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Identification of a putative cellulase gene in the giant freshwater prawn, *Macrobrachium rosenbergii* (De Man, 1879)

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Abstract

Nutrition plays an important role in the development of all organisms and in particular that of farmed aquatic species where costs associated with feed can often exceed 60% of total production costs. Crustacean species in addition, have the added metabolic requirement for regular moulting to allow normal growth and this requires large amounts of energy in the form of sugars (glucose). The current study explored the capacity of the giant freshwater prawn to produce endogenous cellulose-degrading enzymes capable of extracting nutrients (simple sugars) from plant sources in formulated feeds used in the prawn aquaculture industry. We identified a putative cellulase cDNA fragment in the target organism of 1576 base pairs in length of non-microbial origin that after protein modelling exhibited a TM-score of 0.916 with a described cellulase reported from another crustacean species. The functional role of cellulase enzymes is to hydrolyse cellulose to glucose and the fragment identified in GFP was highly expressed in the hepatopancreas, the site of primary food digestion and absorption in crustaceans. Hepatopancreatic tissue from *Macrobrachium rosenbergii* also showed active digestion of cellulose to glucose following an endoglucanase assay. Cellulase gene(s) are present in the genomes of many invertebrate taxa and play an active role in the conversion of cellulose to available energy. Identification and characterization of endogenous cellulase gene(s) in giant freshwater prawn can assist development of the culture industry because the findings confirm that potentially greater levels of low-cost plant-material could be included in artificial formulated diets in the future without necessarily compromising individual growth performance. Ultimately, this development may contribute to more efficient, cost-effective production systems for freshwater prawn culture stocks that meet the animal’s basic nutritional requirements and that also support good individual growth rates.

Keywords: cellulase, cellulose, endoglucanase, *Macrobrachium*, cellulose digestion

Introduction

*Macrobrachium rosenbergii* (De Mans, 1879), otherwise commonly known as giant freshwater prawn (or GFP), is the largest freshwater Caridean species (Chan 1998). GFP is found naturally in freshwater and estuarine river systems across the Indo-Pacific region, from India, Sri Lanka, Bangladesh, Myanmar, Malaysia, Thailand, Cambodia, Vietnam to Indonesia and the Philippines (Ling 1969; Chan 1998). The prawn is highly regarded in Asia and the Far East as a delicacy and supports a large culture industry based largely on unimproved stocks (Ling 1969). According to FAO Fact Sheets (2012), global aquaculture production for this species reached 220 254 tons. Like most cultured
aquatic organisms, cost of production is critical to industry viability and feed costs in particular contribute a significant component to this total. Therefore, development of cost-effective formulated feeds that can supply adequate nutrition and that contribute to good growth rates of prawns can encourage further expansion of the prawn aquaculture industry. Furthermore, by reducing production costs, cost-competitiveness can be improved and the consumer base can be expanded.

The current study aimed to determine if endogenous cellulase genes were present in the GFP genome that encode functional enzymes that could degrade plant material (cellulose) to simple sugars in the gut and so contribute to an individual’s nutrition. Until recently, it was widely believed that herbivorous/omnivorous higher animals (eukaryotes) were incapable of producing functional cellulose-degrading enzymes directly but relied instead, on symbiotic microbes in their gut that possessed functional cellulase genes in their genomes that could digest complex polysaccharides that their host had consumed into simpler molecules (sugars) that were then absorbed from the host gut (Foley 2001). Cellulose is the most common carbohydrate present in nature and is abundant in nature and so forms the main component of plant cell walls, so it is abundant in nature and readily available as a food source providing organisms have the capacity to break complex cellulose molecules down to simple sugars (Goodenough & Goodenough 1993). This view was first documented by Cleveland (1924) when he reported that defaunation of symbiotic cellulose-degrading enzymes secreted by microbial symbionts in the hindgut of eukaryote species could explain their herbivorous/omnivorous nature (Smant, Stokkermans, Van de Boer, Baum, Wang, Hussey, Gommer, Henrissat, Davis, Helder, Schots & Bakker 1998; Foley 2001).

