#### Gene 569 (2015) 203-217

Contents lists available at ScienceDirect

### Gene

journal homepage: www.elsevier.com/locate/gene

### Research paper

# A glycosyl hydrolase family 16 gene is responsible for the endogenous production of $\beta$ -1,3-glucanases within decapod crustaceans



GENE

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#### ARTICLE INFO

Article history: Received 27 November 2014 Received in revised form 18 April 2015 Accepted 22 May 2015 Available online 27 May 2015

Keywords: β-1,3-glucanase Laminarinase Hemicellulase Land crab Gecarcoidea natalis Cherax destructor

#### ABSTRACT

To identify the gene responsible for the production of a  $\beta$ -1,3-glucanase (laminarinase) within crustacea, a glycosyl hydrolase family 16 (GHF16) gene was sequenced from the midgut glands of the gecarcinid land crab, Gecarcoidea natalis and the freshwater crayfish, Cherax destructor. An open reading frame of 1098 bp for G. natalis and 1095 bp for C. destructor was sequenced from cDNA. For G. natalis and C. destructor respectively, this encoded putative proteins of 365 and 364 amino acids with molecular masses of 41.4 and 41.5 kDa, mRNA for an identical GHF16 protein was also expressed in the haemolymph of C. destructor. These putative proteins contained binding and catalytic domains that are characteristic of a β-1,3-glucanase from glycosyl hydrolase family 16. The amino acid sequences of two short 8–9 amino acid residue peptides from a previously purified  $\beta$ -1,3glucanase from G. natalis matched exactly that of the putative protein sequence. This plus the molecular masses of the putative proteins matching that of the purified proteins strongly suggests that the sequences obtained encode for a catalytically active β-1,3-glucanase. A glycosyl hydrolase family 16 cDNA was also partially sequenced from the midgut glands of other amphibious (Mictyris platycheles and Paragrapsus laevis) and terrestrial decapod species (Coenobita rugosus, Coenobita perlatus, Coenobita brevimanus and Birgus latro) to confirm that the gene is widely expressed within this group. There are three possible hypothesised functions and thus evolutionary routes for the  $\beta$ -1,3-glucanase: 1) a digestive enzyme which hydrolyses  $\beta$ -1,3-glucans, 2) an enzyme which cleaves  $\beta$ -1,3-glycosidic bonds within cell walls to release cell contents or 3) an immune protein which can hydrolyse the cell walls of potentially pathogenic micro-organisms.

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#### 1. Introduction

Laminarinase or  $\beta$ -1,3-glucanase is a ubiquitous digestive hemicellulase enzyme that is present at high activities within the digestive fluid of aquatic and terrestrial crustaceans (Sova et al., 1970; Suzuki et al., 1987; Omondi and Stark, 1995; Figueiredo et al., 2001; Linton and Greenaway, 2004; Johnston and Freeman, 2005; Figueiredo and Anderson, 2009; Linton et al., 2009). It catalyses the hydrolysis of  $\beta$ -1,3-glycosidic bonds within  $\beta$ -1,3-glucans such as laminarin and callose. Laminarin is the major storage polysaccharide

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in brown algae (Bull and Chesters, 1966), diatoms (Pesentseva et al., 2008) and protozoans (Piavaux, 1977) while callose is present in the wound tissue of plants (Bacic et al., 1988; Ruiz-Herrera, 1992; Terra and Ferreira, 1994).

Within the decapods,  $\beta$ -1,3-glucanase has been purified and characterised from the midgut glands of the gecarcinid land crab, *Gecarcoidea natalis*, and the freshwater crayfish, *Cherax destructor* (Allardyce and Linton, 2008). *G. natalis* is an herbivorous species that consumes mainly leaf litter (Greenaway and Linton, 1995). It is able to digest substantial amounts of cellulose and hemicellulose (up to 50% of that consumed with a leaf litter diet) using endogenous cellulase and hemicellulase enzymes such as  $\beta$ -1,3-glucanase (Linton and Greenaway, 2007). The  $\beta$ -1,3-glucanase, which is active against laminarin, is thought to exist as a dimer of two 41 kDa subunits (Table 1). It is assumed that it is synthesised endogenously within the midgut gland since high  $\beta$ -1,3-glucanase activities are present in this tissue (Allardyce and Linton, 2008); however, the gene responsible for the endogenous production of this enzyme has yet to be identified in Crustacea. It is likely that the  $\beta$ -1,3-glucanase is the product of a glycosyl



Abbreviations:  $\beta$ -1,3-glucans, Glucose polymer in which the monosaccharides are joined by  $\beta$ -1,3-glycosidic bonds; cDNA, Complementary DNA; GAPDH, Glyceraldehyde 3 phosphate dehydrogenase; GHF16, Glycosyl hydrolase family 16; LC-MS/MS, Liquid chromatography-mass spectrometry; LGBP, Lipopolysaccharide and  $\beta$ -1,3-glucan binding protein; MS-222, Ethyl 3-aminobenzoate methanesulfonate; Nano-HPLC or UPLC, Ultra performance liquid chromatography; PCR, Polymerase chain reaction; RACE, Rapid amplification of cDNA ends.

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#### Table 1

Molecular mass and glycosyl hydrolase family of the  $\beta$ -1,3-glucanase (laminarinase) characterised in various invertebrates.

Species	Glycosyl hydrolase family	Estimated molecular mass (kDa)	Accession number	Reference
Phylum Mollusca				
Class: Gastropoda				
Haliotis discus hannai (abalone)	16	33	AB488493	Kumagai and Ojima (2009)
Haliotis tuberculata (abalone)		60		Lépagnol-Descamps et al. (1998)
Class: Bivalvia				
Spisula sachalinensis (clam)	16	38	AY308829	Kozhemyako et al. (2004)
Chlamys albidus (scallop)	16	37	DQ093347	Kovalchuk et al. (2009)
Perna viridis (mussel)	16	50	FJ623758	Zakharenko et al. (2011)
Mizuhopecten yessoensis (scallop)	16	36	AY848857	Kovalchuk et al. (2006)
Phylum: Arthropoda, Subphylum: Hexapoda, Class:	Insecta			
Tenebrio molitor (meal worm)	16	50	ACS36221	Genta et al. (2009)
Cryptopygus antarcticus (Antartic springtail)	16	29.9	EU559744	Song et al. (2010)
Spodoptera frugiperda (fall armyworm)	16	37.5	EF641300	Bragatto et al. (2010)
Phylum: Arthropoda, Subphylum: Crustacea, Class:	Malacostracta, Order: Decapod	а		
Gecarcoidea natalis		41, 71		Allardyce and Linton (2008)
Cherax destructor		41, 71		Allardyce and Linton (2008)
Phylum: Echinodermata, Class: Holothuroidea				
Stichopus japonicus	16	37.5		Zhu et al. (2008)

hydrolase family 16 (GHF16) gene given that this gene has been confirmed to produce a similar enzyme (of 30–50 kDa) in other invertebrates (Table 1). Interestingly, although a digestive  $\beta$ -1,3-glucanase gene has not yet been identified, crustaceans do possess a similar GHF16 gene that produces a lipopolysaccharide and  $\beta$ -1,3-glucan binding protein (LGBP) (Lee et al., 2000; Sritunyalucksana et al., 2002; Du et al., 2007; Lin et al., 2008; Amparyup et al., 2012). This protein is primarily expressed in the haemocytes and is believed to play an immune role. Specifically, LGBPs bind lipopolysaccharides and  $\beta$ -1,3-glucans and this, via the prophenol oxidase system, stimulates an immune response (Lee et al., 2000; Sritunyalucksana and Söderhäll, 2000; Amparyup et al., 2012).

Although the lipopolysaccharide and  $\beta$ -1,3-glucan binding proteins have quite a different role than the digestive  $\beta$ -1,3-glucanases, there are some clues to suggest that the two classes of proteins and thus their genes are similar. The crustacean GHF16 gene produces a protein with both a catalytic and a binding domain, as seen in  $\beta$ -1,3-glucanases (Lee et al., 2000; Amparyup et al., 2012) and thus is classed as a glycosyl hydrolase. It has been suggested that the catalytic domain is inactive and therefore the protein lacks activity; however, this conclusion is based on a different protein that was initially identified as LGBP. The initial protein described by Cerenius et al. (1994) consists of 1339 amino acids and has a putative molecular mass of 152 kDa. It possesses a β-glucan binding domain but no catalytic domain. In contrast, numerous subsequent studies on crustaceans have since identified LGBPs of 349-376 amino acids (approximately 36-41 kDa) with both catalytic and binding domains (Lee et al., 2000; Roux et al., 2002; Sritunyalucksana et al., 2002; Lin et al., 2008; Liu et al., 2009; Yeh et al., 2009; Zhao et al., 2009; Amparyup et al., 2012). The size discrepancy and presence of a catalytic domain in these proteins suggest that the more recently identified proteins are not, in fact, related to that described by Cerenius et al., 1994. In addition, a number of studies have detected expression of a GHF16 protein within the midgut gland of the crustaceans Eriocheir sinensis, Fenneropenaeus chinensis, Litopenaeus vannamei and Penaeus stylirostris (Gross et al., 2001; Liu et al., 2009; Zhao et al., 2009) (Roux et al., 2002). It is therefore suggested that, like the GHF16 enzymes expressed in the hepatopancreas of molluscs (Table 1), these proteins may actually possess  $\beta$ -1,3-glucanase activity.

The aim of this study was to establish if a GHF16 gene expressed in the midgut gland of decapod crustaceans was responsible for producing an active  $\beta$ -1,3-glucanase that is secreted into the digestive fluid. To confirm this, the GHF16 cDNA derived from the midgut gland of *G. natalis* and *C. destructor* was sequenced. This sequence was then matched to the characteristics of a  $\beta$ -1,3-glucanase that had been

previously purified and characterised from these species (Allardyce and Linton, 2008). To establish the link between the gene and protein, short peptides of  $\beta$ -1,3-glucanase purified from *G. natalis* were sequenced and compared to the putative amino acid sequences. The GHF16 cDNA was also partially sequenced in a range of distantly related decapod crustaceans to establish that crustaceans generally possess and express such a gene. Taken together, this evidence would explain the  $\beta$ -1,3-glucanase activity identified in the digestive fluid and midgut glands of numerous crustaceans.

#### 2. Materials and methods

# 2.1. Strategy for sequencing $\beta$ -1,3-glucanase cDNA from <u>C. destructor</u> and G. natalis

A GHF16 B-1,3-glucanase (laminarinase) cDNA was sequenced from cDNA derived from the midgut gland of the Gecarcinid land crab, G. natalis and the freshwater crayfish, C. destructor. To do this, crabs were euthanised and midgut gland, muscle and gill tissue taken. The midgut gland is a digestive tissue responsible for the production of digest enzymes. In contrast, the muscle and gills are not involved in digestion, and thus were taken as control tissues.  $\beta$ -1,3-glucanase is likely to be expressed in the midgut gland but not in the muscle and gill. Total RNA was extracted from each tissue and used to synthesise cDNA. Degenerate primers, designed from the conserved regions of  $\beta$ -1,3glucanase sequences, were used to partially amplify and sequence the  $\beta$ -1,3-glucanase (laminarinase) cDNA from both *G. natalis* and C. destructor. From these partial sequences, sequence specific primers were designed for 3' and 5' rapid amplification of cDNA ends (RACE). RACE PCR was then used to amplify and sequence the 3' and 5' ends of the  $\beta$ -1,3-glucanase cDNA. Finally, the entire open reading frame sequence was determined in three replicate animals. In addition to the nucleotide sequence, the protein sequence of a number of short fragments of the  $\beta$ -1,3-glucanase (laminarinase) purified previously from G. natalis was determined using Orbitrap mass spectrometry. These sequences were compared to the putative amino acid sequence to support the conclusion that the nucleotide sequence presented encoded for the enzyme that had been previously purified and characterised. To confirm that crustaceans more broadly possess and express a GHF16 β-1,3glucanase, the cDNA was also partially sequenced in a range of amphibious and terrestrial decapods (Mictyris platycheles, Paragrapsus laevis, Coenobita perlatus, Coenobitarugosus, Coenobita brevimanus and Birgus latro).

#### 2.2. Collection and maintenance of animals

Crayfish, *C. destructor* were purchased from a local aquarium supplier; *M. platycheles* and *P. laevis* were collected from the Barwon river estuary (Victoria, Australia); Christmas Island species (*G. natalis, C. perlatus, C. rugosus C. brevimanus*) were collected from the rainforest on Christmas Island. All species were transported back to the laboratory (Deakin laboratory for *C. destructor, M. platycheles* and *P. laevis*; Parks Australia laboratory for Christmas Island species), euthanized and their tissues taken. Tissues taken from the Christmas Island species were stored in RNA later (Ambion #AM7020) and airfreighted back to the laboratory at Deakin. For *B. latro*, tissue was taken from animals which had been recently killed by vehicles driving on the Island's roads.

