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# The last piece in the cellulase puzzle: the characterisation of β-glucosidase from the herbivorous gecarcinid land crab *Gecarcoidea natalis*

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## SUMMARY

**A 160 kDa enzyme with β-glucosidase activity was purified from the midgut gland of the land crab *Gecarcoidea natalis*. The enzyme was capable of releasing glucose progressively from cellobiose, cellotriose or cellotetraose. Although β-glucosidases (EC 3.2.1.21) have some activity towards substrates longer than cellobiose, the enzyme was classified as a glucohydrolase (EC 3.2.1.74) as it had a preference for larger substrates (cellobiose<cellotriose=cellotetraose). It was able to synthesise some cellotetraose by the transglycosylation of smaller substrates – another common feature of glucohydrolases. The interaction between the glucohydrolase described here and the endo-β-1,4-glucanases described previously for *G. natalis* provides a complete model for cellulose hydrolysis in crustaceans and possibly in other invertebrates. After mechanical fragmentation by the gastric mill, multiple endo-β-1,4-glucanases would initially cleave β-1,4-glycosidic bonds within native cellulose, releasing small oligomers, including cellobiose, cellotriose and cellotetraose. The glucohydrolase would then attach to these oligomers, progressively releasing glucose. The glucohydrolase might also attach directly to crystalline cellulose to release glucose from free chain ends. This two-enzyme system differs from the traditional model, which suggests that total cellulose hydrolysis requires the presence an endo-β-1,4-glucanse, a cellobiohydrolase and a β-glucosidase.**

Key words: Crustacea, *Gecarcoidea natalis*, digestion, cellulose, glucohydrolase.

## INTRODUCTION

Herbivorous terrestrial crustaceans, such as gecarcinid land crabs, consume a wide range of plant material, which is rich in cellulose and hemicellulose. Land crabs possess multiple endogenous endo-β-1,4-glucanases, one of the key enzyme classes involved in cellulose hydrolysis (Allardyce and Linton, 2008; Linton et al., 2006), which enable them to digest substantial amounts of cellulose (Greenaway and Linton, 1995). While endo-β-1,4-glucanases have been identified in many invertebrates (reviewed in Watanabe and Tokuda, 2001), a model for total cellulose hydrolysis has yet to be established (Béguin and Aubert, 1994; Enari, 1983). In many species, the enzymes utilised to produce glucose, other than endo-β-1,4-glucanase, have not been identified.

The traditional model for cellulose digestion in invertebrates reflects what is known about fungal cellulase systems. This model suggests that cellulose hydrolysis involves the synergistic activity of endo-β-1,4-glucanases (1,4-β-D-glucan-4-glucanohydrolases, EC 3.2.1.4), exoglucanases, including both cellobiohydrolases (1,4-β-D-glucan cellobiohydrolases, EC 3.2.1.91) and sometimes glucohydrolases (1,4-β-D-glucan glucanohydrolases, EC 3.2.1.74) and β-glucosidases (β-glucoside glucohydrolases, EC 3.2.1.21) (Lynd et al., 2002; Watanabe and Tokuda, 2010). All three enzyme classes must be present in this system in order to produce glucose – endo-β-1,4-glucanases randomly cleave internal glycosidic bonds, releasing chain ends for attack by cellobiohydrolases, which in turn cleave cellobiose units from the non-reducing end of these exposed chains, and β-glucosidases subsequently act on these cellobiose units in order to release glucose (Lynd et al., 2002). Cellobiohydrolases are also considered to be important because they are active towards crystalline cellulose and are able to displace individual chains from the surface of crystalline regions, making it accessible for enzymatic hydrolysis (Schwarz, 2001). By contrast, endo-β-1,4-glucanases primarily act on amorphous regions of native cellulose. β-Glucosidase is necessary not only because it liberates glucose but because it removes cellobiose, a known inhibitor of both endo-β-1,4-glucanase and cellobiohydrolase activity (Lynd et al., 2002; Ward and Moo-Young, 1989).

