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Characterisation and Recognition by Immune Hosts of a Sheep Nematode Parasite *Teladorsagia circumcincta* Chitinase

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Abstract

A 912 bp full length cDNA encoding *Teladorsagia circumcincta* chitinase (*Tci*CHT) was cloned and expressed in *Escherichia coli*. Recombinant *Tci*CHT was purified and its enzyme assays performed. The predicted protein consisted of 304 amino acids and weighed about 34 kDa on sodium dodecyl (lauryl) sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The recombinant *Tci*CHT was expressed as inclusion bodies and treated with 8M urea to denature the protein. Multiple alignments of the protein sequence of *Tci*CHT with homologues from other helminths showed that the highest similarity (88%) to the CHT of Haemonchus sp, and 65-87% similarity to the other nematode CHT. Substrate binding sites and conserved regions were identified and shown to be conserved in other homologues. Enzyme assays were carried out using multiple substrates but failed to produce any activity. Recombinant *Tci*CHT was recognised by antibodies in both serum and saliva from field-immune sheep in ELISA, however, that was not the case with nematode-naïve sheep. Given the importance of the enzyme and its recognition by the immune-sheep, *Teladorsagia circumcincta* chitinase might have potential as a vaccine candidate to control this common sheep parasite.

Introduction

Teladorsagia circumcincta is a mucosal browser and resides in the abomasa of the ruminants. The parasite has a direct life-cycle where the eggs laid by the adult worms are passed on to the pasture through faeces and eggs develop into the infective stage larvae (L3), which are ingested and reside in the abomasa of the ruminants and develop into adult worms. Parasitic nematode worm infection is one of the biggest health problems for farmed ruminants worldwide. Parasitic worm infections are harmful to a host animal for many reasons and cause costly production losses and if left untreated, animals can die causing further economic loss to farmers.

The control and productivity losses caused by parasitic nematodes cost the New Zealand livestock industry ~$700 million annually. Currently, farmers rely on the use of anthelmintics to control parasitic nematodes, however resistance of parasites to one or more of these agents is now widespread. Recent industry-funded surveys in New Zealand found that 64% of sheep farms and 94% of beef farms now have parasites that are resistant to at least one of the anthelmintics [1]. It is really important to understand worm biology and look for the targets that are essential for the worm survival.

Chitinase catalyses the hydrolytic cleavage of the ß-1, 4-glycoside bonds present in biopolymers of N-acetylglucosamine, particularly in chitin. Chitinases are widely distributed in living organisms and are found in fungi, bacteria, parasites, plants and animals. The chitinolytic enzymes are also characterised based in their enzymatic action on chitin substrates. Endochitinases are the enzymes catalysing the random cleavage at internal points in the chitin chain whereas exochitinases catalyse the progressive release of acetylchitobiose from the non-reducing end of chitin.

Chitinases perform a variety of function depending on the organism they are present in, for example, in bacteria chitinases are mainly involved in the nutritional processes [2,3] whereas in yeast and fungi, these enzymes participate in morphogenesis [4,5]. In animals and plants, chitinases primarily play a role to defend the organism against infections [6-8] and regulate innate immunity and tissue function. In parasites, chitinase is a significant component of the eggshell and play a vital role in egg hatching [9].

Chitin is one of the most abundant polysaccharide and in nematode parasite comprises cuticles, egg-shell, pharynx and microfilarial sheath [10-12]. Nematode chitinases consists of multiple genes and it is believed that the enzymes have additional roles in the nematode life cycle because of the presence of stage-specific gene expression Chitinase was detected in the excretory/secretory (ES) protein of the root-knot nematode [13]. Helminth chitinase are induced during T helper- type responses and contribute to asthma, fibrosis and helminth immunity [14,15]. The chitin metabolism can provide unique targets for parasite control because chitin is not found in vertebrates. Because of being central in the chitin metabolism, chitinase has potential as a vaccine candidate. Mice vaccinated with chitinase DNA resulted in partial protection against Onchocerca volvulus [16]. Similarly, rabbits and mice vaccinated with recombinant chitinase-like proteins provided protection against mite *Sacroptes scabiei* and hard tick *Haemaphysalis longicornis* respectively [17,18].

The aim of the present experiments was to determine the full-length sequence of chitinase in the sheep abomasal nematode parasite Teladorsagia circumcincta, express the protein, structural analysis and antigenicity of the recombinant protein.

