitor underwent ten-fold dilution to obtain gradient concentrations, resulted in a final concentration from 10^{-8} to 10^{-4} mol l^{-1} in the reaction mixture. The IC50 of each candidate was then calculated.

Results

*Construction and propagation of the recombinant baculovirus expressing r*SeChi

The *Se*Chi gene was previously identified in our laboratory (Zhang *et al.*, 2012), and the detailed information of this gene was up load to gene bank (ADI24346). To study *Se*Chi, we first inserted the *Se*Chi gene into the pFastbac1 commercial vector under the control of the highly expressed *polyhedrin* promoter.

https://doi.org/10.1017/S0007485319000191 Published online by Cambridge University Press

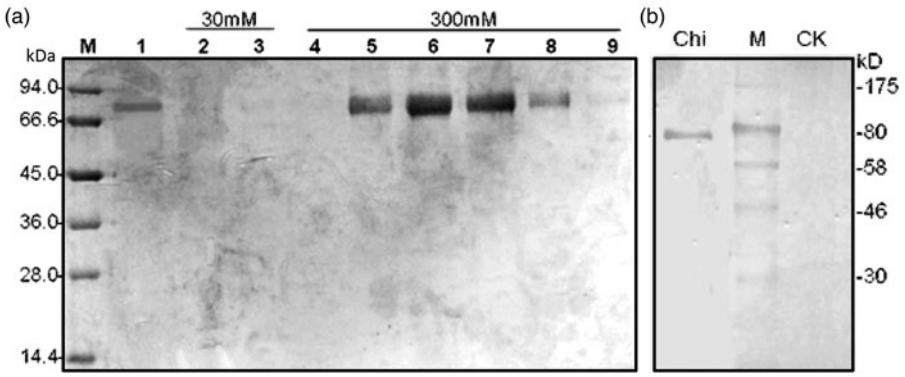

Fig. 2. Purification quality of r*Se*Chi detected by (a) SDS-PAGE and (b) western blotting. (a) Purification of recombinant r*Se*Chi. M, protein molecular weight marker; lane 1, supernatant of Hi5 cells after P4-Chi infection; lanes 2–3, eluted by binding buffer with 30 mM imidazole; lane 4–9, eluted by elution buffer with 300 mM imidazole. (b) Western blot analysis of the purified recombinant protein r*Se*Chi. Chi, purified r*Se*Chi; M, protein molecular weight marker; CK, BSA protein.

For ease of detection and purification, a 6xHis tag was added to the C-terminus of *Se*Chi, and the resulting protein is referred to as r*Se*Chi hereinafter (Supplementary fig. 1). The resulting recombinant bacmid was named Bacmid-Chi.

Bacmid-Chi was transfected into healthy Sf9 cells with the help of Cellfectin II reagent (Invitrogen Life Technologies). At 96 h p.t., most of the cells ruptured, and the virions were released into the culture medium. Thus we collected the culture medium, to obtain the first generation of recombinant virions, P1-Chi. By 3 generations of propagation, high-concentration recombinant virions (10^8 pfu ml^{-1}) were harvested. The presence of the recombinant gene was confirmed by PCR with the DNA from infected cell lysate as the template (data not shown). The results of both SDS-PAGE (fig. 1a) and western blot (fig. 1b) showed a protein band of approximately 74 kDa in the culture supernatant after the Hi5 cells were infected with P4-Chi, whereas no corresponding band appeared in the mock-infected Hi5 cells (fig. 1b).

These results indicated that the recombinant protein r*Se*Chi could be expressed in insect cells and secreted into the culture medium. These also indicated that Bacmid-Chi containing the target gene had been successfully constructed, and the recombinant baculovirus was obtained after transfection into Sf9 cells.

*Expression and purification of r*SeChi

Hi5 cells were subsequently infected with the acquired P4-Chi recombinant virions for r*Se*Chi expression. To explore the optimal expression conditions, different MOIs (0, 2, 5, 10, 20, 30 pfu $cell^{-1}$) and different harvest times (0, 1, 2, 3, 4, 5, 6 days) were tested, and the expression levels of r*Se*Chi in the culture supernatants were detected by SDS-PAGE. The results of the MOI screening assays showed that for an MOI range of 0–10 pfu $cell^{-1}$, r*Se*Chi displayed higher expression for higher MOIs. However, for MOIs of 20 and 30 pfu $cell^{-1}$, no significant increase in expression level but a nonspecific band was observed (fig. 1c), indicated that an MOI of 10 pfu $cell^{-1}$ was the most suitable. On the other hand, the results of the time point screening assays showed that the best harvest time was 3 days p.i., since no increase in expression level but a nonspecific band was observed at 4 days p.i. (fig. 1d). According to the screening results, the optimal expression conditions for r*Se*Chi were determined, namely, the infection of Hi5 cells with an MOI of 10 pfu $cell^{-1}$ and harvest of the culture supernatant at 3 days p.i. Protein expression was performed using these conditions in subsequent experiments.

The r*Se*Chi was constructed with a 6xHis tag at the C-terminus and thus can be purified by Ni^{2+} chelate affinity chromatography. The results showed that miscellaneous proteins were mostly eluted with elution buffer with an imidazole concentration of 30 mM, and r*Se*Chi was eluted with an imidazole concentration of 300 mM in the elution buffer (fig. 2a). The obtained protein was shown to be a single protein band by SDS-PAGE, indicating that the protein was pure.