Like fungal systems, endo-β-1,4-glucanases and β-glucosidases appear to be key components of the cellulase systems of many marine and terrestrial invertebrates (Chararas et al., 1983; Davison and Blaxter, 2005; Ferreira et al., 2001; Linton and Greenaway, 2007; Linton et al., 2006; Pontoh and Low, 2002; Sakamoto et al., 2009; Tokuda et al., 2002; Watanabe and Tokuda, 2010). However, it appears that cellobiohydrolase activity is uncommon among invertebrates (Bärlocher, 1982; McGrath and Matthews, 2000; Sakamoto et al., 2007; Watanabe and Tokuda, 2010), suggesting that many species are capable of producing glucose from crystalline cellulose without requiring a cellobiohydrolase (Scrivener and Slaytor, 1994; Slaytor, 1992; Watanabe and Tokuda, 2010). The characteristics of animal cellulase systems that enable them to function without a cellobiohydrolase are not well understood. One model, as characterised by the wood-eating cockroach *Panesthia cribrata*, involves the interaction of multiple endo-β-1,4-glucanases and β-glucosidases, resulting in total cellulose hydrolysis (Scrivener and Slaytor, 1994). The endo-β-1,4-glucanases in this system are secreted in high concentrations in order to overcome the low activity of these enzymes towards crystalline cellulose. In addition, species that lack a cellobiohydrolase must be capable of converting the soluble oligomers released by the endo-β-1,4-glucanases to glucose

without the intermediate production of cellobiose by a cellobiohydrolase. The β-glucosidases from *P. cribrata* can release glucose from a number of small oligomers, including cellobiose, cellotriose, cellotetraose and cellopentaose (Scrivener and Slaytor, 1994). Therefore, this system might not require the production of cellobiose as the β-glucosidases can directly attack the small oligomers released by the endo-β-1,4-glucanases. The possible absence of a cellobiohydrolase has been noted for other cellulolytic insects such as termites (Watanabe and Tokuda, 2001; Watanabe and Tokuda, 2010), as well as for the freshwater mollusc *Corbicula japonica* (Sakamoto et al., 2009).

Like *P. cribrata*, the terrestrial gecarcinid *Gecarcoidea natalis* possesses multiple endo-β-1,4-glucanases, which are capable of producing small oligomers, including cellobiose and other short oligomers (Allardyce and Linton, 2008). The digestive juice from *G. natalis* is also known to have β-glucosidase activity (Linton and Greenaway, 2004). Given the similarities between this system and that of the *P. cribrata*, it is likely that the β-glucosidase from *G. natalis* is capable of hydrolysing the small oligomers produced by the endo-β-1,4-glucanases and therefore does not require the intermediate activity of a cellobiohydrolase. In order to test this hypothesis, the β-glucosidase from *G. natalis* was isolated from the endo-β-1,4-glucanases described previously (Allardyce and Linton, 2008), and its ability to hydrolyse small glucose oligomers was determined. The presence of a crustacean cellulase system that lacks a cellobiohydrolase would provide further evidence for the presence of such a system among invertebrates.

## MATERIALS AND METHODS

### Preparation of midgut gland homogenate

*Gecarcoidea natalis* Pocock 1888 were collected, maintained and handled as described previously (Allardyce and Linton, 2008). The midgut glands of individual crabs were dissected, pooled and homogenised as described previously (Allardyce and Linton, 2008). A total of three homogenate samples (each consisting of the pooled tissue of three to four animals) were purified as separate repeats; this represented a total of 11 animals. The chromatography results are presented from one of these repeats and are representative of the remaining two repeats.

### Protein separation – ammonium sulphate precipitation

A crude purification of the midgut gland homogenate was performed using ammonium sulphate precipitation, as described previously (Allardyce and Linton, 2008). A 30–60% saturation range was used as it produced the best purification. All liquid-chromatography steps were carried out at 4°C using an Econo Gradient pump and a model 2110 fraction collector (Bio-Rad, Hercules, CA, USA).