#### 2.3. Extraction of tissue and total RNA

Animals were initially anaesthetised; to do this, animals were injected with the anaesthetic MS-222 (50 mg mL<sup>-1</sup> at a dose of

 $250 \text{ mg kg}^{-1}$ ) into the infrabranchial sinus, and monitored until there was no righting reflex (ability of the animals to turn themselves over). The animals were then quickly killed by the destruction of the brain and subesophageal ganglion. Midgut gland, muscle and gill tissue were quickly removed with instruments which had been treated with RNAase Zap (Ambion #AM9780). For all tissues except for the midgut gland from G. natalis and B. latro, between 100 and 250 mg of tissue was homogenised in 1-2.5 mL of TriReagent (Sigma #93289-100ML) using zirconia/silica beads (Daintree scientific #110791110z) in a FastPrep-24 homogeniser (MP biochemical #6004500). RNA was then isolated as per the manufacturer's (Sigma) protocol, reconstituted in 20-200 µL of nuclease free water, and its concentration (absorbance at 260 nm) and purity (ratio of absorbance at 260:280 nm) were determined using a NanoDrop UV-visible spectrophotometer (Nanodrop 2000c Thermoscientific). From previous experience, RNA could not be extracted from the midgut glands of G. natalis and B. latro with enough purity for cDNA synthesis using TriReagent. For this reason, RNA from the midgut gland of these species was extracted using an ISOLATE II RNA mini kit (Bioline #BIO-52071). cDNA from all species was



**Fig. 1.** Alignment of the putative amino acid sequences for β-1,3-glucanases from two molluscs (the abalone, *Haliotis discus* (H\_disc) (accession number BAH84971) and the scallop, *Mizuhopecten yessoensis* (M\_yess) (accession number AAW34372)) and three insects (the fall armyworm, *Spodoptera frugiperda* (S\_frugi) (accession number ABR28478), the American cockroach, *Periplaneta americana* (P\_ameri) (accession number ABR28480) and the orange sulphur butterfly, *Colias eurytheme* (C\_eury) (accession number ACI32831)) that were used to construct degenerate primers. Total conservation is highlighted in black while partial conservation is highlighted in light grey. Sequences used to create the degenerate primers are indicated by blue and red boxes. Forward and reverse arrows and letter and number codes indicate the sequences from which the forward and reverse degenerate primers were constructed.

synthesised from 2 µg of RNA using a Biorad iScript reverse transcription supermix for RT-q PCR (Biorad #170-8840). It was then used in subsequent PCR reactions.

#### 2.4. Amplification of an internal region of the $\beta$ -1,3-glucanase gene

An internal region of the GHF16 gene from G. natalis and C. destructor was initially amplified using degenerate primers and PCR. To design the degenerate primers, the amino acid sequences of GHF16 B-1,3glucanases from two molluscs (the scallop, Mizuhopecten yessoensis, accession number AAW34372 and the abalone Haliotis discus hannai, accession number BAH84971) and three insects (the fall armyworm Spodoptera frugiperda, accession number ABR28478, the American cockroach Periplaneta americana, accession number ABR28480 and the orange the sulphur butterfly Colias eurytheme, accession number ACI32831) were aligned using Clustal Omega (http://www.clustal.org/) (Fig. 1). Degenerate primers were then designed from highly conserved regions of the aligned sequences using the iCODEHOP website (http:// dbmi-icode-01.dbmi.pitt.edu/i-codehop-context/) to reduce their degeneracy (Fig. 1) (Rose et al., 2003). Different combinations of forward and reverse primers were trialled in the PCR reactions (Tables 2, 3). All primers were synthesised by Geneworks Pty Ltd (www.geneworks. com.au).

#### 2.5. PCR reactions

Using the degenerate primers and the polymerase chain reaction, PCR products of fragments of the GHF16 cDNA were amplified (Figs. S1-S3) (Table 3). PCR reactions were performed in a MJ-Research thermocycler using GoTaq Green master mix, primers and cDNA. Each 20  $\mu$ L PCR reaction consisted of 1  $\mu$ L of cDNA, 0.5  $\mu$ L of each 100  $\mu$ M forward and reverse primers (final concentration of primers = 2.5  $\mu$ M), 10  $\mu$ L of GoTaq Green two times master mix (Promega #M7122) and 8  $\mu$ L of nuclease free water. The PCR reaction mixture was initially subjected to 92 °C for 5 min to melt the DNA and primers, before being cycled 40 times through the following; 92 °C for 1-min (melting), 45 °C for 2 min (annealing) and 72 °C for 2 min (extension). The reactions were held at 72 °C for 5 min for a final extension of products, and then maintained at 18 °C and stored at -20 °C.

Following PCR, a 10  $\mu$ L aliquot of the reaction solution was electrophoresed at 100 V for 35 min in a 1% agarose gel containing TBE buffer (89 mM tris borate pH8.3, 2 mM EDTA). Products were visualised under UV light with SYBR safe gel stain (Life Technologies #S33102), which had been incorporated into the gel as per the manufacturer's instructions. The size of the products was determined from their migration distance against that of DNA standards (either E-Gel 1 Kb Plus DNA ladder (Life Technologies #10488-090) or E-gel low range quantitative DNA ladder (Life technologies #12373-031)). PCR products of

#### Table 2

Sense and antisense degenerate primers that were used in various combinations to amplify the parts of the laminarinase cDNA by PCR. Primers were from the output of the iCODEHOP website following the alignment of  $\beta$ -1,3-glucanase (laminarinase) sequences (AAW34372, BAH84971, ABR28478, ABR28480, ACI32831) (Rose et al., 2003).

Primer name	DNA sequence of the primers
Sense primers	
Lam G1F	AGGGCGACTGGATCTGGCCNGCNAT
Lam H3F	CTGGCCCGGCTCNGGNGARATHG
Lam C1 F	GAGATCACCTGCTGGGGNGGNGGNAA
Lam A49F	GCGGCGGCAACTGGGARTTYCA
Antisense primers	
Lam L4R	TGATGAAGTAGAACTTCTGGTCRAANGGNGC
Lam N1R	GCCACTGGTGCCTGSNNTYCCARAA
Lam H4R	CCTGGACTCCATGATGTCDATYTCNCC
Lam A106R	TCAGCACGATGTAGAACTTYTGRTCRAA
Lam B14R	TTGTCGCGGCCGTTCCARAARTC

#### Table 3

Primer combinations which amplified GHF16 PCR products and the number of nucleotide base pairs successfully sequenced from these PCR products. PCR products were amplified using cDNA derived from the midgut gland of *G. natalis* (a) and *C. destructor* (b) and the haemolymph of *C. destructor* (c).

Primer pair	Nucleotide base pairs sequenced
(a) Gecarcoidea natalis (midgut gland)	
C1F, H4R	372 bp
H3F, L4R	340 bp
GN Lam spec F1, GN Lam spec R1	447 bp
3' RACE	695 bp
5' RACE	351 bp
(b) Cherax destructor (midgut gland)	
C1F, H4R	268 bp
G1F, L4R	358 bp
H3F, L4R	308 bp
H3F, N1R	434 bp
A49F, B14R	713 bp
3' RACE	630 bp
5' RACE	469 bp
(c) Cherax destructor (haemolymph)	
A49F, B14R	771 bp
C1F, L4R	718 bp
G1F, L4R	395 bp
H3F, L4R	335 bp
CD Lam 5' S1, CD lam spec R2	493 bp
5' RACE	354 bp

interest were excised from the gel, purified, using a Nucleospin extract II kit (Macherey-Nagel #740 609.50) and directly sequenced using a BigDye Terminator v3.1 cycle sequencing kit (Life Technologies #4337454) and an Applied Biosystems ABI 3730 capillary sequencer as per the manufacturer's protocol.

For each species, the overlapping nucleotides of the partial sequences were aligned using Clustal Omega (www.clustal.org) and assembled to elucidate the majority of the sequence. Finally 3' and 5' rapid amplification of cDNA ends (RACE) was used to sequence the respective ends of the cDNA (Fig. 2). As per the manufacturer's protocol (Takara), sequence specific primers were designed from the partial sequences obtained from the initial PCR reactions (Table 4). For 3' RACE reactions, sense sequence specific primers between 19-25 nucleotides long, with a melting temperature between 50-60 °C, and a GC content between 40-60% were designed (Table 4). 5' RACE reactions required an antisense sequence specific primer (10–14 nucleotides long, melting temperature between 30–40 °C and a GC content between 45–55%) with a 5' phosphorylated primer end and two sets of nested sequence specific PCR primers with the same specifications as the 3' RACE sequence specific primer (Table 4). A Takara 3'-Full RACE core set (Cat #6121) and a 5'-full RACE core set kits (Takara #6122) were used for these reactions. PCR and sequencing protocols used in the RACE reactions were as described above. The open reading frame of the sequence was determined by translating the sequence into the putative amino acid sequence using Gene Runner ver3.05. SMART domain analysis (http://smart.embl-heidelberg.de/) was used to classify the putative proteins into one of the glycosyl hydrolase families. Signal P3.0 programme (http://www.cbs.dtu.dk/services/SignalP/) was used to determine a potential hydrophobic signal sequence, and the ExPasy ProtParam tool (http://web.expasy.org/protparam/) used to estimate the molecular masses of the putative proteins. The tertiary structure of the protein was predicted using homology modelling of the putative amino acid sequences (Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2/)).

2.6. Partial sequencing of a GHF16 protein from the midgut glands of other decapod species

Partial sequences of GHF16 cDNA were also obtained for other amphibious (*M. platycheles* and *P. laevis*) and terrestrial decapods (*C. perlatus, C. rugosus, C. brevimanus* and *B. latro*). To do this, a fragment

**a)** <u>Gecarcoidea natalis</u> nucleotide and putative amino acid sequence of a GHF16  $\beta$ -1,3-glucanase derived from the midgut gland.

- 1 M K V L W L L M L A S G A L A A D I V D P S S C T A F P C L A TGA A GGT GCT GT GGCT GCCT TGCCT CGCGGGCCCT GGCT GCCGA CA TA GT GGA CCCA A GT TCCT GCA CCGCCT TCCCGT GCCT C
- 91 I F N D E F D H L D H E V W E H E I T M S G G G N W E F 0 Ά ATCTTTAACGACGAGTTCGACCACCTGGATCATGAAGTCTGGGAGCATGAGATCACCATGTCGGGAGGCGGGAACTGGGAGTTCCAAGCG C1F
- Y L N N R S V S Y T R D S T L F I K P Q L M S D W K D E G F TACCTCAACAACAGGAGTGTGAGCTACACCCGTGACTCGACCCTCTTCATCAAGCCGCAACTCATGTCTGACTGGAAGGACGAGGGGGTTC GN Lam spec F1
- . ЕТ. ИТ. W. G. M. N. G. R. G. D. V. C. T. G. N. S. Y. G. C. D. R. V. G. 271 т Ġ
- ATNLVNPIMSARL**R**TLNDFAFRYGRIEVR 361 T ACCGCCAACCACCTCGTCAACCCCATCATGAGTGCCAGGCTTAGGACCTCCAACGACTTCGCCTTCAGATACGGCCGCATCGAGGTCCGT
- 451
- R G N D D Y G S L S N A V A G S T M H W G P F 541 S To7 ATCGACATCGTGGAGTCCAGGGGCAACGATGACTATGGTTCCTCTGAGCAACGCAGTGGCCGGTTCCACCATGCACTGGGGACCTTTCTGG
- H3F, H4R N F Y D M T A V E Y N A N S G S F A D D F H V W R V D W P L 631 CCACTCAACTTCTATGACATGACCGCCGTCGAGTACAACGCCAACTCCGGCTCCTTCGCTGACGACTTCCATGTCTGGCGCGCTCGACTGG
- 721
- 811 AACATCTACGACAACCCGTGGGCCTCCGGGGACAAGCTGGCTCCCTTCGACCAGAAGTTCTACATCGTCCTGAACGTCGCTGTGGGCGGC CN Lam 3' RACE F2 1 T N G F F P D G I V S N K P W A N T S P Q A F L D F W N A 1 T N G F F P D G I V S N K P W A N T S P Q A F L D F W N A
- 901 R ACCAACGGCTTCTTCCCTGACGGCATCGTCTCCAACAAGCCCTGGGCCAACACCTCCCCCTCAGGCCTTCCTCGACTTCTGGAACGCACGT
- 991 D S W L P T W E Q G E G K I S E N A A L Q V D Y V K V W K L  ${\tt GACAGCTGGCTGCCTACGTGGGAGCAGGGTGAGGGCAAGATCAGCGAGAACGCCGCCTTGCAGGTGGATTACGTGAAGGTGTGGAAGCTG$

1081 V S A D E -GTGAGCGCGGACGAGTGA

#### **b**) <u>Cherax destructor</u> nucleotide and putative amino acid sequence of a GHF16 $\beta$ -1,3-glucanase derived from the midgut gland.