The purified r*Se*Chi was subjected to western blot analysis using *Se*Chi antiserum (prepared by the laboratory) as the primary antibody. The results showed a specific band with a size of 74 kDa (fig. 2b), which was essentially consistent with the previous analysis with anti-His antibody (fig. 1b). The results indicated that the purified protein obtained by Ni^{2+} chelate chromatography was the target protein. The concentration of the purified r*Se*Chi was determined with Pierce™ BCA Protein Assay Kit (Thermo Scientific™), and the results showed that the concentration was 25 mg l^{-1}.

*Activity detection and enzyme property characterization of r*SeChi

The enzyme activity of r*Se*Chi was measured as described in the experimental procedures. Meanwhile, to assess the viability of r*Se*Chi, a commercial chitinase *Sg*Chi (Sigma, best reaction condition 25°C, pH 6.0 from *Streptomyces griseus*) was used as a positive control. The results showed that the ratio of enzyme activity between *Se*Chi and *Sg*Chi (U_{rSeChi} / U_{SgChi}) was 0.79, suggesting that the activity of r*Se*Chi was comparable to that of the commercial chitinase.

The activity of r*Se*Chi was then measured at reaction temperatures of 10 to 90°C using CM-Chitin-RBV (Loewe Biochemica GmbH, Germany) as the substrate. It can be seen from fig. 3a that the optimum reaction temperature of r*Se*Chi is 40°C.

The activity of r*Se*Chi was then measured at reaction pH values between 3 to 12. The results indicated that r*Se*Chi showed the highest activity at pH 9 (defined as 100%) and pH 6 (relative activity approximately 70%), while it showed lower activity (below 40%) when the pH was less than 4 or higher than 11 (fig. 3b). When the pH is less than 3, the substrate was unstable and thus difficult to measure.

https://doi.org/10.1017/S0007485319000191 Published online by Cambridge University Press

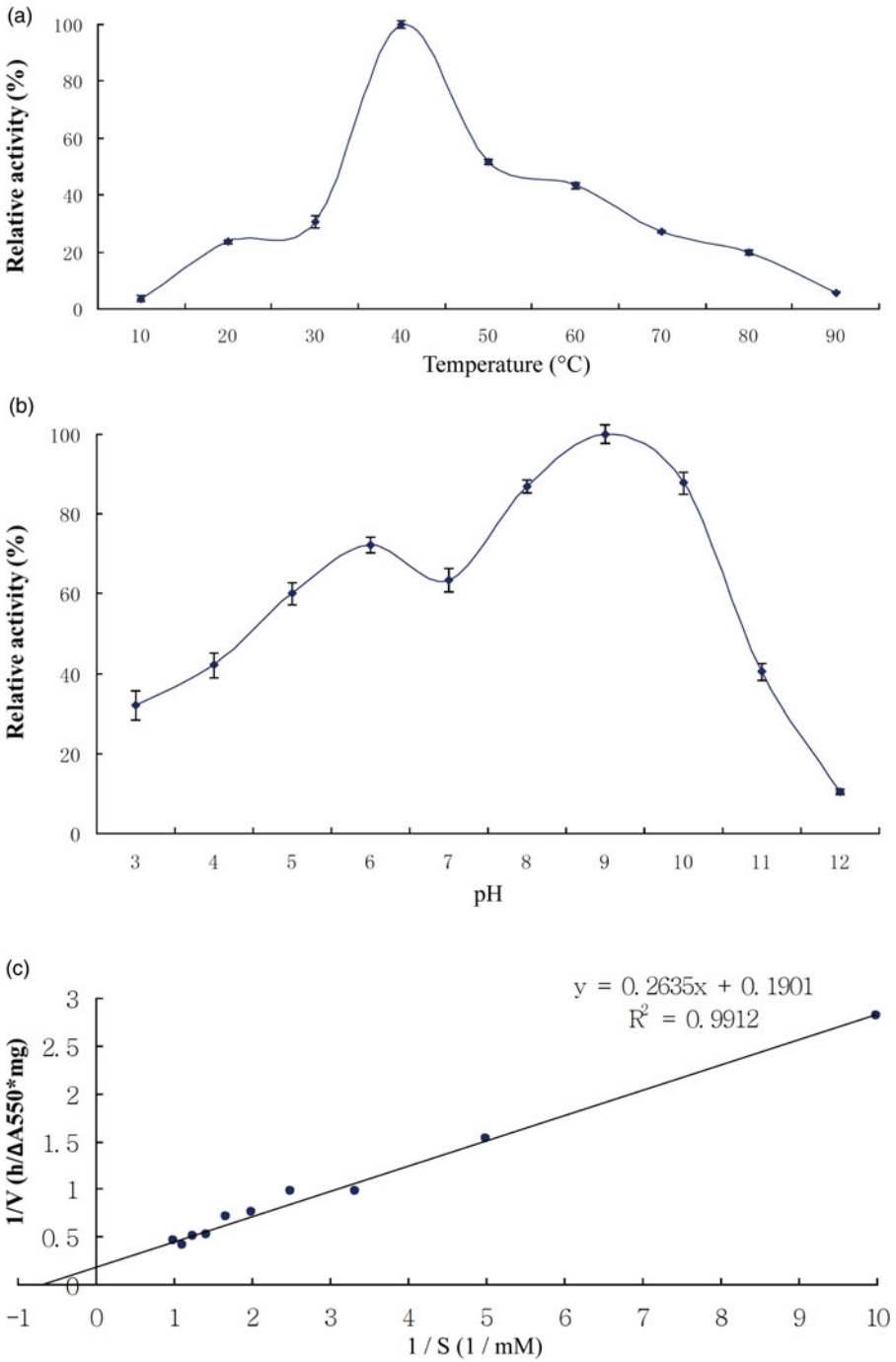

Fig. 3. Enzyme activity and enzymatic property assays of r*Se*Chi. (a, b) Optimum temperature (a) and optimum pH (b) screening of r*Se*Chi. CM-Chitin-RBV (0.5 mM) was used as the substrate. The highest chitinase activity was defined as 100% in these assays. Every value is the mean of three independent assays. All error bars represent the standard error ($n = 3$). (c) Lineweaver-Burk plot of r*Se*Chi.