### Liquid chromatography

#### Anion-exchange chromatography

Samples for anion-exchange chromatography were dialysed overnight against 20 mmol $l^{-1}$ PIPES with two buffer changes. The dialysate was centrifuged to remove fine particles and applied to a 2.5 cm×60 cm Macro-Prep DEAE column (Bio-Rad). Proteins were eluted from the column at a flow rate of 0.3 ml $min^{-1}$ with the following conditions: 0–220 min, isocratic elution with 20 mmol $l^{-1}$ PIPES buffer (pH 5.5); 220–860 min, sodium chloride concentration in the 20 mmol $l^{-1}$ PIPES buffer (pH 5.5) was increased linearly from 0 mol $l^{-1}$ to 1 mol $l^{-1}$; 860–1060 min, sodium chloride concentration was held at 1 mol $l^{-1}$; 1060–1260 min, sodium chloride concentration was decreased linearly from 1 mol $l^{-1}$ to 0 mol $l^{-1}$; 1260–1460 min, column was re-equilibrated with 20 mmol $l^{-1}$ PIPES buffer (pH 5.5). Over the 1460 min of the chromatography run, 5.5 ml fractions were collected every 18.25 min. Collected fractions were stored at –20°C until analysis. Every third fraction was analysed for protein content and β-glucosidase activity. Fractions containing the enzyme of interest were combined and concentrated by centrifugation at 2060 ***g*** for 15 min at 4°C in a 10,000 nominal-molecular-mass limit Ultra-15, Ultracel-10K filter (Amicon, Houston, TX, USA).

#### Hydrophobic-interaction chromatography (HIC)

Concentrated fractions after purification using anion-exchange chromatography were prepared for hydrophobic-interaction chromatography as described previously (Allardyce and Linton, 2008). Samples were eluted from a 1.5×50 cm methyl HIC column (Bio-Rad) with a flow rate or 0.3 ml $min^{-1}$ under the following conditions: 0–220 min, isocratic elution with 20 mmol $l^{-1}$ PIPES buffer (pH 5.5) containing 1.5 mol $l^{-1}$ ammonium sulphate; 220–860 min, ammonium sulphate concentration in the PIPES buffer was reduced linearly from 1.5 mol $l^{-1}$ to 0 mol $l^{-1}$; 860–1060 min, isocratic elution with 20 mmol $l^{-1}$ PIPES buffer (pH 5.5) containing 0 mol $l^{-1}$ ammonium sulphate; 1060–1260 min, ammonium sulphate concentration in the 20 mmol $l^{-1}$ PIPES buffer (pH 5.5) was increased linearly from 0 mol $l^{-1}$ to 1.5 mol $l^{-1}$; 1260–1460 min, re-equilibration of the column with 20 mmol $l^{-1}$ PIPES buffer (pH 5.5) containing 1.5 mol $l^{-1}$ ammonium sulphate; 5.5 ml fractions were collected every 18.25 min for the entire 1460 min of the chromatography run and stored at –20°C until analysis. Every third fraction was assayed for protein content and cellobiase activity. Fractions containing cellobiase activity were combined and concentrated as described above.

#### Superdex gel-filtration chromatography

Concentrated protein samples from the HIC step were further purified using a 1×30 cm Superdex 200 gel filtration column (GE Healthcare, Chalfont St Giles, Bucks, UK). Samples were eluted isocratically using 0.1 mol $l^{-1}$ sodium acetate buffer (pH 5.5) containing 0.05 mmol $l^{-1}$ sodium chloride at a flow rate of 0.3 ml $min^{-1}$; 3.09 ml (1.03 min) fractions were collected from 18 to 100 min. The protein content of the eluant was detected by the absorption at 280 nm using an EM-1 Econo UV monitor (Bio-Rad).

### Total protein concentration and enzyme activity of chromatography fractions

Fractions eluting from the chromatography steps and the concentrates arising from these steps were assayed for both total protein content and β-glucosidase activity. Protein concentrations were determined using the Bradford protein assay (Bio-Rad) as per the manufacturer's instructions. Cellobiase activity of fractions and concentrates arising from the liquid-chromatography steps was measured as the rate of production of glucose from the hydrolysis of cellobiose (Sigma, St Louis, MO, USA, cat. no. C7252). The assay for the production of glucose has been described previously (Linton and Greenaway, 2004). One unit of enzyme was defined as 1 μmol of glucose produced per minute.