It was recognized later, however, that termites in the family Termitidae lacked symbiotic microbes in their digestive tract but were still able to digest cellulose material (Krishna 1969; Martin 1991). A study of the Japanese subterranean termite Reticulitermes speratus (formerly known as Leucotermes speratus), from which the hindgut protozoa had been removed, showed no reduction in cellulase activity (Yokoe 1964). Endogenous cellulase activity has also been detected in the Australian arboreal higher termite, Nasutitermes walkeri, and a mound-building lower termite, Coptotermes lacteus. In these two species, intestinal microbes have been shown to play no role (N. walkeri), or only a relatively minor role (C. lacteus), in overall cellulase enzyme synthesis (O’Brien, Siu & Kevinson 1979).

Further evidence that some higher eukaryote animals are able to produce and secrete cellulase enzymes endogenously came from recognition that the sterilized hepatopancreas of a snail (Helix pomatia) produced both cellulase and chitinase activity. Furthermore, cellulase and chitinase activity was found to be proportional to individual body weight and total protein content in the hepatopancreas. Increases in the activity of both enzymes was also independent of total digestive juice bacterial count, so cellulase and chitinase enzymes were therefore active in the apparent absence of microorganisms (Strasdin & Whitaker 1963).

It remains to be confirmed, however, if cellulase enzymes are produced endogenously by crustacean taxa generally and specifically by GFP, or if they are secreted by symbiotic microorganisms living within the digestive system of eukaryote host organisms as is the case observed for protozoans in the guts of lower termites (Nakashima, Watanebe, Saitoh, Tokuda & Azuma 2002). Cellulase enzymes could also originate from food material consumed by eukaryotes (Walters & Smock 1991; McGrath & Matthews 2000).

Experiments on the Australian wood-eating cockroach Panesthia cribrata demonstrated that cellulases are distributed in the fore- and midgut, but are absent from the protozoa that inhabit the hindgut (Scrivener, Slaytor & Rose 1989). An endogenous endoglucanase gene (RsEG) has also been identified in the termite R. speratus (Watanebe, Noda, Tokuda & Lo 1998). These findings taken together, show that capacity to digest cellulosic material, at least in some higher eukaryotes, does not necessarily depend on presence and activity of symbiotic microbes in the host digestive system. Indeed, some higher animals are capable of synthesising cellulase enzymes endogenously that allows direct digestion of cellulose to simple sugars.

In crustaceans, endogenous cellulase production has been reported in the Australian freshwater redclaw crayfish, Cherax quadricarinatus. The gene
that encodes cellulase, CqEG in redclaw was shown to have originated from the hepatopancreas (Byrne, Lehnert, Johnson & Moore 1999; Crawford, Kricker, Anderson, Richardson & Mather 2004). The redclaw crayfish cellulase is an endoglucanase, more specifically a β-1,4-endoglucanase, and it is a member of Glycosyl Hydrolase Family 9 (GHF9) (Watanabe et al. 1998; Byrne et al. 1999). This endoglucanase has been reported to show homologies with a number of endogenous cellulase enzymes in termites, indicating that it potentially originated from an insect-crustacean progenitor (Watanabe & Tokuda 2001). Since these studies, a number of classes of cellulase enzymes have been identified in various crustacean taxa and other lower eukaryote groups (notably, nematodes and molluscs) (Linton, Greenaway & Towle 2006).