The correlation between the concentration of CM-Chitin-RBV and the activity of r*Se*Chi was determined under the reaction conditions of pH 9.0 at 40°C. In the substrate concentration range of 0.1–1.0 mM and after reaction for 1 h, the mean speed was close to the initial speed of the reaction. Each concentration of CM-Chitin-RBV (S, mM) and the relative mean velocity of reaction (V, ΔA550 mg^{-1} h^{-1}, absorbance of the catalyzed product per μl of r*Se*Chi per hour) were imported to the Lineweaver–Burk equation, 1/S was linearly related to 1/V (fig. 3c). The calculation results showed that the Michaelis constant (K_m) of r*Se*Chi was

https://doi.org/10.1017/S0007485319000191 Published online by Cambridge University Press

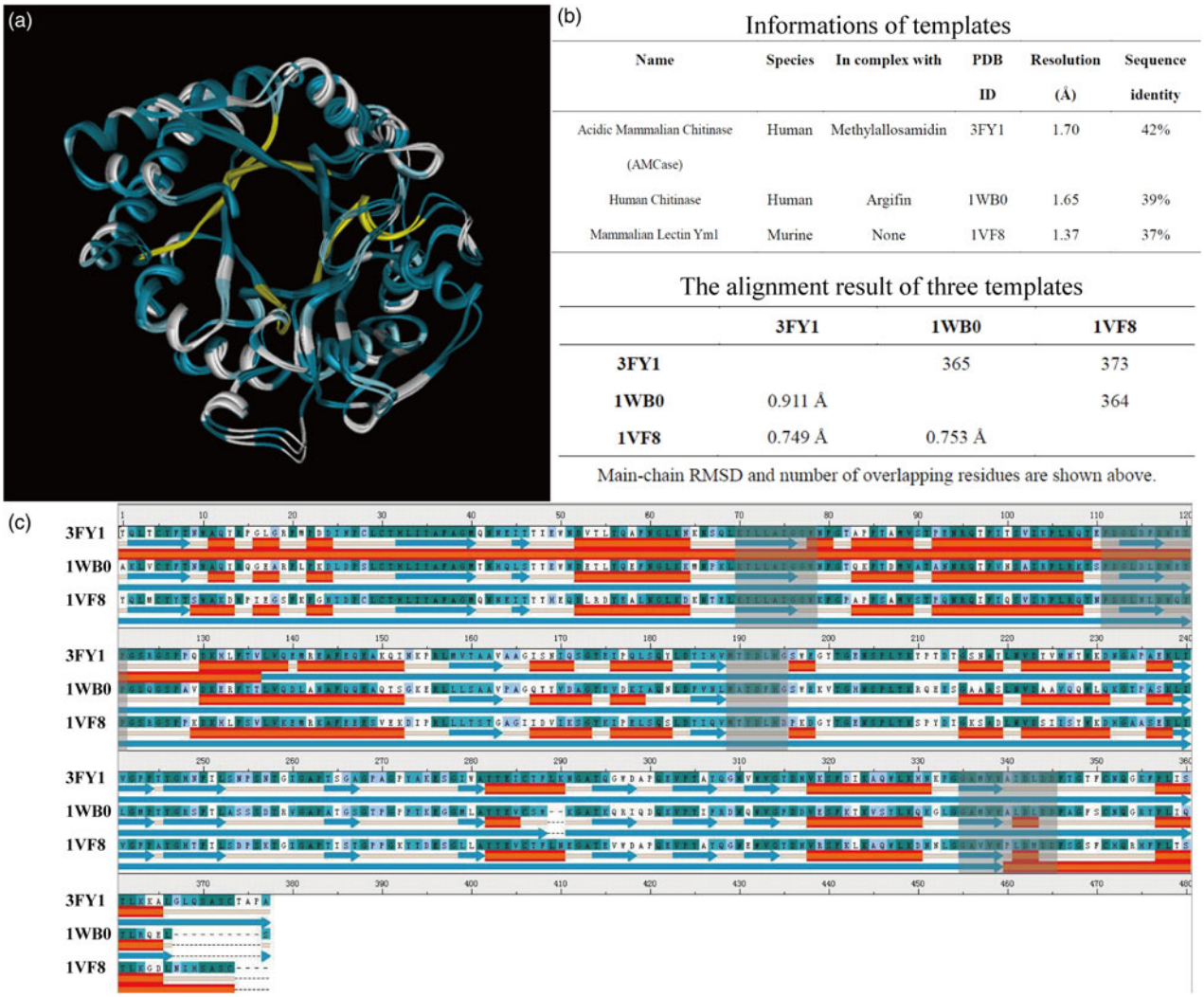

Fig. 4. Structure and sequence alignments of the three homologous templates of *Se*Chi. (a) Structure alignment. The conserved domains (β3: KTLLAIGGW, β4: FDGLD(F/L)DWEYP, β6: M(T/A)YD(L/F)H, and β8: GAMVWA(I/L)DLDD) are colored in yellow. (b) Sequence information of the three templates. (c) Sequence alignment. The conserved domains are marked in gray boxes. The background color of each residue is based on the similarity among the three templates. The darker the color is, the greater the similarity.