- 1
- 91 I F N D D F D Y F D H D V W E H E V T M S G G G N W E F O V ATCTTCAACGATGACTTCGATTACTTCGACCACGATGTGTGGGAACACGAGGTCACCATGTCGGGTGGTGGGAACTGGGAATTCCAGGTC
- C1F
   C1F

   181 Y L N N R S I S Y T R D S T L F I K P D L T S N W
   A49F O T E G F TATCTAAACAACCGGTCTATCAGCTATACCCGAGACTCAACACTCTTCATCAAACCGGACCTGACGTCGAACTGGCAGACTGAAGGCTTC
- CD Lam 5' RACE A2 271 L S S G N L N L W G M N G R G D V C T G N S Y Y G C E R T G  ${\tt CTATCTAGCGGAAAATCTAACTTGTGGGGGAATGAATGGACGTGGGAGACGTGTGTACTGGTAACTCTTACTATGGCTGTGAACGTACAGGC$
- 361 N P V N I I N P V M S A R L R T L S D F A F R Y G R I E V R AACCCTGTTAACATTATCAACCCCGTCATGAGTGCGAGAGCCCAGGACCCCTCTCGGTTTCGCCTTCAGGTACGGTCGTATTGAGGTCGT
- 451
- H3F G P N W GIF S R G N D N Y G N L G N Q Y G G T T V H W G 541 ATCGACATTGTGGAGTCCAGGGGTAATGACAACTATGGCAACCTGGGCAACCAGTATGGAGGCACCACTGTCCACTGGGGGACCTAACTGG H4R
- . MYEKTHSDYTASDGSFANSFHTWRLDW 631 O K N CAGAAGAATATGTACGAGAAGACGCACTCTGACTATACTGCCAGTGATGGATCCTTCGCTAACAGTTTCCACACCTGGAGACTAGATTGG
- 721 TKDNMLFYLDDQLQLTVDPGTNFWDFGG ACCAAGGACAATATGTTGTTCTACCTGGATGACCAACTTCAGCTGACAGTGGACCCAGGCACCAATTTCTGGGACTTCGGTGGATTTGGA
- CD Lam 3' RACE F2 811 NELDNPWKAGSKMAPFDQKFYVV L4R
- 901 VNGFFPDGITDKPWSNVSPQASLDFWNGRG
- B14R, N1R SWLPTWEQGEGRISENAALQVDYVKVWKME 991 TCATGGCTGCCCACTTGGGAGCAAGGCGAGGGCCGCATCAGTGAGAATGCAGCTCTCCAGGTGGACTATGTGAAGGTCTGGAAGATGGAG

1081 S V D Q -AGTGTCGACCAGTAG **c)** <u>Cherax destructor</u> nucleotide and putative amino acid sequence of a GHF16  $\beta$ -1,3-glucanase protein derived from the haemolymph

1	М	R	Т	L	С	L	L	L	L	A	С	G	A	F	A	A	D	L	V	Е	Ρ	Е	D	С	Т	G	F	Ρ	С	L
	ATG	AGG	GACA	CTG	TGC	TTA	CTG	TTG	CTT	GCC	TGT	GGG	GCT	TTT	GCC	GCC	CGAC	CTG	GTG	GAG	CCG	GAP	GAC	TGI	ACA	.GGG	TTC	ccc	TGC	CTC
91	I	F	N NAC	D	D	F	D	Y	F	D	H	D	V	W	Е	H	E	V CTTC	T	M	S	G	G	G	N N	W	E	F	Q	V
	AIC	110	AAC	GAI	GAC	110	GAI	IAC	110	GAC	CAC	GAI	910	199	GAA	CAC	GAG	1910	ACC	AIG	100	661	.661	GGGG		A49F	GAA	110		GIC
181	Y	L	Ν	Ν	R	S	Ι	S	Y	Т	R	D	S	Т	L	F	I	K	Ρ	D	L	т	S	Ν	W	Q	Т	Е	G	F
	TAT	CTA	AAC	AAC	CGG	TCI	ATC	AGC	TAT	ACC	CGA	GAC	TCA	ACA	CTC	TTC	CATC	AAA	1CCG	GAC	CTG	ACG	STCG	AAC	TGG	CAG	ACT	GAA	GGC	TTC
														CD	Lam	5' R,	ACE /	42												
271	L	S	S	G	N	L	N	L	W	G	M	N	G	R	G	D	V	С	Т	G	N	S	Y	Y	G	С	Е	R	T	G
CINICINGCGGUNUCCICUUCIIGIGGGGGUIGUNIGGUCGIGGGGGGGGGIGIGIUCIGGIUNCICIIHCIHCIGIGGGGGGGGAGGGGGGGGGGGGGGGGGG																														
361	Ν	Ρ	V	Ν	Ι	I	Ν	Ρ	V	М	S	A	R	L	R	Т	L	S	D	F	A	F	R	Y	G	R	Ι	Е	V	R
	AAC	CCI	GTI	AAC	ATT	ATC	AAC	ccc	GTC	ATG	AGT	GCG	AGA	CTC	AGG	ACC	CCTC	TCI	'GAT	TTC	GCC	TTC	CAGG	TAC	GGT	CGT	ATT	GAG	GTT	CGT
										_															•	CD La	ım 5'	S1		-
451	A	K	М	P	R	G	D	W	L	M The co	P	A	I	W	L	L	P	R	Y	W	P	Y	G	L	M MCC	P	A	S	G	
	► GCG	AAC	AIG	reeg	CGA	GGA	GAU	166	61	E	ULA	GCI	AIC	IGG	CII	CIC		AGG	JAL	TGG	ICCC	IAI	GGI	CII	IGG		GCC H	AGI	GGI	GAG
541	Ι	D	Ι	V	E	S	R	G	N	D	Ν	Y	G	Ν	L	G	Ν	Q	Y	G	G	т	Т	V	H	W	G	P	Ν	W
	ATC	GAÇ	ATT	GTG	GAG	TCC	AGG	GGT	AAT	GAC	AAC	TAT	GGC	AAC	CTG	GGC	CAAC	CAG	TAT	GGA	GGC	ACC	CACT	GTC	CAC	TGG	GGA	CCT	AAC	TGG
	-																													
631	Q	K	N	М	Y	E	K	T	Н	S	D	Y	T	A	S	D	G	S	F	A	N	S	F	H	T	W	R	L	D	W
	CAG	AAG	AA1	ATG	TAC	GAG	AAG	ACG	CAC	TCT	GAC	TAT	ACT	GCC	AGT	GAI	GGA	TCC	TTC	GCT	AAC	AG1	. TTTC	CAU	ACA	TGG	AGA	CTA	GAT	TGG
721	Т	K	D	Ν	М	L	F	Y	L	D	D	0	L	0	L	т	V	D	Ρ	G	Т	N	F	W	D	F	G	G	F	G
	ACC	AAG	GAC	AAT	ATG	TTG	TTC	TAC	CTG	GAT	GAC	CAA	CTT	CAG	CTG	ACP	AGTG	GAC	CCA	GGC	ACC	AAI	TTC	TGG	GAC	TTC	GGT	GGA	TTT	GGA
811	N	E	L	D	N	P	W	K	A	G	S	K	M	A	P	F	D	Q	K	F	Y	V	V	L	N	V	A	V	G	G
	AAT	GAA	CTG	GAC	AAC	CCC	TGG	AAA	GCT	GGC	TCA	AAG	ATG	GCT	CCA	TTC	GAC	CAG	AAG	TTC	TAC	GTG	GTG	TTG	AAC	GTG	GCT	GTG	GGT	GGC
901	V	N	G	F	F	Ρ	D	G	I	Т	D	K	Ρ	W	S	Ν	V	L4R S	Ρ	0	A	S	L	D	F	W	Ν	G	R	G
	GTT	AAT	GGC	TTC	TTC	CCA	GAT	GGT	ATT	ACA	.GAC	AAG	ccc	TGG	AGT	AAT	GTC	TCT	CCC	CAA	.GCA	TCC	CTG	GAT	TTC	TGG	AAT	GGG	CGT	GGC
																								-		B	14R			-
991	S	W	L	Р	Т	W	E	Q	G	E	G	R	I	S	Е	N	А	Α	L	Q	V	D	Y	V	K	V	W	K	М	Е
	TCA	TGG	CTG	CCC	ACT	TGG	GAG	CAA	.GGC	GAG	GGC	CGC	ATC	AGT	GAG	AA'I	'GCA	GC1	'C'I'C	CAG	GTG	GAC	CTAC	GTG	AAG	GTC	TGG	AAG	A'I'G	GAG
1081	S	v	D	0	_																									
	AGT	GTC	GAC	CAA	TAG																G	GTT	GAC	TAT	CTG	GGT	GAAG	GG		
																					-	_	С	D Lar	n spe	c R2	-			

**Fig. 2.** Nucleotide and putative amino acid sequences of the  $\beta$ -1,3-glucanase cDNA derived from the midgut gland of the gecarcinid land crab, *Gecarcoidea natalis* (accession KJ995764) (a) and the midgut gland (b) (accession KJ995763) and haemolymph (c) (accession KJ995766) of the freshwater crayfish, *Cherax destructor*. The signal peptide is highlighted in yellow; amino acids that are critical to catalysis are highlighted in red; amino acids involved in modifying the pKa of the catalytic amino acids are highlighted in blue; while tryptophan amino acids involved in binding are highlighted in green. Bases highlighted in grey represent the sites of the PCR primers. Arrows and codes represent the forward and reverse primers that were used to generate the nucleotide sequences.

of the GHF16 cDNA derived from the midgut gland was amplified using degenerate primers and PCR (Fig. S3). The PCR product was excised from the gel, isolated and sequenced as described above.

#### 2.7. Expression of GHF16 $\beta$ -1,3-glucanase mRNA within various tissues

Expression of GHF16 mRNA within midgut gland, muscle and gill, was determined by amplifying a  $\beta$ -1,3-glucanase fragment using cDNA derived from these tissues, degenerate primer pairs (C1F H4R for *G. natalis* and H3F L4R *C. destructor*) and PCR. Fragments of either  $\beta$ -actin or GAPDH were also amplified from cDNA derived from each of the tissues. This served as a positive control to ensure that good quality cDNA was used in the reactions. RNA samples did not contain genomic DNA given that control PCR reactions using isolated total RNA in control reverse transcriptase reactions did not yield any PCR products for  $\beta$ -1,3-glucanase fragments.

#### 2.8. Expression of a GHF16 protein within the haemocytes of C. destructor

To determine if a similar GHF16 gene is expressed in the haemocytes of *C. destructor*, the open reading frame of a GHF16 protein was

sequenced using cDNA derived from the haemolymph. To do this, 50–100 µL haemolymph samples from 10 animals were collected and pooled into 1 mL of RNA later. This precipitated the protein and cells, which were then collected by centrifugation (12,000 g for 15 min). After centrifugation, the RNA later was carefully aspirated off and the RNA isolated from the pelleted material using ISOLATE II RNA mini kit (Bioline #BIO-52071). cDNA was synthesised using Biorad iScript reverse transcription supermix for RT-q PCR (Biorad #170-8840) and fragments of the GHF16 protein amplified using degenerate primer pairs, sequence specific primers, PCR and 5' RACE as described above. PCR products were directly sequenced and the sequences assembled as described above (Fig. S2).

#### 2.9. Can the putative protein be translated into a digestive enzyme?

Sequences of short peptides were determined from a  $\beta$ -1,3-glucanase purified previously from the midgut gland of *G. natalis* (Allardyce and Linton, 2008). These sequences were compared to the same sequences from the putative amino acid sequence to indicate if the putative amino sequence derived from the cDNA could potentially match that of a previously purified  $\beta$ -1,3-glucanase. The probability of

the two sequences possessing the same amino acids by chance was calculated from the chance of randomly selecting the correct amino acid out of 20 for each of amino acid residues in the peptide that matched

#### Table 4

Sequence specific primers used for 3' and 5' RACE reactions, for checking the sequences and for positive control PCR reactions for *G. natalis* (a), *C. destructor* (b) and *B. latro* (c). For primers used in the nested PCR reactions of the 5' RACE, the antisense primers pointed towards the 5' end while the sense primers pointed towards the 3' end, as per the 5' RACE kit instructions. GAPDH primers were as described by Tsang et al. (2011). These primers were also used as positive controls for all amphibious and terrestrial decapod species.  $\beta$ actin primers were used as positive controls for *Cherax destructor*.