### Non-denaturing SDS-PAGE and enzyme activity staining

The enzyme activity of both the midgut gland homogenate and partially purified enzyme were measured by staining samples run on a non-denaturing SDS-PAGE gel. Samples were first diluted so that the final amount of protein in each lane would be either 5 μg (for homogenate samples) or 10 μg (for partially purified samples). Fifteen μl of these diluted samples were mixed with 15 μl of SDS-PAGE sample buffer; 20 μl of this final solution were then pipetted

into each lane. Electrophoresis was performed using an 8×10 cm, 12% polyacrylamide gel at a current of 30 mA, as per the method of Laemmli (Laemmli, 1970). To retain enzyme activity, samples were not heated or treated with β-mercaptoethanol. Protein samples were run alongside a lane containing 7 µl of Mark 12 unstained molecular mass standards (Invitrogen, Carlsbad, CA, USA, cat. no. LC5677).

After electrophoresis, gels were stained for either carboxymethyl cellulase activity or β-glucosidase activity. To detect β-glucosidase activity, gels were rinsed briefly with distilled water and washed with 2.5% Triton X-100 dissolved in 0.1 mol $l^{-1}$ sodium acetate buffer (pH 5.5) for 25 min on a rocking platform (in order to remove SDS). Gels were then soaked in 0.1 mol $l^{-1}$ sodium acetate buffer (pH 5.5) for 10 min to renature the enzymes. This buffer was poured off and replaced with the same buffer containing 0.1 mmol $l^{-1}$ of the fluorescent substrate 4-methylumbelliferyl β-D-glucopyranoside (Sigma, cat. no. M3633). Gels were incubated with this solution for 10 min before visualising under ultraviolet light; β-glucosidase activity was identified by the presence of fluorescent bands.

Carboxymethyl cellulase activity was detected using an assay based on that of Xue and colleagues (Xue et al., 1999). Following electrophoresis, gels were removed from the casting plates, rinsed briefly with distilled water and incubated in 2.5% Triton X-100 dissolved in 0.1 mol $l^{-1}$ acetate buffer (pH 5.5) for 25 min. The gel was then incubated for 20 min in 0.1 mol $l^{-1}$ sodium acetate buffer (pH 5.5) before being transferred to a 60°C oven for 2 min to remove excess water. Gels were carefully laid on a thin (approximately 2 mm) 2% agarose gel containing 0.2% carboxymethyl cellulose for 30 min at room temperature to allow the enzymes in the polyacrylamide gel to hydrolyse the carboxymethyl cellulose in the agarose gel. After incubation, the reactions were stopped by icing the two gels. The agarose gel was cut to the exact size of the polyacrylamide gel to allow the accurate location of any bands, after which the two gels were separated. The agarose gel was stained with 0.2% Congo red for 30 min and then destained with 1 mol $l^{-1}$ sodium chloride. Areas of cellulase activity were detected as clear bands against a dark background. To increase contrast between the bands and the background, gels were covered in 5% acetic acid, and the background staining changed from red to dark violet-blue.

After staining for either β-glucosidase or carboxymethyl cellulase activity, the protein bands of the molecular mass standards in the polyacrylamide gel were counterstained by incubating overnight on a rocking platform with 0.05% Coomassie Brilliant Blue R (Sigma, cat. no. B7920) and dissolved in an aqueous solution of 10% acetic acid and 40% methanol. This gel was then destained for 3 h in an aqueous solution of 10% acetic acid and 40% methanol. Photographs of the same activity-stained and Coomassie-stained gel were scaled to the same size within CorelDraw X3, and the size of the activity bands was estimated by plotting the log of molecular mass against the relative migration distance of the standards. All molecular masses are expressed as means ± s.e.m.

### Substrate kinetics

The kinetic parameters of the purified enzyme were calculated by incubating enzyme samples with cellobiose (Sigma), cellotriose (Sigma, cat. no. C1167) or cellotetraose (Sigma, cat. no. C8286). At least six different reactions were performed for each substrate, with final concentrations ranging from 0.075 to 5 mmol $l^{-1}$ (for cellobiose) and 0.075 to 1.5 mmol $l^{-1}$ (for cellotriose and cellotetraose). Each reaction was assayed for the production of glucose, as described previously (Linton and Greenaway, 2004). The $V_{max}$ and $K_m$ values for each substrate were calculated by plotting a regression based on the Michaelis–Menten model using the software Sigmaplot, version 11 (Systat Software, San Jose, CA, USA). The $K_m$ value for each substrate was averaged over three replicates, and the mean value for each substrate compared using a one-way ANOVA, followed by a Tukey's highly significant post-hoc analysis (Zar, 1999), using Sigmaplot. $K_m$ values are expressed as means ± s.e.m.