1.37 mM, and the maximum enzymatic reaction rate (V_{max}) was 5.26 (ΔA550 h^{-1} mg^{-1}). These results showed that r*Se*Chi has strong catalytic activity.

Homology construction of SeChi *3D model*

We analyzed the amino acid sequence of *Se*Chi obtained in our laboratory. The results showed that *Se*Chi belongs to the insect group I chitinase (Zhu *et al.*, 2008) and has a typical chitinase structure, including an N-terminal signal peptide sequence, a catalytic domain, a connection domain, and a C-terminal chitin binding domain. Moreover, we further confirmed that the enzyme belongs to the glycoside hydrolase 18 family, since the catalytic domain (amino acid 24–376) is conserved as in other members of this family.

With the software 'Discovery Studio v2.5', the amino acid sequence of *Se*Chi was compared with 51,388 protein sequences in the PDB database. The results showed that *Se*Chi had high homology (*E*-value less than 10^{-5}) with 46 sequences, and the highest identity was 42%. According to the principle of low *E*-value, high consistency, and high coverage, three sequences were screened, and their high-resolution crystal structures with ligands were acquired from the PDB database for multi-template homology modeling (fig. 4b, c).

Using prestructural analysis, water molecules, ligands, and metal ions were removed from the three template structures. It can be seen from the template overlay (fig. 4a) that all three templates contain a typical (α/β) 8-barrel structure similar to that of other family 18 chitinases, where the central part is the inner barrel composed of β1-β8, and the peripheral part is the outer barrel composed of α1-α8. The core structures of the three templates are very similar in that the inner barrel is closely attached to the outer bucket, whereas some differences are observed in the random coil between α and β.

The three sequences were compared with the target protein sequence to produce the first sequence profile, and the

https://doi.org/10.1017/S0007485319000191 Published online by Cambridge University Press