Recognition of endogenous cellulase enzymes and their associated encoding genes in a number of crustacean groups suggests the possibility that these genes are widely conserved across crustacean taxa and potentially that M. rosenbergii could also possess them, which would allow endogenous digestion of cellulosic material. The current study aimed to investigate this possibility and to determine if GFP possessed an endogenous gene(s) that encode cellulase activity. In addition, assay studies were also carried out to quantify the relative levels of cellulase activity in hepatopancreatic tissue in the prawn. Presence of endogenous cellulase activity in GFP would show that this species is capable of digesting cellulose directly, and therefore potentially does not require symbiotic microorganisms within the digestive tract to convert cellulose to simple sugars. Furthermore, confirmation of endogenous cellulase enzymes in GFP could assist development of new low-cost formulated diets for GFP that incorporate appropriate plant material as a source of energy. Potentially, this development could reduce the cost of rearing prawns in culture and hence drive further expansion of the GFP aquaculture industry.

Methods

Genomic analysis

RNA sequencing and analysis

Live M. rosenbergii specimens were obtained from an aquaculture farm in Pantai, and another farm in Sri Sedayan, both farms are in the state of Negeri Sembilan, Malaysia. Individuals were reared in aquaria, equipped with circulating air pumps. RNA from hepatopancreatic tissue from 10 prawns was isolated and pooled using a standard phenol extraction method, and sent to the Beijing Genomics Institute in Shenzhen, China for sequencing. Raw sequencing data were compiled using the Short Oligonucleotide Analysis Package version 2 (SOAP2) (Li, Li, Kristiansen & Wang 2008; Li, Yu, Li, Lam, Yiu, Kristiansen & Wang 2009). Potential cellulase sequences from the processed data were identified via BLAST software analysis of the GenBank database. Prosites were identified using the ScanProsite application in the prosite domain database, PROSITE (de Castro, Sigrist, Gattiker, Bulliard, Langendijk-Genevaux, Gasteiger, Bairoch & Hulo 2006).

Phylogenetic analysis

Phylogenetic trees were constructed using amino acid sequences from identified cellulase fragments [Cluster1859_Consensus1 (Cluster1859), Singletons52022 and Singletons54665]. Sequences were aligned with homologous cellulase gene fragments from other species via the ClustalW Multiple Alignment application (Larkin, Blackshields, Brown, Chenna, McGettigan, McWilliam, Valentin, Wallace, Wilm, Loperz, Thompson, Gibson & Higgins 2007), followed by phylogenetic analysis with MEGA5 software (Tamura, Peterson, Peterson, Stecher, Nei & Kumar 2011). A Maximum Likelihood method, based on the Jones-Taylor-Thornton matrix-based model was used. Maximum bootstrap value was 2000 replications (Felsenstein 1987; Jones, Taylor & Thornton 1992). A number of cellulase sequences from various species were used as outgroups in the phylogenetic analysis.

Presence of cellulase RNA

Quantitative PCR (qPCR) was carried out to validate the presence of cellulase expression in M. rosenbergii specimens. This is to determine whether M. rosenbergii will produce cellulase when cellulosic material is consumed. Experimental GFP individuals were fed with pellets containing 20% microcrystalline cellulose (Avicel PH-102; FMC BioPolymer, Philadelphia, PA, USA) for 3 weeks, while a second control group of specimens were fed with standard commercial feed pellets (containing approximately 35% of protein). Primers for qPCR were designed from the transcriptome sequencing data with β-actin used as the control...
for the gene expression analysis. The RNA required for the process was obtained from hepato-pancreas, gill, muscle and intestine tissues, and converted to cDNA with reverse transcriptase prior to qPCR. The primer pair used was designed from a fragment identified from the transcriptome data that showed homology with cellulase.

qPCR reactions were conducted using an Applied Biosystems Real-Time Thermal Cycler 7500. The standard reaction procedure was conducted as follows; initial denaturation at 95°C for a period of 10 min, followed by a further denaturation at 95°C for 1 min, and then annealing at 55°C for 30 s. qPCR data were collected and compiled into average C_T. Relative gene expression pattern data were deduced using the 2^(-ΔC_T) method outlined by Livak and Schmittgen (2001). ΔC_T was estimated from the difference between average C_T of tissue reacted with Mr52/2 primer pair against average C_T of tissue reacted with beta-actin primer pair. For example:

\[ ΔC_{T,H1} = \text{Average } C_{T,H1} \text{ Mr52/2} - \text{Average } C_{T,H1} \text{ Beta-actin} \]