Materials and methods

All chemicals used in these experiments were purchased from the Sigma Chemical Co. (Mo, USA) unless stated. Use of lambs for parasite culturing and harvesting adult worms for molecular biology studies has been approved in protocol # 13502 by the AgResearch Grasslands Animal Ethics Committee.

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Parasite culture and collection

Pure cultures of *T. circumcincta* were obtained by passaging larvae through sheep and adult worms recovered as described previously [19].

RNA isolation and cDNA synthesis

RNA was isolated from adult worms and first strand cDNA synthesised from 1 ug total RNA using the iScript Select cDNA Synthesis Kit (Bio-Rad) as as described previously [19].

Cloning and expression of *T. circumcincta* recombinant *TciC*HT in *E. coli*

A partial *T. circumcincta* chitinase sequence (TELCIR_01681) was obtained from AgResearch's internal database. In order to obtain the full length chitinase gene sequence, Rapid Amplification of cDNA Ends (RACE) using the SMARTer RACE cDNA Amplification Kit (Clontech) was carried out. Both 3' end and 5' RACE primers were designed but failed to get a full-length gene. Nested PCR reactions were carried out using SL1 and SL2 primers to detect the presence of longer transcripts. The gene sequence was sent to GenScript (Hong Kong) for gene synthesis and insertion into PUC57. The *Tci*CHT gene were cloned, using protocols described previously [20] into the expression vector AY2.4. Restriction enzymes Ndel and Notl were used in cloning and the resulting protein was N-terminal His tagged recombinant.

E. coli strain BL21 (DE3) transformed with AY2.4 *Tci*CHT as described previously [21] was grown in Luria Broth (LB). L-Arabinose was added as inducer and the culture grown for an additional 2h at 30 oC and 250rpm. Bacteria were harvested as described before. Briefly, The pellet was weighted and resuspended in 10ml per gram of pelleted bacteria of equilibration buffer (20mM sodium bi-phosphate, 0.5M NaCl, 20mM Imidazole, pH 7.4). Protease inhibitors were added to the suspension, which was then passed through, the chamber of a MP110 Microfluidizer® (Microfluidics, USA) seven times consecutively under ice, at 20,000psi to ensure the full lysis of E.coli as recommended by the manufacturer. The crude lysate was centrifuged at 15,000g for 20min at 4°C to remove all cell debris and the supernatant collected and filtered through a 0.22µm to insure the removal of further impurities.

Recombinant *Tci*CHT was expressed as totally insoluble protein and the protein was purified and folded as inclusion bodies as described [22]. Briefly, purified recombinant poly-histidine *Tci*CHT was obtained by Fast protein liquid chromatography (FPLC) under native conditions using from a Ni-NTA column (Qiagen), completed with a BIO-RAD chromatography system (Bio-Rad, USA). Sodium bi-phosphate buffer was used as an equilibration buffer, sodium bi-phosphate containing 20mM imidazole and 8M urea as the wash buffer and sodium bi-phosphate containing 500mM imidazole and 8M urea as elution buffer. The recombinant *Tci*CHT was purified as inclusion bodies, therefore, dialysed in the buffer containing 8M, 6M, 4M, 2M and no urea for 12h in each buffer at 4°C. The protein concentration was determined by the Nanodrop (A280nm assay) using extinction coefficient (92610M-1cm-1) and molecular weight (34KDa).

Purification and gel Electrophoresis

Recombinant *Tci*CHT was produced as recombinant poly-histidine protein and was obtained by FPLC under native conditions using a Ni-NTA column (Qiagen), and a Biologic DUO-FLOW BIO-RAD chromatography system (Bio-Rad, USA) as described before [22].

Protein Structure Modelling

CHT sequences from several closely related helminth species including *H. contortus* were collected from NCBI. Protein alignments were performed using the Muscle multiple alignment option in Geneious 8 (Biomatters Ltd) with the Blosum 62 similarity matrix. Iterative threading assembly refinement (I-TASSER) [23], was used to construct a structural model of TeciCHT. For each target, I-TASSER simulations generate a large ensemble of structural conformations, called decoys. The confidence of each model is quantitatively measured by the C-score that is calculated based on the significance of threading template alignments and the convergence parameters of the structure assembly simulations. Another important metric is TM-score which is estimated from C-score and is used for measuring the similarity of two protein structures/ Scores higher than 0.5 assumes the parent structure and modelled protein share the same fold while below 0.17 suggests a random nature to the produced model.