(a) Gecarcoidea natalis	
Primer name	DNA sequence of the primers
3' RACE reactions	
Sense primers	
GN lam 3' F1	CGACGAGCTGAAGATGACC
GN Iam 3' F2	CGGGCGTGGACAACATCTAC
5' RACE reactions	
Phosphorylated sequence specific primer	
GN lam 5' RT-P	GCTTGTTGGAGACG (Primer
Antionno muimono	phosphorylated at the 5' end)
Antisense primers	TACCACCCCACCCTCCATAC
CN Lam 5/ A2	
Sense primers	Additioedditienened
GN Lam 5' S1	CGACGAGCTGAAGATGACC
GN Lam 5' S2	CGGGCGTGGACAACATCTAC
Sequence specific primers	
Sense primers	CCAACCCTACCTCAACAACAC
Anticonso primors	CLAAGEGTACCICAACAACAG
CN Lam spec R1	ACAACTTCACTCCCCACAAAC
Giv Lain spee Ki	Normananananananana
Positive control primers for GAPDH (Tsang et a	ıl., 2011)
Sense primers	
GAPDH GA	ATGGTGTATATGTTCAAGTAYGAYTC
Antisense primers	TCCCTACATACAACATCATCATC
GAPDH GK	ICGCIAGAIACAACAICAICYICRGI
(b) Cherax destructor	
D.'	
Primer name	DNA sequence of the primers
3' RACE reactions	DNA sequence of the primers
3' RACE reactions Sense primers	DNA sequence of the primers
3' <i>RACE reactions</i> Sense primers CD Lam 3' F2	DNA sequence of the primers GGGACTTCGGTGGATTTGG
3' RACE reactions Sense primers CD Lam 3' F2 5' RACE reactions	DNA sequence of the primers
Yrimer name 3' RACE reactions Sense primers CD Lam 3' F2 5' RACE reactions Phosphorylated sequence specific primer	DNA sequence of the primers
3' RACE reactions         Sense primers         CD Lam 3' F2         5' RACE reactions         Phosphorylated sequence specific primer         CD Lam 5' RT-P	DNA sequence of the primers GGGACTTCGGTGGATTTGG CAAATCCACCGAAG (Primer
<i>3' RACE reactions</i> Sense primers CD Lam 3' F2 <i>5' RACE reactions</i> Phosphorylated sequence specific primer CD Lam 5' RT-P	DNA sequence of the primers GGGACTTCGGTGGATTTGG CAAATCCACCGAAG (Primer phosphorylated at the 5' end)
Yrimer name 3' RACE reactions Sense primers CD Lam 3' F2 5' RACE reactions Phosphorylated sequence specific primer CD Lam 5' RT-P Antisense primers	DNA sequence of the primers GGGACTTCGGTGGATTTGG CAAATCCACCGAAG (Primer phosphorylated at the 5' end)
Yrimer name 3' RACE reactions Sense primers CD Lam 3' F2 5' RACE reactions Phosphorylated sequence specific primer CD Lam 5' RT-P Antisense primers CD Lam 5' A1	DNA sequence of the primers GGGACTTCGGTGGATTTGG CAAATCCACCGAAG (Primer phosphorylated at the 5' end) GGCGAAATCAGAGAGGGGTC
<i>Yrimer name</i> <i>3' RACE reactions</i> Sense primers CD Lam 3' F2 <i>5' RACE reactions</i> Phosphorylated sequence specific primer CD Lam 5' RT-P Antisense primers CD Lam 5' A1 CD Lam 5' A2	DNA sequence of the primers GGGACTTCGGTGGATTTGG CAAATCCACCGAAG (Primer phosphorylated at the 5' end) GGCGAAATCAGAGAGGGTC CGGTTTGATGAAGAGTGTTGAG
<i>Y T T T T T T T T T T</i>	DNA sequence of the primers GGGACTTCGGTGGATTTGG CAAATCCACCGAAG (Primer phosphorylated at the 5' end) GGCGAAATCAGAGAGGGTC CGGTTTGATGAAGAGGGTTGAG
<i>Y</i> rimer name <i>3' RACE reactions</i> Sense primers CD Lam 3' F2 <i>5' RACE reactions</i> Phosphorylated sequence specific primer CD Lam 5' RT-P Antisense primers CD Lam 5' A1 CD Lam 5' A2 Sense primers CD Lam 5' S1	DNA sequence of the primers GGGACTTCGGTGGATTTGG CAAATCCACCGAAG (Primer phosphorylated at the 5' end) GGCGAAATCAGAGAGGGTC CGGTTTGATGAAGAGGGTTCGAG CGGTCGTATTGAGGTTCGTG
<i>Y</i> rimer name <i>3' RACE reactions</i> Sense primers CD Lam 3' F2 <i>5' RACE reactions</i> Phosphorylated sequence specific primer CD Lam 5' RT-P Antisense primers CD Lam 5' A1 CD Lam 5' A2 Sense primers CD Lam 5' S1 CD Lam 5' S2	DNA sequence of the primers GGGACTTCGGTGGATTTGG CAAATCCACCGAAG (Primer phosphorylated at the 5' end) GGCGAAATCAGAGAGGGTC CGGTTTGATGAAGAGGTTCGAG CGGTCGTATTGAGGTTCGTG TGACAACTATGGCAACCTGG
<i>Y</i> rimer name <i>3' RACE reactions</i> Sense primers CD Lam 3' F2 <i>5' RACE reactions</i> Phosphorylated sequence specific primer CD Lam 5' RT-P Antisense primers CD Lam 5' A1 CD Lam 5' A2 Sense primers CD Lam 5' S1 CD Lam 5' S2 Sequence specific primers	DNA sequence of the primers GGGACTTCGGTGGATTTGG CAAATCCACCGAAG (Primer phosphorylated at the 5' end) GGCGAAATCAGAGAGGGTC CGGTTTGATGAAGAGGGTTGAG CGGTCGTATTGAGGTTCGTG TGACAACTATGGCAACCTGG
Primer name         3' RACE reactions         Sense primers         CD Lam 3' F2         5' RACE reactions         Phosphorylated sequence specific primer         CD Lam 5' RT-P         Antisense primers         CD Lam 5' A1         CD Lam 5' A2         Sense primers         CD Lam 5' S1         CD Lam 5' S2         Sequence specific primers         Antisense primers	DNA sequence of the primers GGGACTTCGGTGGATTTGG CAAATCCACCGAAG (Primer phosphorylated at the 5' end) GGCGAAATCAGAGAGGGTC CGGTTTGATGAAGAGTGTTGAG CGGTCGTATTGAGGTTCGTG TGACAACTATGGCAACCTGG
Primer name         3' RACE reactions         Sense primers         CD Lam 3' F2         5' RACE reactions         Phosphorylated sequence specific primer         CD Lam 5' RT-P         Antisense primers         CD Lam 5' A1         CD Lam 5' A2         Sense primers         CD Lam 5' S1         CD Lam 5' S2         Sequence specific primers         Antisense primers         CD Lam 5' S2         Sequence specific primers         Antisense primers         CD Lam 5' S2         Sequence specific primers         Antisense primers         CD lam spec R2	DNA sequence of the primers GGGACTTCGGTGGATTTGG CAAATCCACCGAAG (Primer phosphorylated at the 5' end) GGCGAAATCAGAGAGGGTC CGGTTTGATGAAGAGTGTTGAG CGGTCGTATTGAGGTTCGTG TGACAACTATGGCAACCTGG CCTTCACCCAGATAGTCAACC
Primer name         3' RACE reactions         Sense primers         CD Lam 3' F2         5' RACE reactions         Phosphorylated sequence specific primer         CD Lam 5' RT-P         Antisense primers         CD Lam 5' A1         CD Lam 5' A1         CD Lam 5' A2         Sense primers         CD Lam 5' S1         CD Lam 5' S2         Sequence specific primers         Antisense primers         CD Lam 5' S2         Sequence specific primers         Antisense primers         CD Lam 5' S2         Sequence specific primers         Antisense primers         CD lam spec R2	DNA sequence of the primers GGGACTTCGGTGGATTTGG CAAATCCACCGAAG (Primer phosphorylated at the 5' end) GGCGAAATCAGAGAGGGTC CGGTTTGATGAAGAGTGTTGAG CGGTCGTATTGAGGTTCGTG TGACAACTATGGCAACCTGG CCTTCACCCAGATAGTCAACC
Primer name         3' RACE reactions         Sense primers         CD Lam 3' F2         5' RACE reactions         Phosphorylated sequence specific primer         CD Lam 5' RT-P         Antisense primers         CD Lam 5' A1         CD Lam 5' A2         Sense primers         CD Lam 5' S1         CD Lam 5' S2         Sequence specific primers         Antisense primers         CD Lam 5' S1         CD Lam 5' S2         Sequence specific primers         Antisense primers         CD Lam 5' S2         Sequence specific primers         Antisense primers         CD lam 5' S2         Sequence specific primers         Antisense primers         CD lam 5' S2	DNA sequence of the primers GGGACTTCGGTGGATTTGG CAAATCCACCGAAG (Primer phosphorylated at the 5' end) GGCGAAATCAGAGAGGGTC CGGTTTGATGAAGAGGGTCGTGAG CGGTCGTATTGAGGTTCGTG TGACAACTATGGCAACCTGG CCTTCACCCAGATAGTCAACC
Primer name         3' RACE reactions         Sense primers         CD Lam 3' F2         5' RACE reactions         Phosphorylated sequence specific primer         CD Lam 5' RT-P         Antisense primers         CD Lam 5' A1         CD Lam 5' A2         Sense primers         CD Lam 5' S1         CD Lam 5' S2         Sequence specific primers         Antisense primers         CD Lam 5' S2         Sequence specific primers         Antisense primers         CD lam 5' S2         Sequence specific primers         Antisense primers         CD lam 5' S2         Sequence specific primers         Antisense primers         CD lam 5' S2         Sequence specific primers         Antisense primers         CD lam spec R2         Positive control primers for β-actin         Sense primers         Θ-actin F	DNA sequence of the primers         GGGACTTCGGTGGATTTGG         CAAATCCACCGAAG (Primer phosphorylated at the 5' end)         GGCGAAATCAGAGAGGGTC CGGTTTGATGAAGAGGGTCTGGGTTTGATGAAGAGTGTTGAG         CGGTCGTATTGAGGTTCGTG TGACAACTATGGCAACCTGG         CCTTCACCCAGATAGTCAACC         GGCTACTCCTTCACCACCAC
Primer name 3' RACE reactions Sense primers CD Lam 3' F2 5' RACE reactions Phosphorylated sequence specific primer CD Lam 5' RT-P Antisense primers CD Lam 5' A1 CD Lam 5' A2 Sense primers CD Lam 5' S1 CD Lam 5' S2 Sequence specific primers Antisense primers CD lam spec R2 Positive control primers for $\beta$ -actin Sense primers $\beta$ -actin F Antisense primers	DNA sequence of the primers         GGGACTTCGGTGGATTTGG         CAAATCCACCGAAG (Primer phosphorylated at the 5' end)         GGCGAAATCAGAGAGGGTC CGGTTTGATGAAGAGTGTTGAG         CGGTCGTATTGAGGTTCGTG TGACAACCTGG         CCTTCACCCAGATAGTCAACC         GGCTACTCCTTCACCACCAC
Primer name 3' RACE reactions Sense primers CD Lam 3' F2 5' RACE reactions Phosphorylated sequence specific primer CD Lam 5' RT-P Antisense primers CD Lam 5' A1 CD Lam 5' A2 Sense primers CD Lam 5' S1 CD Lam 5' S2 Sequence specific primers Antisense primers CD lam spec R2 Positive control primers for $\beta$ -actin Sense primers $\beta$ -actin F Antisense primers $\beta$ -actin R	DNA sequence of the primers         GGGACTTCGGTGGATTTGG         CAAATCCACCGAAG (Primer         phosphorylated at the 5' end)         GGCGAAATCAGAGAGAGGGTC         CGGTTTGATGAAGAGGGTC         CGGTCGTATTGAGGTTCGTG         TGACAACTATGGCAACCTGG         CCTTCACCCAGATAGTCAACC         GGCTACTCCTTCACCACCAC         AGCTGTGGACGGTTCCATG
Primer name 3' RACE reactions Sense primers CD Lam 3' F2 5' RACE reactions Phosphorylated sequence specific primer CD Lam 5' RT-P Antisense primers CD Lam 5' A1 CD Lam 5' A2 Sense primers CD Lam 5' S1 CD Lam 5' S2 Sequence specific primers Antisense primers CD lam spec R2 Positive control primers for $\beta$ -actin Sense primers $\beta$ -actin F Antisense primers $\beta$ -actin R	DNA sequence of the primers         GGGACTTCGGTGGATTTGG         CAAATCCACCGAAG (Primer phosphorylated at the 5' end)         GGCGAAATCAGAGAGGGGTC CGGTTTGATGAAGAGTGTTGAG         CGGTCGTATTGAGGTTCGTG TGACAACCTGGG         CCTTCACCCAGATAGTCAACC         GGCTACTCCTTCACCACCAC         AGCTGTGGACGGTTCCATG
Primer name 3' RACE reactions Sense primers CD Lam 3' F2 5' RACE reactions Phosphorylated sequence specific primer CD Lam 5' RT-P Antisense primers CD Lam 5' A1 CD Lam 5' A2 Sense primers CD Lam 5' S1 CD Lam 5' S2 Sequence specific primers Antisense primers CD lam spec R2 Positive control primers for $\beta$ -actin Sense primers $\beta$ -actin F Antisense primers $\beta$ -actin R (c) Birgus latro	DNA sequence of the primers GGGACTTCGGTGGATTTGG CAAATCCACCGAAG (Primer phosphorylated at the 5' end) GGCGAAATCAGAGAGGGGTC CGGTTTGATGAAGAGGGTTCGAG CGGTCGTATTGAGGTTCGTG TGACAACTATGGCAAGCTGG CCTTCACCCAGATAGTCAACC GGCTACTCCTTCACCACCAC AGCTGTGGACGGTTTCATG
Primer name 3' RACE reactions Sense primers CD Lam 3' F2 5' RACE reactions Phosphorylated sequence specific primer CD Lam 5' RT-P Antisense primers CD Lam 5' A1 CD Lam 5' A1 CD Lam 5' A2 Sense primers CD Lam 5' S1 CD Lam 5' S1 CD Lam 5' S2 Sequence specific primers Antisense primers CD lam spec R2 Positive control primers for $\beta$ -actin Sense primers $\beta$ -actin F Antisense primers $\beta$ -actin R (c) Birgus latro Primer name	DNA sequence of the primers GGGACTTCGGTGGATTTGG CAAATCCACCGAAG (Primer phosphorylated at the 5' end) GGCGAAATCAGAGAGGGTC CGGTTTGATGAAGAGGGTTCGTG TGACAACTATGGCAACCTGG CCTTCACCCAGATAGTCAACC GGCTACTCCTTCACCACCAC AGCTGTGGACGGTTTCATG DNA sequence of the primers
Primer name 3' RACE reactions Sense primers CD Lam 3' F2 5' RACE reactions Phosphorylated sequence specific primer CD Lam 5' RT-P Antisense primers CD Lam 5' A1 CD Lam 5' A2 Sense primers CD Lam 5' S1 CD Lam 5' S2 Sequence specific primers Antisense primers CD lam spec R2 Positive control primers for $\beta$ -actin Sense primers $\beta$ -actin F Antisense primers $\beta$ -actin R (c) Birgus latro Primer name 3' RACE reactions	DNA sequence of the primers GGGACTTCGGTGGATTTGG CAAATCCACCGAAG (Primer phosphorylated at the 5' end) GGCGAAATCAGAGAGAGGGTC CGGTTTGATGAAGAGGGTTCGTG TGACAACTATGGCAACCTGG CCTTCACCCAGATAGTCAACC GGCTACTCCTTCACCACCAC AGCTGTGGACGGTTTCATG DNA sequence of the primers
Primer name 3' RACE reactions Sense primers CD Lam 3' F2 5' RACE reactions Phosphorylated sequence specific primer CD Lam 5' RT-P Antisense primers CD Lam 5' A1 CD Lam 5' A2 Sense primers CD Lam 5' S1 CD Lam 5' S2 Sequence specific primers Antisense primers CD lam spec R2 Positive control primers for $\beta$ -actin Sense primers $\beta$ -actin F Antisense primers $\beta$ -actin R (c) Birgus latro Primer name 3' RACE reactions Sense primers	DNA sequence of the primers GGGACTTCGGTGGATTTGG CAAATCCACCGAAG (Primer phosphorylated at the 5' end) GGCGAAATCAGAGAGGGGTC CGGTTTGATGAAGAGGGTTGAG CGGTCGTATTGAGGTTCGTG TGACAACTATGGCAACCTGG CCTTCACCCAGATAGTCAACC GGCTACTCCTTCACCACCAC AGCTGTGGACGGTTTCATG DNA sequence of the primers