The ΔΔC_T of each tissue sample was calculated in relative ΔC_T compared with control hepatopancreas tissue (H1): ΔΔC_T,H1. Therefore, for example:

\[ ΔΔC_{T,G2} = ΔC_{T,G2} - ΔC_{T,H1} \]

Following this, relative gene expression patterns of each sample were calculated using 2^(-ΔΔC_T). This allowed comparison of the levels of cellulase expression in cellulose-fed specimens with the control group. A graph was then constructed from the 2^(-ΔΔC_T) data, showing cellulase gene expression.

Protein modelling

Identified cellulase fragments were modelled into proteins using the I-TASSER application, a server-based modelling application maintained at the University of Michigan (Wu, Skolnick & Zhang 2007; Zhang 2007, 2008; Roy, Kucukural & Zhang 2010; Roy & Zhang 2012). The .pdb result files were displayed using the PyMOL Molecular Graphics System version 1.5 (Schrödinger, LLC, New York, USA). The top scoring TM-align results (Zhang & Skolnick 2005) generated from the protein modelling were visualized using PyMOL software, to observe similarities between cellulase proteins.

Cellulase activity assays

Assays were conducted to determine the presence of cellulase activity in GFP hepatopancreatic tissue. Hepatopancreatic tissue was dissected and pooled from individuals fed with food pellets containing 20% (w/v) microcrystalline cellulose over a period of 3 weeks. Homogenates were prepared from the tissue using 1X Tris-buffered saline. Endoglucanase or carboxymethylcellulase (CMCase) activity was determined using a method as described by Wood and Bhat (1988). Assays were conducted by adding 0.2 mL of homogenate to 1.8 mL of 1% (w/v) carboxymethylcellulose (CMC) in 0.05 M citrate buffer. The mixture was then homogenized via vortexing and samples incubated at 40°C for 30 min. 3.0 mL of DNS reagent was then added to measure reducing sugars liberated from enzymatic reactions (Miller 1959). The reaction mixture was boiled at 100°C for 15 min and allowed to cool before 1 mL of 40% Rochelle salt was added. Finally, absorbance was read using a spectrophotometer at 575 nanometer (nm). A glucose standard curve was used as the standard comparison.

One unit of endoglucanase activity is defined as 1 μmol of glucose liberated per mL of enzyme per minute [Eqn (1)]:

\[ \text{Glucose(μmole) } = \frac{OD \times 1000}{M \times RMM} \]  

where OD, absorbance reading of the sample; M, slope of the glucose standard curve; RMM, relative molecular mass of glucose (which is 180).

Glucose results were then calculated from the endoglucanase activity equation [Eqn (2)]:

\[ \text{Endoglucanase activity(U mL}^{-1}) = \frac{\text{Glucose(μmole)}}{V \times 60 \text{ minutes}} \]

where V, volume of sample (mL).

Results

Identification of cellulase transcriptome fragments in GFP

Sequencing of RNA derived from the hepatopancreas of M. rosenbergii and subsequent sequence analysis revealed the presence of three cellulase transcriptome fragments referred to as
Cluster1859_Consensus1 (Cluster1859), Singletons52022 and Singletons54665, based on the names assigned to each cluster and singleton (Mohd-Shamsudin, Kang, Zhao, Tan, Kwong, Liu, Zhang, Othman & Bhassu 2013) using SOAP2 software. Following BLAST analysis, the three fragments were found to show strong sequence homologies with cellulases reported from other eukaryote species, including a crustacean (*C. quadricarinatus* – 59% homology) and a termite (*Coptotermes acinaciformis* – 59% homology) (Wu 2001; Crawford et al. 2004).