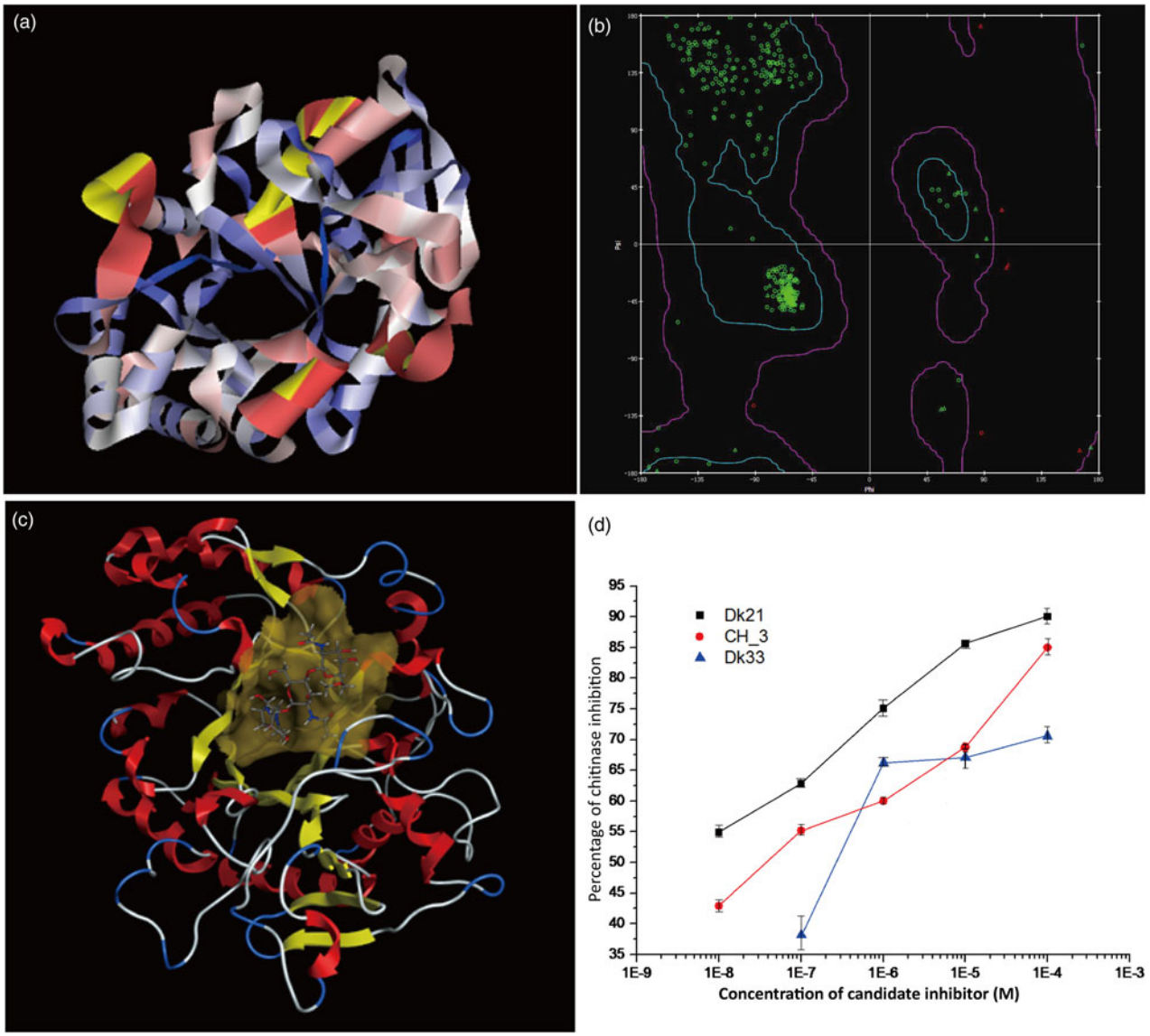

Fig. 5. The 3D modeling and inhibitor screening of *Se*Chi. (a) A solid ribbon picture of the *Se*Chi.B99990003 model. Blue, high verify score; white, average verify score; red, low verify score; yellow, lowest verify score (less than 0). (b) Ramachandran plot of the *Se*Chi model. Green regions, the most favored regions; purple regions, favored regions; red dots, amino acids with unreasonable conformations. (c) The ligand binding pocket of the *Se*Chi model (displayed as the yellow region). (d) The inhibitory effects of 3 molecules (Dk21, C_H3, and Dk33) at different concentrations. CM-Chitin-RBV was used as the substrate. Each value is the mean of three independent assays. All error bars represent the standard error ($n = 3$).

similarity of the four sequences was 93.2%. As the catalytic domain plays a key role in chitinase, we then analyzed the catalytic domains of the four sequences, resulting in a second sequence profile. The resulting identity was 50%, and the four conserved sequences in chitinase, especially the important amino acid residues in the β4 chain, glutamate (E), aspartic acid (D), and tryptophan (W), were retained.

Using the two resulted profiles, three-dimensional models of the target sequence were constructed and evaluated based on PDF Total Energy, DOPE, and Profile-3D scores. *Se*Chi.B99990003 showed the highest credibility (fig. 5a).

The score of each amino acid from the *Se*Chi.B99990003 model was obtained. The results showed that a low-score region (Verify Score below 0) was located in the outer area of the model (a/β) 8-barrel structure, while the core region was conserved. The Ramachandran diagram was then used to manually adjust the unsatisfactory amino acids, and the 'Side-chain Refinement' function was used for optimization. The final model was named *Se*Chi, and its Ramachandran diagram is shown in fig. 5b. The number of amino acids with unreasonable conformations has been reduced to seven (red dots).

https://doi.org/10.1017/S0007485319000191 Published online by Cambridge University Press

Table 2. List of the inhibition rates of seven candidate inhibitors.

Compound	Inhibition rate (%)*
Dk21	96.98 ± 1.95
C_H3	90.94 ± 0.62
Dk33	83.89 ± 0.15
D_H3	80.11 ± 0.25
Dk16	77.18 ± 0.2
Dk31	70.81 ± 0.37
Dk30	51.68 ± 4.31

*The values are means of 3 independent assays. All error bars represent standard error.

Virtual screening of SeChi *inhibitors*

We evaluated five commonly used molecular docking tools, MOE, Ligandfit, Cdocker, Surflex Dock, and FlexX, and selected MOE and Surflex for subsequent screening work. The *Se*Chi model obtained by homology modeling was then applied as the receptor, and the binding domain of methylallosamidin in the complex was defined as the docking domain.

The compound library of the School of Pharmacy at Sun Yat-sen University was used as the candidate ligands and analyzed by MOE software. According to the molecular docking and binding free energy, 1000 candidates were selected for the second round of docking analysis. In the second round of screening, the 1000 compounds were subjected to scoring in the software SYBYL-X, and 80 compounds were selected.

Inhibitory activity measurement of candidate SeChi *inhibitors*

Twenty pure samples of the 80 candidate inhibitors were available and were used for inhibition tests with CM-Chitin-RBV as the substrate. The inhibition rates were calculated, and the results showed that 7 compounds had inhibitory activity at a concentration of 10^{-3} M (table 2), among which the compound Dk21 showed the highest inhibitory activity with an inhibition rate of 96.98%, followed by C_H3 (90.94%) and Dk33 (83.89%). The other 13 compounds did not show obvious inhibitory activity.

Compounds Dk21, C_H3, and Dk33, which showed the most significant inhibitor activity, were selected to determine the correlation between inhibitor concentration (from 10^{-8} to 10^{-4} M) and inhibitory rate (fig. 5d). Consistent with the inhibition rate assays (table 2), Dk21 has the highest *Se*Chi inhibition activity, followed by C_H3 and Dk33. The IC50 for each inhibitor was then calculated. At r*Se*Chi concentration of 250 mg l^{-1}, the IC50 for Dk21 C_H3, and Dk33 are showed in table 3.

Table 3. List of the IC50 for the top three candidate inhibitors of *Se*Chi.

Compound	IC50 (mol l^{-1})	95% Confidence interval (mol l^{-1})
Dk21	5.97×10^{-9}	7.30×10^{-10}–2.11×10^{-8}
C_H3	5.71×10^{-8}	1.16×10^{-8}–1.66×10^{-7}
Dk33	2.92×10^{-7}	2.83×10^{-13}–3.74×10^{-6}

Discussion

The chitinase of *Spodoptera exigua* was firstly discovered and studied in our laboratory. Two chitinse genes, namely *Se*Chi and *Se*Chi-h, were identified and silenced through specific dsRNA injection respectively (Zhang *et al*., 2012). In the meantime, we up loaded the detailed information of *Se*Chi to gene bank (ADI24346). In recent years, no further researches on *Se*Chi were reported ever since. In this study, we managed to clone and express the *Se*Chi gene for the first time.