the putative sequence. For *G. natalis* the calculated probability was

Probability 
$$= \left(\frac{1}{20}\right)^{17} = 7.63 \times 10^{-23}.$$

To do this, a  $\beta$ -1,3-glucanase sample, previously purified from the midgut gland of G. natalis, was run on a 12% polyacrylamide gel and stained with Coomassie blue as per the method of Allardyce and Linton(2008) and Linton and Shirley(2011). A protein band corresponding to the  $\beta$ -1,3-glucanase was excised from the gel and processed by in-gel tryptic digestion. Obtained peptides were eluted from the gel, lyophilised and resuspended in 0.1% formic acid. For LC-MS/ MS analysis of peptides a nano-HPLC system (nanoAquity, Waters) online-coupled to an ion source into a LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific) via a nanoelextrospray ion source (TriVersa NanoMate, Advion) was used. Peptides were injected on a trapping column (nanoAquity UPLC column, C18, 180  $\mu$ m  $\times$  20 mm, 5 µm, Waters) and separated on a C18 UPLC column (nanoAquity UPLC column, C18, 75  $\mu$ m  $\times$  100 mm, 1.7  $\mu$ m, Waters) in a 45 min gradient. Full scan MS spectra were acquired in a positive ion mode in the LTQ-Orbitrap XL using a CID top 6 method as described in Rockstroh et al. (2011).

#### 2.10. Phylogenetic analysis

Full length amino acid sequences for GHF16 proteins such as  $\beta$ -1,3-glucanase and lipopolysaccharide and  $\beta$ -glucan binding proteins from insects, molluscs and crustaceans were downloaded from GenBank (http://www.ncbi.nlm.nih.gov/genbank) and aligned using the Clustal W algorithm within the MEGA6 software package (http://www.megasoftware.net). A neighbourhood joining phylogenetic tree was then constructed using MEGA6 and assuming the Jones–Taylor–Thornton substitution model (Jones et al., 1992). The reliability of the phylogeny was tested with the bootstrap model with a 1000 replicates. Branches with boot strap values lower than 50 were collapsed to form polyphyletic groups.

#### 3. Results

#### 3.1. G. natalis and C. destructor GHF16 sequence

The GHF16 mRNA sequence for *G. natalis* had an open reading frame of 1098 bp while that for *C. destructor* had an open reading frame of 1095 bases. Translated, this would produce a putative protein of 365 amino acids with an estimated molecular mass of 41.4 kDa for *G. natalis*, and a 364 amino acid protein with an estimated molecular mass of 41.5 kDa for *C. destructor* (Fig. 2).

The putative amino acid sequences from both species contained a hydrophobic signal sequence that was 15 amino acids long (Fig. 2, marked in yellow) and the correct catalytic and binding residues to be a catalytically active β-1,3-glucanase (Juncosa et al., 1994; Henrissat and Davies, 1997; Genta et al., 2009; Kovalchuk et al., 2009; Song et al., 2010). Both sequences contained features that are characteristic of glycosyl hydrolase family 16 enzymes, in particular the amino acid sequence "Glu-Ile-Asp-Ile-Val-Glu" (Fig. 2, marked in red); the first glutamate residue acts as the nucleophile, the second glutamate acts as the general acid base catalyst, while the aspartate acts to modify the pKa of the catalytic residues (Juncosa et al., 1994; Genta et al., 2009). The sequences also contain tryptophan substrate binding residues (Fig. 2, marked in green) and 2 arginine residues and 1 histidine residue (Fig. 2, marked in blue) that help to modify the pKa of the catalytic glutamate residues (Genta et al., 2009; Kovalchuk et al., 2009; Song et al., 2010; Zakharenko et al., 2011). The predicted tertiary structure, produced from homology modelling of the putative amino acid sequences, had a β-jelly roll motif, which is also typical of family 16 glycosyl hydrolases (Genta et al., 2009) (Fig. 3). Like the  $\beta$ -1,3-glucanases from



**Fig. 3.** Predicted tertiary structure of the β-1,3-glucanase proteins expressed in the midgut glands of *G. natalis* (a and b) and *C. destuctor* (c and d). Whole protein (b and d) and the active site (a and c) depicting the secondary structure of the protein plus the side chains of the amino acids involved in the catalysis and substrate binding. Critical catalytic residues (coloured red), the amino acid residues which aid these (coloured blue) and the tryptophan residues involved in substrate binding (coloured white) are labelled in the panels a and c. These residues have the same colour scheme in panels b and d. Structures were predicted from the putative amino acid sequence by homology modelling using the Phyre2 server (http://www.sbg.bio.ic.ac.uk/ phyre2/). Figure prepared using Pymol (http://www.schrodinger.com/pymol/).

Tenebrio molitor and Cryptopygus antarcticus, the secondary and tertiary structures of the  $\beta$ -1,3-glucanase from both *G. natalis* and *C. destructor* were highly similar to that of an endo- $\beta$ -1,3-glucanase from Nocardiopsis sp strain f96 (Protein data bank code c2hykA) (Genta et al., 2009; Song et al., 2010). Compared to the amino acid sequence from Nocardiopsis, the respective similarity for that of *G. natalis* and *C. destructor* was 42 and 40%.



Fig. 4. Short peptide sequences (P) from a  $\beta$ -1,3-glucanase (laminarinase) purified from the midgut gland of the gecarcinid land crab, *Gecarcoidea natalis* aligned with the putative amino acid sequences (A) of a  $\beta$ -1,3-glucanase (laminarinase) from *Cherax destructor* and *Gecarcoidea natalis*. Totally conserved amino acid residues are coloured red while highly conserved amino acid residues are coloured green. The peptide sequences for *G. natalis* were determined by orbitrap mass spectrometry of a purified  $\beta$ -1,3-glucanase which had undergone a tryptic digest while the putative amino acid sequences for *C. destructor* and *Gecarcoidea natalis* were derived from the cDNA sequence.

3.2. Amino acid sequences of short peptides from a previously purified  $\beta$ -1,3-glucanase

In addition to the putative amino acid sequence determined from the cDNA sequence, two partial sequences of 8 and 9 amino acids long were obtained by Orbitrap mass spectrometry from a  $\beta$ -1,3-glucanase purified previously from the midgut gland of G. natalis. These fragments aligned perfectly with the putative sequence derived from the same tissue in *G. natalis* with the probability of the two proteins being the same by chance alone and thus being misidentified is  $7.63 \times 10^{-23}$  (Fig. 4). Similarly the amino acid residues were also highly conserved (first peptide) and totally conserved (second peptide) when compared with the putative amino acid sequence from C. destructor (Fig. 4). The probability that these two proteins share the same amino acids by chance is  $6.10 \times 10^{-19}$ . Furthermore, the estimated molecular masses of the  $\beta$ -1,3-glucanase proteins (41.4 kDa for G. natalis and 41.5 kDa for C. destructor) match that determined from  $\beta$ -1,3-glucanase purified previously (Allardyce and Linton, 2008) (Table 1). Thus, there is strong evidence that the GHF16 cDNA sequences presented here encode the β-1,3-glucanase enzymes which have been previously purified and characterised.

#### 3.3. Alignment of sequences

A BLAST search of the putative amino acid sequences from *G. natalis* and *C. destructor* revealed that they were similar to that of  $\beta$ -glucan