Alignment analysis revealed that Cluster1859 and Singletons54665 were very similar in sequence to each other, with Cluster1859 being the larger fragment. Cluster1859 and Singletons52022 possessed an overlapping sequence region, and therefore were considered to constitute sequence components of a larger single gene fragment: Cluster1859+Singletons52022. Both fragments contained active sites belonging to GHF9 (Zhou, Smith, Oi, Koehler, Bennett & Scharf 2007), following prosite pattern analysis.

Phylogenetic analysis applying a Maximum Likelihood approach (Felsenstein 1987), based on the Jones-Taylor-Thornton matrix-based model (Jones et al. 1992), revealed that the identified fragments were more closely related to cellulases reported from crustacean taxa, than to those from bacteria, echinoderms or insects (Fig. 1).

Identified GFP cellulase sequence fragments were translated into protein models using the I-TASSER application (Wu et al. 2007; Zhang 2007, 2008; Roy et al. 2010; Roy & Zhang 2012). Protein modelling results showed that the models of the identified fragments were homologous to a

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**Figure 1** Phylogeny tree of Cluster1859+Singletons52022 (Singletons52022+Cluster1859), in relation with endoglucanases of other species. The accession numbers are: Thermonospora (1JS4), *Cellulomonas flavigena* (AAW62376.2), *Micromonospora* sp. (ADU07460.1), *Micromonospora aurantiaca* (EFA33082.1), *Chloroflexus aggregans* (YP_002464713.1), *Paenibacillus barcinonensis* (CAB38941.1), *Strongylocentrotus purpuratus* (XP_782526.2), *Eisenia andrei* (ACE75510.1), *Coptotermes acinaciformis* (AAK12339.1), *Coptotermes formosanus* (ADB12483.1), *Mastotermes darwiniensis* (CAD54730.1), *Teleogryllus emma* (ABV32557.1), *Limnoria quadripunctata* (ADB85441.1), *Austrothelphusa transversa* (ABA87134.1), *Cherax quadricarinatus* (AAD38027.1), *Euastacus* sp. (AAD87135.1). The analysis was carried out with ClustalW (Larkin et al. 2007) and the Maximum Likelihood method based on Jones-Taylor-Thornton model (Felsenstein 1987; Jones et al. 1992).
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**Cellulase gene expression**

The primary function of cellulase is to hydrolyse cellulose to glucose (Wood 1985). In other animal taxa capable of digesting cellulose endogenously, hydrolysis of cellulose occurs within the digestive tract as reported in termites (O’Brien et al. 1979). Quantitative PCR (qPCR) was employed therefore, to determine cellulase gene expression patterns in a variety of GFP tissues. Tissue samples were taken from the hepatopancreas, gill, muscle and intestine and were analysed to determine relative levels of cellulase expression. qPCR estimates of gene expression patterns were undertaken using the $2^{-\Delta\DeltaCT}$ method (Livak & Schmittgen 2001) and relative expression levels in tissues were then compiled into Fig. 3.

**GFP cellulase activity**

Cellulase activity in the GFP hepatopancreas was determined using CMC as the substrate for the cellulase reaction. Sugar amount in samples were determined by absorbance using a spectrophotometer set at 575 nm compared against a glucose standard. Endoglucanase activity was then calculated. Based on the result calculated and shown in Fig. 4, it was determined that hepatopancreatic homogenates produced endoglucanase activity of 0.695 U mL$^{-1}$.