Insect chitinase undergoes a high degree of posttranslational modification such as glycosylation and signal peptide excision, and it exhibits complex secondary and three-dimensional structure (Kramer & Muthukrishnan, 1997). Therefore, the correct posttranscriptional modification, processing, transport and protein folding are the key to obtaining active insect chitinase. Due to the differences between prokaryotic and eukaryotic expression systems, expression of insect chitinase in insect cells might be a better option. In a previous study on the apple leaf miner moth, the *Lithocolletis ringoniella* chitinase *Lr*Cht5 was expressed in both *E. coli* and Sf9 cells, and the *Lr*Cht5 expressed in insect cells exhibited chitinolytic activity (Fan *et al*., 2015). Similarly, *Se*Chi expression in this laboratory using *E. coli* system showed that the resulting protein was present as inclusion body (data not shown). Thus in this study, the recombinant expression of *Se*Chi was performed using the insect cell system. Since Sf9 and Hi5 cells are both derived from Lepidoptera insects, the process of posttranscriptional modification is similar to that of *Spodoptera exigua*.

Multiple factors affect the expression level, including protein properties, including cell status, MOI, and infection time course (Bernard *et al*., 2001; Kim *et al*., 2007). In this study, we optimized the protein expression conditions with a selection of screening procedures and eventually selected the Hi5 cell line and used an MOI of 10 for 72 h as the expression conditions. The resulting concentration of r*Se*Chi protein was 25 mg l^{-1}. Western blot analysis with anti-His antibody and anti-r*Se*Chi antiserum confirmed the successful expression of r*Se*Chi with a molecular weight of approximately 74 kDa, which was larger than the predicted 64 kDa. This phenomenon was also found in other studies (Arakane *et al*., 2003), and the predicted reason was that group I chitinase is rich in S/T residues in the attachment region that are usually O-glycosylated (Zheng *et al*., 2002). The r*Se*Chi constructed with a 6xHis tag at the C-terminus was then purified by Ni^{2+} chelate affinity chromatography. As in other studies (Reynolds & Samuels, 1996), the optimum temperature and pH of r*Se*chi were then screened, and the chitin hydrolysis activity was measured. The enzyme activity of r*Se*chi was similar to that of the commercial chitinase *Sg*Chi, indicating that r*Se*Chi exhibits high chitinase activity.

Computer-aided molecular docking technique (Shoichet, 2011) is an efficient way for virtual screening of the *Se*Chi inhibitor. Previous studies have shown that the catalytic mechanism of family 18 chitinases is a substrate-supporting mechanism. In the substrate binding process, the substrate changes conformation into a boat shape to assist the catalytic process (Fukamizo, 2000). In this study, molecular docking technique based on the substrate structure theory was used to study the inhibitors of *Se*Chi. Two rounds of docking (Shoichet, 2011) were performed, and 80 candidate inhibitors were selected. However, virtual screening is not sufficient to verify inhibitory activity. Thus 20 among the 80 candidates were subsequently tested with the purified r*Se*Chi. As a result, we found 7 candidate compounds that showed inhibitory activity against r*Se*Chi. These results suggested that the molecular docking via 3D modeling in this study is helpful for

https://doi.org/10.1017/S0007485319000191 Published online by Cambridge University Press

inhibitor screening. These also indicated that the purified r*Se*Chi displays chitinase activity and is similar to the native *Se*Chi. In conclusion, we harvested active *Spodoptera exigua* chitinase in this study for the first time. Furthermore, compounds with inhibitory activity against *Se*Chi were discovered using 3D modeling, molecular docking, and *in vitro* analysis with the purified r*Se*Chi. In the previous study of this lab, we found that the RNA silencing against *Se*Chi led to cuticle and pupa defects of *Spodoptera exigua* and a death rate of around 60% in *in vivo* experiments (Zhang *et al.*, 2012). Regrettably, we are currently not able to test whether the *Se*Chi inhibitors can kill *Spodoptera exigua*, since we have to give a break to this study on the *Se*Chi due to a funding situation. However, we believe that similar to the *Se*Chi dsRNA, the inhibitors screened in this work may cause defect on *Spodoptera exigua*. Besides, the compounds with inhibitory activity are small molecules. Someday we may find useful compounds that are more stable and lower in cost compared to dsRNA. Thus we believe that this study provided information for the *Se*Chi and is useful for the development of insect chitinase inhibitors.

Supplementary material

The supplementary material for this article can be found at https://doi.org/10.1017/S0007485319000191.

Acknowledgments

The authors acknowledge Prof. Weihua Xu (State Key Laboratory of Biocontrol, Sun Yat-sen University) for providing the Hi5 cell strain, bacmid bMON14272, and plasmid pFastBac1. This work is supported by National Natural Science Foundation of China (No. 81603026). We acknowledge the President Fund of Guangdong Academy of Agricultural Sciences (201601) and Science and Technology Plan Project of Guangdong Province (2016A020210039).

Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions

WZ designed the study and revised the paper. LZ expressed and purified the r*Se*Chi, and built the 3D model. ZG wrote the paper. ZP set up the expression system in insect cells. HG screened the inhibitors and tested for inhibitory activity. DZ coordinated the design of this study. JX guide the virtual screening with the small molecule library.

References

Andersen, S.O. (2003) Biochemistry of Insect Cuticle. *Annual Review of Entomology* **24**, 29–59.

Arakane, Y. & Muthukrishnan, S. (2010) Insect chitinase and chitinase-like proteins. *Cellular and Molecular Life Sciences* **67**, 201–216.