			20			40			60	*		
B latro			20			40			60			_
C brevi	;										;	_
C perlat	:										:	-
C_rugo	:										:	-
C_vari	:										:	-
M_platy	:										:	-
P_laevis	:										:	_
C_des_MG	÷	MR'	LCL-LI	LACGAFAA		-DLVEPI	EDCTGFPC	LIFNDDF	-DYFDHDV	WEHEVIMS	:	51
C_des_H	:	MR	TW-TTN	LACGAPAA			EDCTGF PCI	LIENDDE-		NEHEVIMS	:	51
P clarkii	:	MTR	LCFLLL	LASGALAA		-DVVAP	EDCTGEPCI	LIENDEE-	-DFLDHEV	WEHEITINS	:	53
P mono	:	MKG	VASVVI	LACGALAA		-DIVEPH	EDCTSFPC	IFEDNE-	-DYLDNDI	WEHEITMS	:	53
H_gamm	:	M	IC-LLI	LACGVFAA		-NVVDPI	KDCTAFPC	IFSDDF-	-DYLDHDA	WEHEITMS	:	50
L_styli	:	MKTVLLSLSRMKG	VASVVI	LACGALAA		-DIVQPI	EDCASFPC	MIFEDNF-	-DYLDNDV	WEHELTMS	:	63
M_rosen	:	MR'	LA-TII	LATCAFAA		-DIVDPI	KDCTSFPCI	LIFNDDF	-DFLDHEV	WEHEVTMS	:	51
L_vann	:	MKG	VASVLI	LACGALAA		-DIVEPH	EDCASFPC	MIFEDNF-	-DYLDNDV	WEHELTMS	:	53
M_jap	÷	MKR	VPSVEI	LPCGPLPA		-HIVQPI	EVCASIAC.	LVFQ	NOTNONU	CEICPS	:	41
P_ieni F_sine	÷	MR	ALW-LLT	LACGALAV			DOCTAPDO	LIENDDE	-DELDHDVI	WEFETTIS	1	51
S frugi	:	MWS	TAGVIA	TASLGAAC	TPSLTTV	SGTHAP	-VTVCSGA	LIFADGE-	-DTEDLEK	NOHENTLA	:	60
Ceury	:	MWA	LL-GVVA	LATSASAC	WSSITTV	SGTHAP	-ETVCSGS	IIFADDE-	-OEFDLEK	OHENTLA	:	59
Acarda	:	MWV	L-CVVA	LATSATAC	FTSITTV	SGTHAP	-ETVCSGAI	LIFADDF-	-EEFDLEK	QHENTLS	:	59
C_antarc	:	MN	AFTFPLI	LAFCAFAH			GAWVI	LDWEDEFN	GNL-ADR	WNFELGCN	:	44
M_yesso	:		M	DPLLC	LV	LLP-	-LVAGA	AGFRDDF-	-TTWDPSD	YQIEVSAW	:	37
H_disc	:	MEYSVKYLSASGD	VYHV	DGVFT	IPAA	-SSLSP-	-RLYRRGN	TVFEDSFN:	SHQLNPKH	WHHEITCW	:	63
		80	*	100		*	120	*	14	40		
B_latro	:										:	-
C_brevi	:										:	-
C_perlat	:			<b>T</b> LFIR	FELLSR-	-WKDEA	FLSSCELDI	WGMNGRGI	DVCIGNSY	YGCDRVGT	:	48
C_rugo	÷		F	-GSTLFIR	E LSR-	-WKDEA	ISSCELN	WGMNGRG	DVCIGNSY	YGODRVGT	:	51
C_vari	÷	MERCANTNIN	ovevm	TLFIR	E LSR-	WKDEA	ISSCELNI	WGMNGRGI	DVCIGNSY	TGODRVGT	:	48
P laevie	:	WEFQAILINN	NOVO11P	-DSILFIK		-WKDEG	TSCELN	WGMNGRGI	DVCTANSE	VGODRVGN	:	41
C des MG	÷	GGGNWEFCVYLNN	RSISYTE	-DSTLFIK	D TSN-	WOTEG	ISSCNLNI	WGMNGRGI	DVCIIGNSY	YGOERTGN	÷	121
C des H	:	GGGNWEFQVYLNN	RSISYTE	-DSTLFIK	PDI TSN-	-WOTEG	FLSSGNLNI	WGMNGRGI	OVCIGNSY	YGCERTGN	:	121
G_nat_MG	:	GGGNWEFQAYLNN	RSVSYTE	-DSTLFIK	PQLMSD-	-WKDEG	FL <mark>SS</mark> G <mark>E</mark> LNI	WGMNGRGI	DVCIGNSY	YGC <mark>DRV</mark> G <b>T</b>	:	121
P_clarkii	:	GGGNWEFQMYINN	RSISYTE	-DSTLFIR	PDLTSN-	-WQTTD	FL <mark>SS</mark> G <mark>D</mark> VNI	WGMNGRGI	DVCIGNSY	YGCER <mark>V</mark> GN	:	123
P_mono	:	GGGNWEFQAYVNN	RSISYTE	-DSTLFIK	FDLTSN-	-WKGED	FLSSCTLDI	WGMNGRGI	DVCIGNSY	YGC <mark>S</mark> RVG <mark>S</mark>	:	123
H_gamm	:	GGGNWEFQVYVNN	RSVSYTE	-DSTLFIK	GITSE-	-WKSEE	TSCDLN	WGMNGRG	DVCIGNSY	YGCKRVG <b>T</b>	:	120
L_styli	1	GGGNWEFQAYVNN	SISTIF	-DSTLFIK	E TAN-	WKGDD	TISCILDI	WGMNGRG	DVCIGNSY	IGOSRIGS	:	133
M_rosen	:	GGGNWEFQVIVNN	STSYTE	-DSTLFIK	F TAN-	WKGDD	TTSCNLN	WGMNGRGI	DVCUGNSE	YCOSETICS	:	121
Miap	;	GGGNWEFCAYVNN	RSISYTE	-DSTLFIK	D TSN-	WKGEE	ISSCNLD	WGMNGRGI	DVCUGNSY	YGOSRVGS	:	111
Pleni	:	GGGNWEFQMYLNN	SLGYTE	-DSTIIIK	FELTSK-	-WYSEH	FI FNDELNI	GI	KCTDHRD	YGCVRKGT	:	115
E_sine	:	GGGNWEFHAYLNN	SVSYTE	-DSTLFIK	FQLTSD-	-WRGEA	FLTSGELN	WGMNGRG	SVCT SNAF	YGC <mark>NR</mark> VGT	:	121
S_frugi	:	GGGNWEFQYYGNN	RTNSFVF	-SGSLFIR	PSLTSD-	-EFGEA	FL <mark>SS</mark> G <mark>HW</mark> NV	EGG-APA	DRCTNPQW	NGCERTGT	:	129
C_eury	:	GGGNWEFQYYSNN	RTNSYTE	-DGILYIK	FSLTSD-	-QFGEH	FLTSGLLN	EGG-APA	DRCINPQW	YGCERQGT	:	128
A_carda	:	GGGNWEFQYYNNN	RTNSYTE	-NGILYIK	TITSD-	-QFGEG	TSEVIN	EGG-APA	DRCHNPQW	IGOERIGS	:	128
C_antarc	÷	GWGNNELQCITDN	GANARQ	EDGKLVIS	AVKE-	-WWGDG-		NOT	IRCHOUDNE	A COLLORY	:	106
H_yesso	:	GGGNGEFOMYTPE:	ANTYTE	-NGVLYLK	ETFTAD-	-KEGDD	FORCYLDY	KOOW	SCHAAOD	NGORBOG-	:	129
n_dibe	•	obolion grint i b		1	pl		fl q	1	ct	ac a	•	200
					-	_	-		_			
D lates		* 16	×	*	180		*	200	X*	22		16
C brevi	:		1						PGSGEID.	IVE SKOND	:	17
C perlat	;	ATNLVNPTMSA	RIBTHK	FSFRYGRT	EVRAKMP	RGDWIW	PAIWMLPH	WPYGP	PASCETD	IVESBOND	:	117
C_rugo	:	ATNLVNPVMSA	RIFTHKE	FSFRYGRI	EVRAKMP	RGDWIW	PAIWLLPH	HWPYGP	PASCEID	IVESROND	:	120
C_vari	:	ATNLVNPIMSA	RI <mark>R T</mark> HKI	FAFRYGRI	EVRAKMP	RGDWIW	PAIWLLP <mark>H</mark>	HWPYGP	PASGEID:	IVESROND	:	117
M_platy	:	PTNLVNPIMSA	RIFSLSI	FAFKYGRI	EVRA <mark>QT</mark> P	RGDWIW	PAIWLLP <mark>RI</mark>	NWPYGG	PASGEID:	IFESROND	:	135
P_laevis	:	NGRGDVCTANS	YCCDRV	VAFKYGRI	EIRAKMP	RGDWIW	PAIWMLPQ	YWPYEG	PASGEID.	IVESROND	:	110
C_des_MG	÷	PVNIINPVMSA	RIRTLSI	FAFRYGRI	EVRAKME	RGDWIWI	PAIWLLPR	YWPYCI	PASCEID.	IVESROND	:	190
C_des_H	1	PVNIINPVMSA	AL FILSE	PAPRIGRI	EVRANME	RGDWLWI	PAIWLLPRI	IWPICL	PASSEID.	IVESROND	:	190
P clarkii	;	PVNTINPVISA	RTLEN	FAFKYGRI	EVRAKLE	RGDWIW	PAIWLLPR	WPYCP	PASCEID:	IMESRUND	;	192
P mono	:	SSNIINPVTSA	RIRTMSN	FAFRYGRI	EVRAKME	RGDWIW	PAIWMLPR	NWPYGL	PASCEID	ILESROND	:	192
H gamm	:	ATNIVNPITSA	RIRTLSE	FAFRYCCI	EIRAKMP	SGDWIW	PAMWLLP <mark>KN</mark>	NWPYGP	PASCEID	IVESHONS	:	189
L_styli	:	SSNLVNPVLSA	RI <mark>R</mark> TMSN	FAFRYGRI	EIRAKMP	RGDWI WI	PAIWMLP <mark>RI</mark>	NWPYGA	PASGEID:	ILESROND	:	202
M_rosen	:	ADNLINPVMSA	RIFTLSI	FAFKYGRI	EVRAKMP	RGDWI WI	PAIWLLP <mark>RI</mark>	NWPYGA	PASGEID:	IVEIRGND	:	190
L_vann	:	SSNLVNPVLSA	RTMSN	PAFRYGRI	EIRAKMP	RGDWIWI	PAIWMLP <mark>RI</mark>	NWPYCA	PAS GEID	ILFERGND	:	192
M_jap	:	SSNIINPITSA	TTTUD	FAFRYGRL	EIRAKMP	RGDWIWI	PAIWMLPR	WPYCL	PASCEID:	TTE SROND	:	180
F_ieni E_sine	:	ATNLINGIMSA	TRTTOT	PAFRIGRV	EVRAKME	RGDWL WI	PAIWLMEKI	SKIEG	PASEID	TVF TROMP	:	190
S frugi	;	PTNIINPIKSA	RTVNS	FSFRYGRI	EVRAKME	AGDWIW	PAIWIMPA	NTYGT	PASSETD	VESBONE	:	198
Ceury	:	PTNIINPIKSA	RIRTVNS	FSFRYGRV	EVRAKME	AGDWIW	PAIWLMPAY	NAYGS	PSSGEID	LVES RONR	:	197
A_carda	:	PDHIINPIKSA	RI <mark>RTVNS</mark>	FSFRYGRV	EVRAKMP	AGDWIWI	PAIWMMPAI	FNSYGT	PASGEID	IVESRONR	:	197
C_antarc	:	VNPDKEFTSA	RM <mark>I</mark> TK	ANWLHGKF	EMRARLP	KGKHI W	PA <mark>F</mark> WMMP <mark>Q</mark> I	ISEYGG	PRSGEID	ITEIRCOR	:	147
M_yesso	:	GGDSEILEPVMSG	T TN	FAMTYGRV	NVRAKIP	KGDWIWI	PAIWMLP <mark>R</mark> I	DWSYGG	PRSGEID	IMESRONT	:	175
H_disc	:	AQUPPIMSS	VBSV	ASITHGRV	EVVAKIE	KGDWIW	PAIWLLEPO	WPWKYCA	PASCETD	E E ECE	:	196
		p sa		r Að	era p	gaw wi	paw p	Ad I	PASSEID	C E IGN		