Chitinase assay

Recombinant chitinase activity was measured as described. The kit contains three different substrates to measure endo- and exo-chitinase activity. Each substrate was dissolved in DMSO and diluted 1:20 in the assay buffer (100 mM citric acid, 200 mM sodium phosphate, pH 5.5) prior to the assay. The assay started after the addition of recombinant *Tci*CHT and incubated for 30 min at 37 °C. The reaction was stopped by the addition of stop solution (500mM sodium carbonate, 500 mM sodium bicarbonate, pH 10.5) and the liberated 4-MU was measured at 450nm.

Host Recognition

To test for the presence of antibodies in the blood and saliva that react with the recombinant enzyme, saliva and serum samples taken from parasite-exposed and -naïve sheep as described previously. The pooled serum and saliva samples used for ELISA were collected from 18 male Romney lambs 6-7 months-old and previously exposed in the field to multiple species of parasites and had developed immunity to *T. circumcincta* infection. *Tci*CHT (5 µg/ml) was immobilised onto ELISA plates (Maxisorp, Thermo Scientific) overnight. Free binding sites were then blocked with Superblock (Thermo Scientific, USA) and then incubated with serial dilutions (200- to 6400-fold for serum or 20- to 160-fold for saliva) in ELISA buffer for 2h at room temperature. Bound serum immunoglobulins were then detected with rabbit anti-sheep Ig-HRP (Dako, Denmark), diluted 1:5000 by incubation for 1h at 37 °C and the colour developed with 3,3',5,5' tetramethylbenzidine (AppliChem, Germany). Saliva IgA was detected with rabbit anti-sheep IgA-HRP, which was diluted, incubated and the colour developed, as described for serum Ig.

Results

***Tci*CHT gene sequence and structure**

The 912 bp full length *T. circumcincta* cDNA sequence has been

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deposited in Genbank as Accession No. KX452945. The predicted protein consisted of 314 amino acids (Figure 1). Multiple alignments, using Alignment Geneious 8, of the protein sequences of *Tci*CHT with homologues from published *T. circumcincta*, *H. contortus*, *H. placei*, A ceylanicum, *N. americanus*, *N. brasiliensis*, *O. dentatum* and *C. elegans* are shown in Figure 1.

Figure 1. Multiple sequence alignment of *Tci*CHT with homologues from *H. contortus* (GI: CDJ82138), *H. placei* (GI: VDO51030), *A. ceylanicum* (GI: EYC03522), *N. americanus* (GI: XP013294292), *N. brasiliensis* (GI: VDL62424), *O. dentatum* (GI: KHJ90958), *C. elegans* (GI: NP508588) homologues. Amino acid residues indicated in bold are essential to the enzyme activity.

The predicted 3D structure of *Tci*CHT is shown in Figure 2. It has the highest structural similarity with 5WUP which is insect group III chitinase (CAD1) from *Ostrinia furnacalis* [24]. The best model predicted by I-TASSER has a C-score 1.36 and a TM-score of 0.90 ± 0.06 which indicates high quality of the prediction. Figure 2 shows the model quality in terms of Z-score by ProSA-web. The plot shows the Z-scores of all experimentally determined protein chains in current PDB and the position of the predicted *Tci*CHT model.

Figure 2. The predicted tertiary structure of *Tci*CHT. Coiled ribbons represent alpha helix whereas flat ribbons beta sheets whereas colours represent protein subunits.