Arakane, Y., Zhu, Q., Matsumiya, M., Muthukrishnan, S. & Kramer, K.J. (2003) Properties of catalytic, linker and chitin-binding domains of insect chitinase. *Insect Biochemistry & Molecular Biology* **33**, 631–648.

Arora, N., Ahmad, T., Rajagopal, R. & Bhatnagar, R.K. (2003) A constitutively expressed 36 kDa exochitinase from Bacillus thuringiensis HD-1. *Biochemical & Biophysical Research Communications* **307**, 620–625.

Bernard, A., Payton, M. & Radford, K.R. (2001) Protein expression in the baculovirus system. *Current protocols in Neuroscience* Chapter 4, Unit 4 19.

Carlini, C.R. & Grossi-De-Sá, M.F. (2002) Plant toxic proteins with insecticidal properties. A review on their potentialities as bioinsecticides. *Toxicon* **40**, 1515–1539.

Chen, L., Zhou, Y., Qu, M., Zhao, Y. & Yang, Q. (2014) Fully deacetylated chitooligosaccharides act as efficient glycoside hydrolase family 18 chitinase inhibitors. *Journal of Biological Chemistry* **289**, 17932.

Chen, L., Liu, T., Duan, Y., Lu, X. & Yang, Q. (2017) Microbial secondary metabolite, phlegmacin B1, as a novel inhibitor of insect chitinolytic enzymes. *Journal of Agricultural and Food Chemistry* **65**, 3851–3857.

Fan, Y., Zhang, Y., Yang, X., Pei, X., Guo, S. & Pei, Y. (2007) Expression of a Beauveria bassiana chitinase (Bbchit1) in Escherichia coli and Pichia pastoris. *Protein Expression & Purification* **56**, 93–99.

Fan, X.J., Mi, Y.X., Ren, H., Zhang, C., Li, Y. & Xian, X.X. (2015) Cloning and functional expression of a chitinase cDNA from the apple leaf miner moth lithocolletis ringoniella. *Biochemistry* **80**, 242–250.

Fan, X.J., Yang, C., Zhang, C., Ren, H. & Zhang, J.D. (2018) Cloning, site-directed mutagenesis, and functional analysis of active residues in lymantria dispar chitinase. *Applied Biochemistry and Biotechnology* **184**, 12–24.

Fitches, E., Wilkinson, H., Bell, H., Bown, D.P., Gatehouse, J.A. & Edwards, J.P. (2004) Cloning, expression and functional characterisation of chitinase from larvae of tomato moth (Lacanobia oleracea): a demonstration of the insecticidal activity of insect chitinase. *Insect Biochemistry and Molecular Biology* **34**, 1037–1050.

Fukamizo, T. (2000) Chitinolytic enzymes: catalysis, substrate binding, and their application. *Current Protein and Peptide Science* **1**, 105–124.

Gu, Q., Xu, J. & Gu, L. (2010) Selecting diversified compounds to build a tangible library for biological and biochemical assays. *Molecules* **15**, 5031–5044.

Hao, C.J., Chai, B.F., Wei, W., Yi, S. & Liang, A.H. (2005) Polyclonal antibody against Manduca sexta chitinase and detection of chitinase expressed in transgenic cotton. *Biotechnology Letters* **27**, 97–102.

Henrissat, B. & Bairoch, A. (1993) New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochemical Journal* **293**(Pt 3), 781.

Hirose, T., Maita, N., Gouda, H., Koseki, J., Yamamoto, T., Sugawara, A., Nakano, H., Hirono, S., Shiomi, K. & Watanabe, T. (2013) Observation of the controlled assembly of preclick components in the *in situ* click chemistry generation of a chitinase inhibitor. *Proceedings of the National Academy of Sciences of the United States of America* **110**, 15892.

Hu, S.B., Liu, P., Ding, X.Z., Yan, L., Sun, Y.J., Zhang, Y.M., Li, W.P. & Xia, L.Q. (2009) Efficient constitutive expression of chitinase in the mother cell of Bacillus thuringiensis and its potential to enhance the toxicity of Cry1Ac protoxin. *Applied Microbiology & Biotechnology* **82**, 1157.

Kim, J.S., Choi, J.Y., Roh, J.Y., Lee, H.Y., Jang, S.S. & Je, Y.H. (2007) Production of recombinant polyhedra containing Cry1Ac fusion protein in insect cell lines. *Journal of Microbiology and Biotechnology* **17**, 739–744.

Kitts, P.A. and Possee, R.D. (1993) A method for producing recombinant baculovirus expression vectors at high frequency. *Biotechniques* **14**, 810–817.

Kramer, K.J. & Muthukrishnan, S. (1997) Insect chitinases: molecular biology and potential use as biopesticides. *Insect Biochemistry & Molecular Biology* **27**, 887.

Lehane, M.J. (1997) Peritrophic matrix structure and function. *Annual Review of Entomology* **42**, 525.

Lertcanawanichakul, M., Wiwat, C., Bhumiratana, A. & Dean, D.H. (2004) Expression of chitinase-encoding genes in Bacillus thuringiensis and toxicity of engineered B. thuringiensis subsp. Aizawai toward Lymantria dispar larvae. *Current Microbiology* **48**, 175–181.

Liang, T.W., Chen, Y.Y., Pan, P.S. & Wang, S.L. (2014) Purification of chitinase/chitosanase from Bacillus cereus and discovery of an enzyme inhibitor. *International Journal of Biological Macromolecules* **63**, 8.