**Discussion**

Genetic analysis of DNA sequences from _M. rosenbergii_ revealed fragments with homologies to published cellulase genes (Wu 2001; Crawford et al. 2004; Zhang, Lax, Bland & Allen 2011). Furthermore, the fragments showed high similarities with endoglucanases described from other invertebrate species, in particular certain crustacean and insect taxa. The analysis also identified two prosites (de Castro et al. 2006), that are active sites for the GH9, a class of enzymes that include endoglucanases (Zhou et al. 2007). Phylogenetic analysis of the GFP cellulase transcriptome fragments showed clear evolutionary relationships with identified endoglucanase fragments and other cellulases. As Fig. 1 shows, Cluster1859+Singletons52022 showed greatest homology with published crustacean cellulase sequences (Crawford et al. 2004; Linton et al. 2006). These crustacean cellulases were also phylogenetically divergent from cellulases identified in bacteria, annelids and insects. The crustacean cellulase subgroup was however, more closely related to the insect cellulase subgroup than to annelid or bacterial cellulose subgroups, reflecting the more
recent shared ancestry of insects and crustaceans. Given the tissue source and the relationship to eukaryote cellulase sequences, it is therefore highly unlikely that the cellulase identified in the GFP hepatopancreas was sourced from symbiotic microorganisms in their gut.

The protein model developed for Cluster1859+Singletons52022 in GFP showed similarities with other reported cellulases, in particular a cellobiose from Thermomonospora fusca, a filamentous soil bacterium (Sakon, Irwin, Wilson & Karplus 1997). Similarities to the cellulase of a soil bacterium may result from the lack of availability of cellulase gene sequences from other species in the RCSB Protein Data Bank, which the I-TASSER cross referenced during the model generation (Berman, Westbrook, Feng, Gilliland, Bhat, Weissig, Shindyalov & Bourne 2000). The C. quadricarinatus cellulase (Crawford et al. 2004) was also modelled as a protein, and compared with Cluster1859+Singletons52022. The TM-align (Zhang & Skolnick 2005) result showed a homologous region in the upper half of the aligned protein models.

The qPCR results illustrated in Fig. 3 show an increase in cellulase mRNA within GFP hepatopancreatic tissue when specimens were fed with cellulosic material. Cellulase was expressed exclusively in the range of 10² to 10⁴ fold higher compared with a control specimen. Intestinal tissue from GFP fed with cellulose, however, did not apparently express cellulase. These results suggest that cellulase is expressed primarily in the GFP hepatopancreas and that secretion of the enzyme(s) result from consumption of plant-derived cellulosic material (inducible expression of the enzyme).

Assays of enzyme titres resulted in highest expression of cellulase in the GFP hepatopancreas, indicating conversion of cellulose to simple sugars. Results presented in Fig. 4 show presence of glucose when hepatopancreatic tissue homogenate was incubated with CMC due to hydrolyzing activity by cellulase enzymes.

Figure 3 Graph illustrating the cellulase gene expression in relative to the hepatopancreas control samples.

Figure 4 The endoglucanase activity of hepatopancreas after incubation with carboxymethylcellulose (CMC) as substrate.
Conclusions

The current study assessed the potential for \textit{M. rosenbergii} to synthesize cellulase enzymes endogenously, and examined GFP’s ability to digest cellulosic material effectively. Phylogenetic analysis confirmed that the cellulase enzyme identified in \textit{M. rosenbergii} was divergent from bacterial forms, and was most closely related to endogenous cellulases reported recently from other crustacean taxa. Thus, there is strong evidence that GFP cellulase does not originate from any potential symbiotic microorganisms that may be present in the digestive tract of the prawn. Results also indicate that GFP cellulase is expressed primarily in the hepatopancreas and that the enzyme is capable of converting cellu-lose to simple sugars.

Results of the analyses here have potential applications in artificial diet formulation for the \textit{M. rosenbergii} culture industry. New feed formulations that incorporate plant material can reduce the cost of providing nutrients and energy to cultured prawns. This in turn, should decrease the cost of rearing \textit{M. rosenbergii}, and allow for future expansion of the GFP aquaculture industry. As endogenous cellulases have also been identified in other cultured species of crustaceans, there is also potential to apply these findings more widely in the crustacean culture industry and in particular, for the design of improved low-cost diets based on inclusion of cheap plant compounds in aquafeeds for other cultured crustacean taxa as well.

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