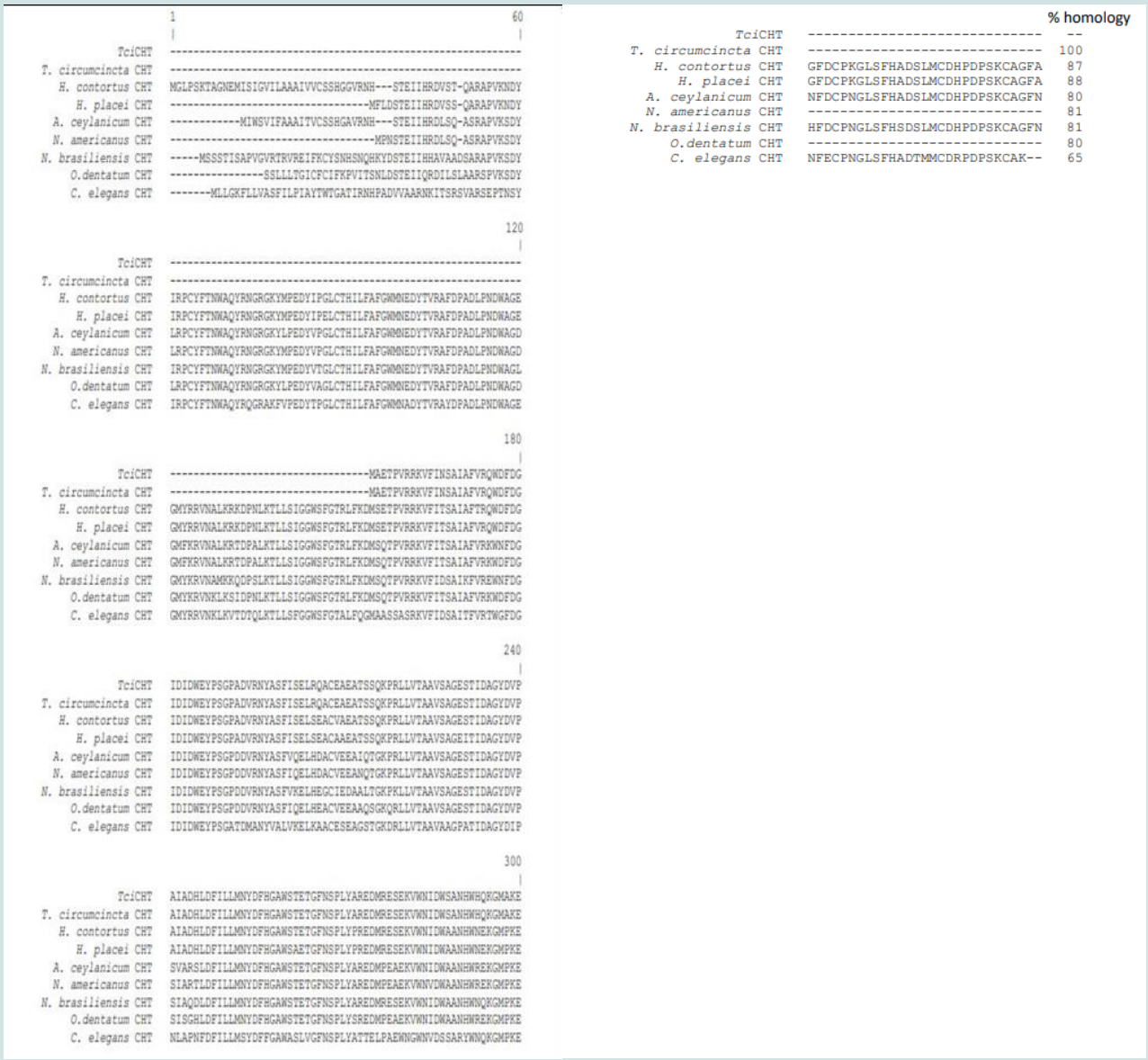

Figure 1: Multiple sequence alignment of *Tci*CHT with homologues from *H. contortus* (GI: CDJ82138), *H. placei* (GI: VDO51030), *A. ceylanicum* (GI: EYC03522), *N. americanus* (GI: XP013294292), *N. brasiliensis* (GI: VDL62424), *O. dentatum* (GI: KHJ90958), *C. elegans* (GI: NP508588) homologues. Amino acid residues indicated in bold are essential to the enzyme activity.

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Recombinant protein expression

A number of varying conditions were used in the trial expression and based on which maximal production of functional recombinant *Tci*CHT was obtained in the *E. coli* strain BL21 (DE3) when expression was induced with 0.2% L-arabinose for 3h at 37 ˚C. The recombinant *Tci*CHT was expressed as inclusion bodies and the inclusion bodies were treated using urea. The protein was dialysed in decreasing urea concentrations to facilitate the folding. Recombinant *Tci*CHT was purified as N-terminal His-tagged protein with weight of about 34 kDa (Figure 3). The presence and the purity of recombinant *Tci*CHT were confirmed by Western blotting.

*Tci*CHT assay

No enzyme activity was detected with either substrate over pH 6-10 or enzyme concentration between 50-250 µg.

Host recognition

Recombinant *Tci*CHT was recognised in by antibodies in saliva and serum samples from the parasite-exposed animals whereas that was not the case of the samples collected from parasite-naïve animals (Figure 4).

Discussion

This study showed the close relationship between a *T. circumcincta* chitinase (*Tci*CHT) to that from other helminth homologues.