Liu, T., Chen, L., Zhou, Y., Jiang, X., Duan, Y. & Yang, Q. (2017) Structure, catalysis and inhibition of OfChi-h, the Lepidoptera-exclusive insect chitinase. *Journal of Biological Chemistry* **292**, 2080–2088.

Luckow, V.A., Lee, S.C., Barry, G.F. & Olins, P.O. (1993) Efficient generation of infectious recombinant baculoviruses by site-specific transposon-mediated insertion of foreign genes into a baculovirus genome propagated in Escherichia coli. *Journal of Virology* **67**, 4566.

Lv, M. (2007) Study on chitinase inhibitors. Doctoral dissertation. Zhejiang University of Technology.

Maccari, G., Deodato, D., Fiorucci, D., Orofino, F., Truglio, G.I., Pasero, C., Martini, R., De, L.F., Docquier, J.D. & Botta, M. (2017) Design and synthesis of a novel inhibitor of T. Viride chitinase through an in silico target fishing protocol. *Bioorganic & Medicinal Chemistry Letters* **27**, 3332–3336.

Merzendorfer, H. & Zimoch, L. (2003) Chitin metabolism in insects: structure, function and regulation of chitin synthases and chitinases. *Journal of Experimental Biology* **206**, 4393–4412.

Müller, M. (1992) Proteolysis in protein import and export: signal peptide processing in eu- and prokaryotes. *Experientia* **48**, 118–129.

Paek, A., Park, H.Y. & Jeong, S.E. (2012) Molecular cloning and functional expression of chitinase-encoding cDNA from the cabbage moth, mamestra brassicae. *Molecules & Cells* **33**, 439.

Reynolds, S.E. & Samuels, R.I. (1996) Physiology and biochemistry of insect moulting fluid. *Advances in Insect Physiology* **26**, 157–232.

Rinaudo, M. (2007) Chitin and chitosan – properties and applications. *Cheminform* **38**, 603–632.

Sakuda, S., Isogai, A., Matsumoto, S. & Suzuki, A. (1987) Search for microbial insect growth regulators. II. Allosamidin, a novel insect chitinase inhibitor. *Journal of Antibiotics* **40**, 296.

Saville, G.P., Thomas, C.J., Possee, R.D. & King, L.A. (2002) Partial redistribution of the Autographa californica nucleopolyhedrovirus chitinase in virus-infected cells accompanies mutation of the carboxy-terminal KDEL ER-retention motif. *Journal of General Virology* **83**, 685–694.

Schrempf, H. (2001) Recognition and degradation of chitin by streptomycetes. *Antonie van Leeuwenhoek* **79**, 285–289.

Shen, Z. & Jacobs-Lorena, M. (1998) A type I peritrophic matrix protein from the malaria vector Anopheles gambiae binds to chitin. Cloning, expression, and characterization. *Journal of Biological Chemistry* **273**, 17665.

Shinoda, T., Kobayashi, J., Matsui, M. & Chinzei, Y. (2001) Cloning and functional expression of a chitinase cDNA from the common cutworm, Spodoptera litura, using a recombinant baculovirus lacking the virus-encoded chitinase gene. *Insect Biochemistry & Molecular Biology* **31**, 521–532.

Shoichet, B.K. (2011) Virtual screening of chemical libraries. *Nature* **432**, 862.

Somers, P.J., Yao, R.C., Doolin, L.E., Mcgowan, M.J., Fukuda, D.S. & Mynderse, J.S. (1987) Method for the detection and quantitation of chitinase inhibitors in fermentation broths; isolation and insect life cycle effect of A82516. *Journal of Antibiotics* **40**, 1751–1756.

Wu, W., Lin, T., Pan, L., Yu, M., Li, Z., Pang, Y. & Yang, K. (2006) Autographa californica multiple nucleopolyhedrovirus nucleocapsid assembly is interrupted upon deletion of the 38K Gene. *Journal of Virology* **80**, 11475–11485.

Wu, Q., Liu, T. & Yang, Q. (2013) Cloning, expression and biocharacterization of OfCht5, the chitinase from the insect Ostrinia furnacalis. *Insect Science* **20**, 147–157.

Xie, J., Zhou, Z., Zhang, X., Lan, X. & Hu, H. (2006) Preliminary screening of chitinase inhibitor producing strains. *Chinese Journal of Antibiotics* **31**, 39–41.

Yan, J., Cheng, Q., Narashimhan, C.B., Li, S. & Aksoy, S. (2002) Cloning and functional expression of a fat body-specific chitinase cDNA from the tsetse fly, Glossina morsitans morsitans. *Insect Biochemistry & Molecular Biology* **32**, 979–989.

Zhang, D., Chen, J., Yao, Q., Pan, Z., Chen, J. & Zhang, W. (2012) Functional analysis of two chitinase genes during the pupation and eclosion stages of the beet armyworm Spodoptera exigua by RNA interference. *Archives of Insect Biochemistry & Physiology* **79**, 220–234.

Zheng, Y., Zheng, S., Cheng, X., Ladd, T., Lingohr, E.J., Krell, P.J., Arif, B.M., Retnakaran, A. & Feng, Q. (2002) A molt-associated chitinase cDNA from the spruce budworm, Choristoneura fumiferana. *Insect Biochemistry and Molecular Biology* **32**, 1813–1823.

Zhu, Q., Arakane, Y., Banerjee, D., Beeman, R.W., Kramer, K.J. & Muthukrishnan, S. (2008) Domain organization and phylogenetic analysis of the chitinase-like family of proteins in three species of insects. *Insect Biochemistry & Molecular Biology* **38**, 452–466.