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B latro				83
C brevi	;		:	84
Cperlat	2	NYGDICN YGGSTLL YG HWPEN FYGMITACYAAN -DGSTAN SE TWRY WANTNYE YYD A W TV	-	184
C rugo	:	NYGDICNCAGGSTI WCHWPINFYGMTTACYTAN-DGSBANSPETWRVIWNTNYLFYWDIVITY	:	187
C vari	:	NYGDICNCAGGSTIHWGEHWPINFYGMUTACYTAN-DGSDANSPETWRVDWNNNNLFYVDDALVLTV	:	184
M_platy	:	NYGTLENTMAGSTLHWGFYWPINFYDMTHKEYTGDEAINFHVWRDIWISTDWKFYVDIVIQITV	:	199
P_laevis	:	YGSISNOVGSSTN <mark>E</mark> NGEFWPYNFYDM TSEYSADFALSFHVWRVWISTDIKFYVD DIKITV	:	174
C_des_MG	:	YGNLCNQYGGTTVHWGFNWQKNMYEKTHSDYTAS-DGSEANS <mark>FFTMRLDWF</mark> KDN <mark>M</mark> LFYLDIQUQLTV	:	257
C_des_H	:	NYGNLCN <u>C</u> YGGTTV <mark>H</mark> NGENWQKNMYEKTHSDYTAS-DGSBANS <mark>FH</mark> TKRUDWIKDNMIFYLDIQUQUTV	:	257
G_nat_MG	:	IYGSISNAVAGSTMINGEFWPLNFYDMIAVEYNAN-SGSBAID <mark>FH</mark> VMRVDWISTDIRFYVDIEIKMTV	:	257
P_clarkii	:	IYSNLCNCYAATTENGENWQTNLYERTHADYSAN-DGSYANSEITMRM/WHKDNIQVEVDIQIQITV	:	259
P_mono	:	IFGTICNCYGGTTI GFWPYNFEEKTHAEYSAN-TGSEAIDFEVMRU.WAKDNYEEYYDIVIQITV	:	259
H_gamm	:	FYGTICNCYGSAKI TYC:YWQQNMYQKAHADYQAP-TGSYATNSTTKRVNMAKDDYKAYYDI EI KI'AY	:	256
L_styli	÷	NFGTHENCYGGUULTWESFWPYNFSEKAHVEYSAN-EGSEANDER WARDGYESYW EVIOLTH	:	269
M_rosen	÷	NYGEI'EN YGGINN WE FWPIN RYDLAHVEYRAN -DGSGAT SDETTRE WARDRIEAN IN V W TA	:	257
L_vann	÷	FGTICKNIGGTTEWGFFWPINFERHVEISAN-GGSGALDERWRDWRDWRDGXEIVIEVQOTT	÷	259
M_Jap	:		:	251
Fsine	:		:	254
S frugi	;	ME-HNGVH CTOFAGSTILL GEVEAN GOFRAHWUR-RN-PAGMSNAR RNCLEWAPTVIR STDIMFIGR	;	268
Ceury	÷	NMF-SNGVHIGTOEAGSTITTCHYPEINGVERAHWLR-RN-SNGVDRAGERWGPEYMPESTDIVELGRV	÷	267
A carda	:	NML-SNGVNIGTOEAGSTITYCFFPGINGWERAHWLR-RN-NOCYDRNFFRYCHEWIPDHURFLIDIOEIGFI	:	267
C antarc	:	PQQILGUL FCAAPDNKGDVGAGERDFPILESALFETEGLWSPDSMOXILDFQVYHTE	:	206
M yesso	:	KAI-LGGONSEVNYVASTI HVGEAYNHNA AKTHASKRKYGGDDN-HCWHTYSLDWAADHIITYVDNVEM RI	:	246
H_disc	:	HLSEANGATOCVDRVLSTIFYCASPSCHRCQGDSKTSKTGTTWALSFHTYSVIWTAGHIRMDIDNCPVMAW	:	267
-		g q t H5Gp t 5a 5H 5 6 W3 6 6D		
D 1-6		300 * 320 * 340 * 360		150
B_latro	:	DEC-SSEWDYSELGD-Q-YDNEW VAEDKMAPED OKFYFILNLAVEGTNGE PDEVT-ADPPKPWANTSPQAFL	:	152
C_previ	:	DECTINAMENGELGD-Q-IDNEW VACDAWAFFDQAFTFIN	:	212
C_periat	÷		÷	212
C_rugo	÷		÷	223
C_vari M_platy	:		:	256
P laevis	:		:	193
C des MG	÷	DPC-TN STORGEGNELDNPWKAGSKMAPEDOKEYVVLNVAVCGVNGEPPDGITDKPWSNVSPOASL	÷	323
C des H	;	DPC-TNFNDFGGFGNELDNPWKAGSKMAPFDCKFYVVLNVAVGGVNGFFPDGITDKPWSNVSPCASL	:	323
G nat MG	:	DPG-TNFNEHAGVDN-I-YDNPWASGDKLAPFDCKFYIVLNVAVGGTNGFPPDGIVSNKPWANTSPOAFL	:	324
Pclarkii	:	DPC-TNFND-GGFDNSLDNPWKAGSKMAPFDCKFYVVLNVAVCGVNGYPDGVP-SNPAKPWSNTSPQAFL	:	328
P mono	:	DPC-TSFNDFAGMGPSFDNPWAAGAKMAPFDCKFYLILNVAVCGTNCFFPDGIASKPWSNLSPTAFL	:	325
H_gamm	:	DPC-TNFNDFGCFGNSYDNPWVAGGKMAPFDCKFYIVLNLAVCGTNGFFPDDVP-SNPPKPWNNVSPQALL	:	325
L_styli	:	DPC-NSFADESCMDSVYDNPWSAGSKMAPFDQKFYDILNVAVCGTNGFPDDDVASKPWSNLSPTAFL	:	335
M_rosen	:	DPC-SSFAEFGCFGDNIDNIWNSGEKMAPFDQKFYVILNLAVCGTGGFFPGGIP-GA-NKPWSNTSPTAFK	:	325
L_vann	:	DPC-SSEADFACMDS-ALYDNPWAAGSKMAPFDQKFYLILNVAVCGTNGFFPDDVAAKPWSNLSPTAFL	:	326
M_jap	:	DPC-SSSTDFSCMDPSFDNPWVACSKMAPFDQKFYDILNVAVCGTNGF5PDDIGPKPWSNLSPTAFL	:	313
P_leni	:	DPC-SNAXDAGCLGN-S-QNNEWRDGSKMAPFDQKFYLTLNLAVCGTNGYAPDGVS-SNPAKPWNNASPHASR	:	320
E_sine	÷	DPG-TNSWN-AGLDS-T-YDNFWVDGDKMAPPDEKFYIIINLAVGGVNGFAPDGIVANKPWENHSPHAPR	:	321
S_frugi	÷	TPENGGENETGEFNSNPNIENPERFYSERAPFIEKFYEINWAVGGTNGFIPDGVS-NPSPRPWWNGSPTAPR	-	340
C_eury	1	TPENGGENEL GETNENUNTLINPIKIGSKAPFIQKETINLAVGETING PDGVV-NPSPEPWWNGSPTAAK	:	330
C antarc	:		:	254
M vesso	:	NTPSCERCIC FDGNN TO ASCC NA PETK PHT I NVAVG RDYDGNGF-YDDFBWGNHNDMR	;	309
H disc	2	TTPSOCYNSYSHOSGTNWNSOCGNDAPFLCCMSLLLNVAVCATNCYTODSWHNTPHAKPWKNNSPTAMM	;	336
	-	pg 5W g npw g apfd kf n avg f		
		* 380 * 400		
B_latro	:	DFWNGRGDWLPSWEQGEGRISENAALQVDYVKVWKMESIEQ : 193		
C_brevi	:			
C_perlat	-	: -		
C_rugo	÷			
C_vari	:			
M_praty				
P_Idevis	:			
C des H	:	DEWNGRGSWIDEWEGEGETSENAALQVDIVKWKMESVDQ · 364		
G nat MG	:	DEWNARDSWLPTWEOGEGKISENAALOVDYVKVWKLVSADE : 365		
P clarkii	:	DFWNARDSWLPSWENGEGRVSENAALQVDYVKVWKMESVEO : 369		
P mono	:	DFWNARDEWLPSWKAGEDRISEGAAMQVDYVRVWKMESTEQ : 366		
Hgamm	:	DFWNGHSSWLPTWEQGEGRISEKAALQVDYVKVWKMENIDQ : 366		
L_styli	:	DFWNARDEWLPSWQAGEGRISEGAAMQVDYVRVWKMESAEQ : 376		
M_rosen	:	DFWNGRGDWLPTWQQGESRISEKAALQIDYVKVWKMESAKQ : 366		
L_vann	:	DFWNARDEWLPSWQAGEDRISEGAAMQVDYVRVWKMESAEQ : 367		
M_jap	:	DFWNARNEWLPTWQAGESRISEGAAMQVDYVRVWKMESVEQ : 354		
P_leni	:	DFWNARGSWLPSWEHGEGHISENAALKVDYVKVWKMESVEQ : 361		
E_sine	:	DFWKGRDSWLPSWEQGEGKISEGAALKVDYVKVWKMESVIQ : 362		
S_frugi	:	DFWNARSAWLNTWNLNVN-DGQDASMQVDYVRIWAL : 375		
C_eury	1	DEWNARNNWL PTWNENVN-NGE-ALQVDIVRVWAL : 3/2		
A_carda	:	DEWNARGAWERTWNINVN-NGEDAANQVDIVRIWAE : 3/4		
M veeso	:	SEWEARHSWEHTWOGDEVALUTDYTEMIDH 339		
H disc		DFWKSKOOWOSTWHGEDVAMKVKSVKMTOY : 366		
		and a second sec		

Fig. 5 (continued).





**Fig. 6.** Expression of  $\beta$ -1,3-glucanase (laminarinase) mRNA and a control mRNA, either beta actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in different tissues indicated for *G. natalis* (a) and *C. destructor* (b). In *G. natalis*, the C1F H4R primer combination was used to amplify a fragment ( $\approx$ 350 bp) of  $\beta$ -1,3-glucanase (laminarinase) while in *C. destructor*, a fragment ( $\approx$ 300 bp) of the  $\beta$ -1,3-glucanase (laminarinase) cDNA was amplified using a H3F L4R primer combination. Control fragments for beta actin ( $\approx$ 200 bp) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) ( $\approx$ 300 bp) were respectively amplified from cDNA derived from all tissues from *C. destructor* and *G. natalis*. Fragment size standards with the sizes indicated were run alongside the samples.

binding and lipopolysaccharide proteins from other crustaceans, and  $\beta$ -1,3-glucanase sequences from other invertebrates. Similarly a BLAST search using the nucleotide sequence from *G. natalis* revealed that it

#### 3.4. Sequences from other crabs

Partial GHF16 cDNA sequences, ranging in size from 581 to 768 base pairs, were also obtained from cDNA prepared from the midgut gland of seven other crustaceans including amphibious and terrestrial brachyuran, and anomuran decapods. Like the sequences from *C. destructor* and *G. natalis*, the putative amino acid sequences contained the characteristic catalytic and binding domains of a glycosyl hydrolase family 16 protein (Juncosa et al., 1994; Genta et al., 2009) (Fig. 5). Thus, the cDNA may encode for a  $\beta$ -1,3-glucanase. Similarly a BLAST search of these putative amino acid sequences suggested that they were similar to that of  $\beta$ -glucan binding proteins from crustaceans and  $\beta$ -1,3glucanases from other species.

#### 3.5. Tissue expression

For *G. natalis*,  $\beta$ -1,3-glucanase mRNA was expressed in the midgut gland but not in gill and muscle (Fig. 6). In contrast,  $\beta$ -1,3-glucanase mRNA was expressed in the midgut gland, gill and haemolymph of *C. destructor*, but not in the muscle (Fig. 6). In all other species examined (*C. perlatus, C. rugosus* and *Coenobita variabilis*),  $\beta$ -1,3-glucanase mRNA was expressed in the midgut gland but not the gill or muscle (data not shown).

### 3.6. GHF16 $\beta$ -glucan binding protein sequence from the haemolymph of C. destructor

Using the same degenerate and sequence specific GHF16  $\beta$ -1,3glucanase primers, a 1095 bp open reading frame was obtained from the cDNA prepared from the haemolymph of *C. destructor* (Fig. 2c). Translated this would produce a 364 amino acid residue protein with a molecular mass of 41.5 kDa (Fig. 2c). The nucleotide sequences of the open reading frames from both tissues, midgut gland and haemolymph, were 99% similar and the putative amino acid sequences were identical. The putative amino acid sequence derived from the haemolymph contained the same catalytic and binding amino acid residues as the putative amino acid sequence determined from the midgut gland (Figs. 2, 5).

#### 3.7. Phylogenetic analysis of sequences

All of the sequences for the GHF16 proteins from insects, crustaceans and molluscs grouped together within a single branch of the phylogenetic tree. In contrast, sequences for true crustacean  $\beta$ -glucan binding proteins that are much larger (152 kDa compared to 30–50 kDa) and possess a binding but no catalytic domain formed an out-group away

**Fig. 5.** Alignment of the putative amino acid sequences for  $\beta$ -1,3-glucanases and lipopolysaccharide and  $\beta$ -glucan binding proteins. Black highlighting indicates total conservation while dark grey and light grey indicate partial conservation. Amino acid sequences that are characteristic of glycosyl hydrolase family 16 proteins and which are critical to catalysis are boxed by a red rectangle. The circle above the alignment indicates the glutamic acid residue which is critical to catalysing the reaction. Amino acids involved in catalysis that aid the critical glutamic acid residue are indicated by a black star above the alignment and blue rectangles around the amino acids. Amino acids involved in substrate binding are indicated by a black square above the alignment and green rectangles around the amino acids. C\_perotagues around the amino acids. Sequences for glycosyl hydrolase family 16 $\beta$ -1,3-glucanase were determined in this study and were from Birgus *latro* (B\_latro accession KJ995771), *Coenobita brevimanus* (C\_brevi accession KJ995770), *Coenobita perlatus* (C\_perlat accession KJ995773), *Cherox destructro* (GHF16 sequence derived from the midgut gland (C\_des\_MG accession KJ995763) and haemolymph (C\_des\_H accession KJ995766)) and *Gecarcoidea natalis*(G\_nat\_MG accession KJ995764). These sequences were aligned with the  $\beta$ -1,3-glucan binding sequences from other crustaceans (*Procambarus clarkii* (P\_clarkii), accession ACR20474; *Penaeus mondon* (P\_mono), accession AEX08659; *Homarus gammarus* (H\_gamm), accession AEX08659; *Litopenaeus stylirostris* (L\_styli), accession ABY89089; *Pacifastacusleniusculus* (P\_leni) accession ACB63533; *Eriocheir sinensis* (E\_sine) accession ACR56716) and  $\beta$ -1,3-glucanase sequences from representative insects (*S\_podoptera frugiperda* (S\_frugi), accession ABR28478; *Colias eurytheme* (C\_eury), accession ACR32831; *Anthocharis discus hannai* (H\_disc), accession ACR682671; *Derna viridis* (P\_viri) accession ACM68926).



**Fig. 7.** Phylogeny of the beta- $\beta$ -1,3-glucanase sequences from the midgut glands of various decapod species determined in this study with that of beta-glucan binding proteins and  $\beta$ -1,3-glucanases of glycosyl hydrolase family 16 (GHF16) from other crustaceans, molluscs and insects. Phylogeny was constructed by the maximum likelihood method assuming Jones–Taylor– Thornton model and the MEGA 6 software package. The credibility of the phylogeny was tested using the bootstrap method with a 1000 replicates and a cut off at 50. The values at each node represent the percentage bootstrap value. Accession numbers are given after the species name. BGBP indicates that the sequence was initially described as a lipopolysaccharide and  $\beta$ -glucan binding protein while gluc indicates that the sequence was initially described as a  $\beta$ -1,3-glucanase (laminarinase). Crustacean sequences determined in this study and were derived from either the haemolymph (H) or midgut gland (M) which are boxed. Branches containing either the  $\beta$ -1,3-glucanase or  $\beta$ -glucan binding protein sequences of the various invertebrate taxa are labelled.

from the GHF16 proteins (Fig. 7) (Cerenius et al., 1994). Within the phylogenetic tree, the crustacean sequences determined in this study were found to group with sequences of GHF16  $\beta$ -glucan binding proteins from other crustaceans (Fig. 7). Also, there was no clear delineation between the sequences expressed in the haemolymph and midgut gland given that they did not form separate branches (Fig. 7). Indeed for *C. destructor* in particular, the amino sequences derived from the midgut and haemolymph were identical, and thus clustered on the same branch (Fig. 7). The amino acid sequences from *C. destructor* were most closely related to a putative  $\beta$ -glucan binding protein sequence from the crayfish *Procambarus clarkii*, which was specifically expressed in the midgut gland. Given the similarity of the sequences from the two tissues and thus the cross reactivity of the primers, it is likely that this sequence from *P. clarkii* is a  $\beta$ -1,3-glucanase and hence has been mis-identified.

Like the crustacean sequences, the molluscan  $\beta$ -1,3-glucanase sequences are grouped with  $\beta$ -glucan binding protein sequences (Fig. 7). Most notably and like C. destructor, the sequences for  $\beta$ -glucan binding protein and  $\beta$ -1,3-glucanase from an abalone species, *Haliotis* formed a monophyletic group (Fig. 7). In contrast for insects, the  $\beta$ glucan binding protein sequences formed a separate branch to that for the  $\beta$ -1,3-glucanases (Fig. 7). The insect  $\beta$ -glucan binding protein lacks the catalytic amino acids present in the  $\beta$ -1,3-glucanases (Pauchet et al., 2009). The two genes are related but evolutionary distinct. Three  $\beta$ -1,3-glucanase sequences, one from the cockroach P. americana, the Antarctic spring tail, C. antarcticus and the gastropod Biomphalaria glabrata did not group with other species of their phyla (Fig. 7). This may indicate a separate evolutionary origin. Indeed it has been suggested that the  $\beta$ -1,3-glucanase gene for *C. antarcticus* may have been acquired by horizontal transfer from bacteria (Song et al., 2010).