Figure 2: The predicted tertiary structure of *Tci*CHT. Coiled ribbons represent alpha helix whereas flat ribbons beta sheets whereas colours represent protein subunits.

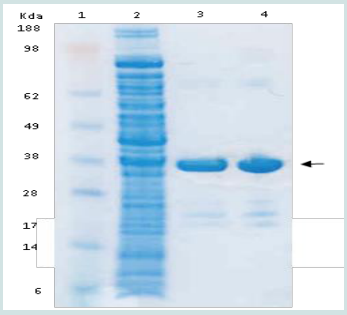

Figure 3: Purified recombinant *Tci*CHT on Bis-Tris protein gel stained with SimplyBlue safe stain. Lane 1: Standards in KDa; Lane 2: Filtered soluble lysate; Lane 3: recombinant *Tci*CHT; Lane 4: recombinant *Tci*CHT after dialysis and refolding.

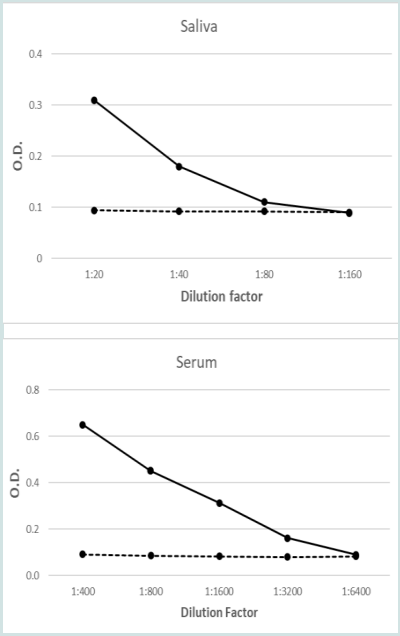

Figure 4: Recognition of *Tci*CHT by diluted parasite-exposed saliva (IgA) (top) or serum samples (IgG) (bottom) (-) but not by serum or saliva samples naïve to parasite infection (---).

*Tci*CHT gene, which encoded 912 bp was cloned, expressed in *E. coli*. Recombinant *Tci*CHT consisted of 30 4 amino acid and the protein was recognised by antibodies using ELISA in the saliva and serum from the sheep that were immune to parasites, but not nematode-naïve animals.

A 912 bp full length cDNA sequence encoding *T. circumcincta* chitinase (*Tci*CHT) was amplified from adult *T. circumcincta* cDNA, cloned and expressed in *E. coli*. The 304 amino acid *Tci*CHT protein expressed in *E. coli* was typical of CHT identified and characterized in several helminths. The *Tci*CHT protein had 87% homology to *H. contortus* homologue and 65-80% similarity to other nematode and trematode homologues (Figure 1). Our analysis showed a high similarity of the *Tci*CHT protein with CHT form other closely related species. We identified the most likely *Tci*CHT model and the analysis revealed high similarity with insect group III chitinase (CAD1) from *Ostrinia furnacalis*.

No enzyme activity was detected with either of the three substrates or varying pH or enzyme concentrations. The recombinant *Tci*CHT protein was expressed in *E. coli* as inclusion bodies and lacked biological activity. Proteins expressed as inclusion bodies may not be folded correctly and be functional enzymes [26-28]. Although, the *Tci*CHT sequence appears to be truncated with both 3' and 5' ends incomplete, his doesn't seem to be the case as both 3' and 5' RACE was performed, which failed to detect any longer sequence (Figure 1). It is interesting to note that *Tci*CHT fully matched with the sequence available in the database indicating the possibility of a shorter sequence compared to other helminth CHT sequences.

Previous studies on the potential of fungal chitinase have shown very promising results and the enzyme has captured and completely destroyed nematode eggs [29] and animals vaccinated with a

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recombinant chitinase resulted in high levels of protection [30-32]. Native *T. circumcincta* CHT is part of worm's ES products, highly antigenic and antibodies in both serum and saliva from field-immune sheep recognised recombinant *Tci*CHT in an ELISA (Figure 4). These findings are very promising and further studies will validate the protective efficacy of recombinant *Tci*CHT.

Summary

A 912 bp cDNA encoding *Teladorsagia circumcincta* chitinase was cloned and expressed in *Escherichia coli*. Multiple alignments of the protein sequence of *Tci*CHT with homologues from other helminths showed good similarity to the other helminth CHT. Recombinant *Tci*CHT was recognised by antibodies in both serum and saliva from field-immune sheep in ELISA.

Acknowledgments

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