#### 4. Discussion

#### 4.1. Sequence presented is a GHF16 $\beta$ -1,3-glucanase

The cDNA sequenced from the midgut glands of G. natalis and *C. destructor* encoded a β-1,3-glucanase from glycosyl hydrolase family 16. The putative proteins from G. natalis and C. destructor respectively were 365 and 364 residues long, and had estimated molecule masses of 41.4 and 41.5 kDa (described previously in Allardyce and Linton, 2008). The proteins also contained the catalytic and binding domains of GHF16 proteins. Both the amino acid sequence of small peptides from G. natalis and the molecular masses of previously purified proteins,  $\beta$ -1,3-glucanases, from both species matched that determined from the putative amino acid sequences. The sequence and molecular mass similarities to active  $\beta$ -1,3-glucanases, taken together with the identification of putative catalytic domains, provide strong evidence that the cDNAs isolated here encode a protein with  $\beta$ -1,3-glucosidase activity rather than just a binding protein. This reasoning is similar to that proposed for the molluscs, where the  $\beta$ -1,3-glucanase protein within the crystalline style is most likely the product of the GHF16 gene expressed in the hepatopancreas, the tissue that synthesises the digestive enzymes (Kozhemyako et al., 2004; Kovalchuk et al., 2006, 2009; Kumagai and Ojima, 2009; Zakharenko et al., 2011). This is the first description of the sequence which is responsible for the endogenous production of a β-1,3-glucanase (laminarinase) in decapod Crustacea. This completes the characterisation of the  $\beta$ -13,-glucanase (laminarinase) which had been previously purified and characterised from G. natalis and C. destructor (Allardyce and Linton, 2008). The putative GHF16 amino acid sequence derived from the haemolymph of C. destructor was 100% identical to that from the midgut gland of the same species. Both sequences contained the catalytic and binding domains of GHF16 proteins. Thus, the protein expressed in the haemocytes may be an active  $\beta$ -1,3-glucanase.

### 4.2. $\beta\text{-1,3-glucanase}$ synthesised by the midgut gland and secreted into the digestive fluid

The  $\beta$ -1,3-glucanase was expressed in the midgut gland, the organ responsible for the production of digestive enzymes, and is secreted into the digestive fluid (Allardyce and Linton, 2008). A GHF16 protein was expressed in the gills and haemolymph of C. destructor. It is likely that this represents expression of a GHF16 protein within the haemocytes that are present in both of these tissues. Perhaps the expression of the GHF16 protein within the gills of C. destructor was due to a bacterial infection in the animal sampled as expression of the GHF16 protein within the gills was not observed in other animals. A GHF16 B-1,3-glucanase was also expressed within the midgut gland of other amphibious and terrestrial decapods species, and this suggests that expression of this gene is widespread amongst the decapod crustacea. This would explain the high β-1,3-glucanase activities observed in the digestive fluid and midgut glands of numerous crustacean species (Sova et al., 1970)(Suzuki et al., 1987; Omondi and Stark, 1995; Figueiredo et al., 2001; Johnston and Freeman, 2005; Figueiredo and Anderson, 2009).

# 4.3. GHF16 genes expressed in the midgut gland of other species may also be $\beta$ -1,3-glucanases that have been misidentified

Expression of a similar GHF16 protein within the midgut gland of the decapod crustaceans, *P. stylirostris, F. chinensis, M. rosenbergii* and *E. sinensis*, has also been described previously (Roux et al., 2002; Liu et al., 2009; Yeh et al., 2009). Based on sequence similarity, the putative protein from these crustaceans was identified as a  $\beta$ -glucan binding protein with no hypothesised  $\beta$ -1,3-glucanase activity. Given that these proteins, like the ones described in this study, have conserved binding and catalytic domains of a GHF16 enzyme, they may also be catalytically active. As demonstrated for the  $\beta$ -1,3-glucanase from *G. natalis*, these sequences may produce an active  $\beta$ -1,3-glucanase that is secreted into the digestive fluid and thus they may have been misidentified. In contrast, true  $\beta$ -glucan binding proteins lack a catalytic domain and hence lack activity (Cerenius et al., 1994).

#### 4.4. Function of the GHF16 protein

Given that the  $\beta$ -1,3-glucanase contains a catalytic site, is expressed in the midgut gland, and is secreted into the digestive fluid, it is reasonable to assume that it is involved in the digestive process. However, its similarity to the immune binding proteins and the expression of the protein within the haemocytes of *C. destructor*raise some doubt as to its role within the midgut. If the enzyme performs a purely digestive role, it could be used to hydrolyse dietary  $\beta$ -1,3-glucans to release glucose in much the same way as the crustacean cellulases release glucose from dietary cellulose. Alternatively, it may assist indirectly in the digestive process by hydrolysing plant and fungal cell walls in order to release the highly digestible cell contents. Finally, it may act as an immune protein to potentially detect and inactivate ingested pathogens. It should also be noted that these options are not mutually exclusive and the enzyme may have a number of putative roles, which are discussed below.

#### 4.4.1. Hydrolysis of $\beta$ -1,3-glucans as a source of glucose

β-1,3-glucanase is an enzyme that can hydrolyse β-1,3-glucans such as laminarin and callose. Laminarin is the major storage polysaccharide within brown algae and callose and is produced in plants in response to wounding (Bull and Chesters, 1966; Bacic et al., 1988). β-1,3-glucans are also present in other organisms such as diatoms, protozoans and fungi (Bull and Chesters, 1966; Piavaux, 1977; Bacic et al., 1988; Ruiz-Herrera, 1992; Terra and Ferreira, 1994; Pesentseva et al., 2008). In species such as *Petrolisthes elongatus* and *Metapenaeus bennetae* that consume algae and possess high activities of β-1,3-glucanase, this enzyme would hydrolyse and thus digest  $\beta$ -1,3-glucans to produce mainly glucose (Johnston and Freeman, 2005; Allardyce and Linton, 2008; Figueiredo and Anderson, 2009).

#### 4.4.2. Physical disruption of fungal and plant cell walls

Alternatively, the  $\beta$ -1,3-glucanase may help to break open the cell walls of fungal and plant material. In particular, the enzyme may work with endo- $\beta$ -1,4-glucanase to hydrolyse the  $\beta$ -1,3 and  $\beta$ -1,4-glycosidic bonds within the cellulose and hemicellulose that comprise the cell walls. This, in conjunction with the mechanical fragmentation by the gastric mill, would rupture the cell walls of plants and fungi to release the highly digestible cell contents that then could be easily digested and absorbed. This may partially explain the function of the  $\beta$ -1,3glucanase and endo-β-1,4-glucanase within the anomuran land crab, B. latro. This species consumes seeds and fleshy fibrous fruits that are high in either protein, carbohydrate or lipid (Wilde et al., 2004). It also possesses both  $\beta$ -1,3-glucanase and endo- $\beta$ -1,4-glucanase activities, but would derive little energy from the digestion of cellulose and hemicellulose, compared to the other nutrients (Wilde et al., 2004; Linton et al., 2014). B. latro also lacks a  $\beta$ -1,4-glucosidase and thus the ability to hydrolyse cellulose to glucose (Linton et al., 2014).

#### 4.4.3. Immune protein

A potential third option is that the protein acts as an immune protein. The  $\beta$ -1,3-glucanase may work like lysozyme by hydrolysing the β-1,3-glucans within cell walls of fungi and bacteria to potentially render them inactive. Indeed, the  $\beta$ -1,3-glucanases secreted into the midgut by insects such Tenebrio and lepidopteran, Helicoverpa armigera can hydrolyse the cell walls of these organisms (Genta et al., 2009; Pauchet et al., 2009). As described for the GHF16 β-glucan binding proteins in the haemolymph, the GFH16 protein in the digestive fluid may also bind to the  $\beta$ -1,3-glucans in the cell walls of micro-organisms and stimulate the prophenol oxidase system to activate a humoral immune response (Lee et al., 2000; Sritunyalucksana and Söderhäll, 2000; Amparyup et al., 2012). However stimulation of the prophenol oxidase system within the digestive fluid has yet to be demonstrated and thus the primary function of the protein may be to hydrolyse cell walls. β-1,3-glucanase activities are present in the digestive fluid of the carnivorous decapods Scylla serrata and Portunus pelagicus (Figueiredo and Anderson, 2009). S. serrata and P. pelagicus consume animal material in the form of molluscs, crustaceans and polychaete worms (Figueiredo and Anderson, 2009). In these species, the  $\beta$ -1,3-glucanase would not function as a digestive enzyme as these species do not consume significant amounts of  $\beta$ -1,3-glucans. It is more likely that the enzyme would act as an immune protein as described above, particularly if they may eat rotting material that may contain significant amounts of bacteria and fungi. Terrestrial hermit crabs (Coenobita sp.) commonly feed on animal faeces and may utilise the  $\beta$ -1,3-glucanase present within the digestive fluid as an immune enzyme to inactivate potentially pathogenic micro-organisms (Greenaway, 2003).

### 4.5. Evolutionary relationship of the $\beta$ -1,3-glucanases and $\beta$ -glucan binding proteins

The evolution of the  $\beta$ -1,3-glucanase gene within both crustaceans and molluscs is unclear since the GHF16 gene for  $\beta$ -1,3-glucanase and  $\beta$ -glucan binding proteins are very closely related given they possess highly conserved catalytic and binding domains (Figs. 5, 7). Indeed, these two classes of protein are so similar that they are grouped together by phylogenetic analysis and thus may be the same protein. Most notably, the sequences for  $\beta$ -1,3-glucanase and  $\beta$ -glucan binding protein are clustered within one branch for both *Haliotis* and *C. destructor*. The putative amino acid sequences for the GHF16 protein from the haemolymph and midgut gland of *C. destructor* were also identical. Similarly the conservation of the catalytic and binding domains between sequences also suggests that the GHF16 protein from other crustaceans may have  $\beta$ -1,3-glucanase activity, and therefore deserves further investigation. The conservation of domains also explains the amplification of a GHF16 gene from the midgut gland and haemocytes of *F. chinensis* and *E. sinensis* (Liu et al., 2009; Zhao et al., 2009).

Given the proposed phylogeny, the original function of the protein is unclear; was the GHF16 protein initially an immune protein which then took on a digestive role? This would be similar to lysozymes in ruminant artiodactyl mammals. These animals express a chicken like lysozyme in the acid stomach. This enzyme evolved from an immune protein to be a digestive enzyme which digests bacteria derived from the rumen (Dobson et al., 1984; Callewaert and Michiels, 2010). Since the GHF16 protein cannot actively select its substrate, it is likely that the real function might be a mix of digestive and immune roles. As an extension of this, the protein may have evolved in two directions. In animals that consume increasing amounts of  $\beta$ -1,3-glucans (laminarin, callose etc.) the activity of B-1,3-glucanase may increase to digest these polysaccharides. Alternatively, it may be an immune protein that deals with potentially pathogenic micro-organisms, particularly in animals that consume rotting material or are long lived. Furthermore, the protein may have co-evolved with the prophenol oxidase system giving rise to the situation where the function of the protein is to amplify the signal of a potentially pathogenic micro-organism (Sritunyalucksana and Söderhäll, 2000). In this system, the binding of a  $\beta$ -1,3-glucan to a binding protein stimulates the activation of pro-phenol oxidase to phenol oxidase. Phenol oxidase catalyses the oxidation of monophenol compounds such as tyrosine that leads to the production of melanin, the products of which are cytotoxic and antimicrobial (Sritunyalucksana and Söderhäll, 2000; Amparyup et al., 2012). This may have occurred in insects, with the protein initially being a  $\beta$ -1,3-glucanase. The protein expressed within the haemolymph may have then lost the catalytic amino acids and evolved into a separate  $\beta$ -glucan binding protein whose function is to amplify the signal of a potentially pathogenic micro-organism (Pauchet et al., 2009). Given these hypotheses, the  $\beta$ -1,3-glucanase from species that do not consume significant amounts of  $\beta$ -1,3-glucans needs to be isolated and characterised in terms of its immune function; that is, its potential to hydrolyse the cell walls of micro-organisms and its ability to activate the prophenol oxidase system in both the haemolymph and digestive fluid. Furthermore, the potential of a GHF16 lipopolysaccharide and  $\beta$ -glucan binding protein expressed within the haemocytes and containing catalytic and binding domains requires re-examination for enzyme activity.

Research into cellulase and hemicellulase enzymes focusses on the characterisation of the enzymes for the digestion of cellulose and hemicellulose within plant cell walls. However what has yet to be addressed is the evolutionary origin of these enzymes. Perhaps like that suggested here, the enzymes original function may have been to hydrolyse the polysaccharides within the cell walls of micro-organisms. The same enzyme may then be utilised to digest plant material as the animal has adopted a leaf litter diet.

#### Acknowledgements

This work was funded by the Molecular and Medical Strategic Research Centre of Deakin University. The authors would also like to thank Peter Greenaway for the help with the collection of the tissue samples.

#### Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx. doi.org/10.1016/j.gene.2015.05.056.

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