## RESULTS

### Partial purification of β-glucosidase using liquid chromatography

#### Anion-exchange chromatography

Two peaks with cellobiase activity were eluted after DEAE anion-exchange chromatography of ammonium-sulphate-precipitated midgut gland homogenate (Fig. 1). The first of these peaks (labelled Peak 1) eluted within fractions 32–36, with peak activity in fraction 34, whereas the second peak (Peak 2) eluted within fractions 38–48, with a peak in fraction 40. Peak 2 was chosen for further purification as it had higher β-glucosidase activity when incubated with cellobiose. Fractions 38–48 were combined, concentrated and applied to the methyl-HIC column.

#### Hydrophobic-interaction chromatography

Separation of the concentrated Peak 2 sample using HIC revealed a single peak contained within fractions 31–43, with the highest activity recorded in fraction 34 (Fig. 2). These fractions were combined, concentrated and applied to a Superdex gel-filtration column.

#### Superdex gel-filtration chromatography

A single β-glucosidase peak was detected from the Superdex gel filtration chromatography, with peak activity in fraction 41 (Fig. 3). The molecular mass of this protein was calculated to be 169.3±59.1 kDa ($N$=9 replicates total, over three purification runs) when compared with the elution of the protein standards. Fractions 37–45 were combined and concentrated; this concentrate had a specific activity of 0.228 µmol glucose $min^{-1}$ mg $protein^{-1}$, with a purification factor of 3.4 (Table 1).

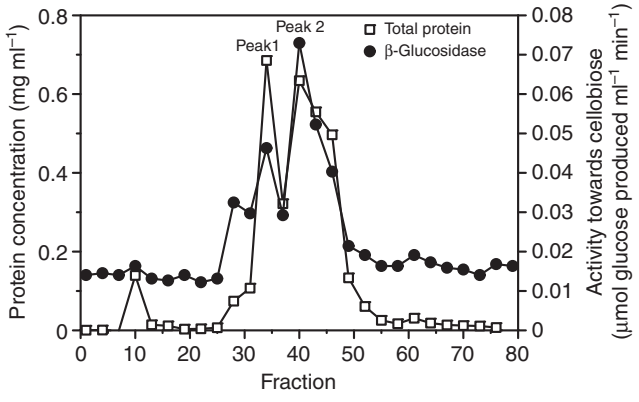


Fig. 1. Anion-exchange chromatography plot of resuspended midgut gland homogenate after precipitation with 30–60% ammonium sulphate. Plot shows protein concentration (mg $ml^{-1}$) and β-glucosidase activity in the collected fractions. β-Glucosidase activity was measured as the rate of production of glucose from the hydrolysis of cellobiose (µmol glucose produced $ml^{-1}$ $min^{-1}$).

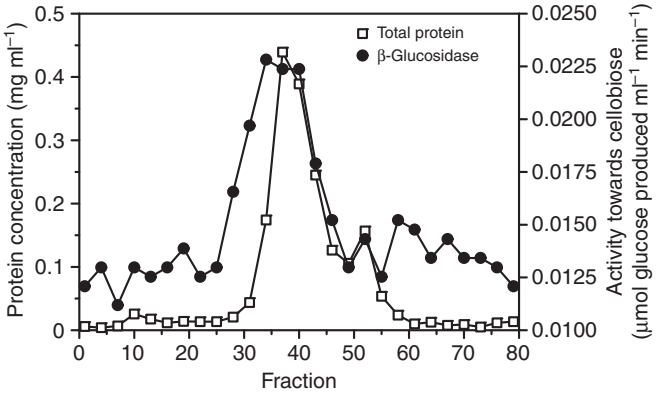


Fig. 2. Hydrophobic-interaction chromatography of combined fractions 38–48 from the anion-exchange chromatography for *G. natalis*. Plot shows protein concentration (mg $ml^{-1}$) and β-glucosidase activity in the collected fractions. β-Glucosidase activity was measured as the rate of production of glucose from the hydrolysis of cellobiose (μmol glucose produced $ml^{-1}\,min^{-1}$).

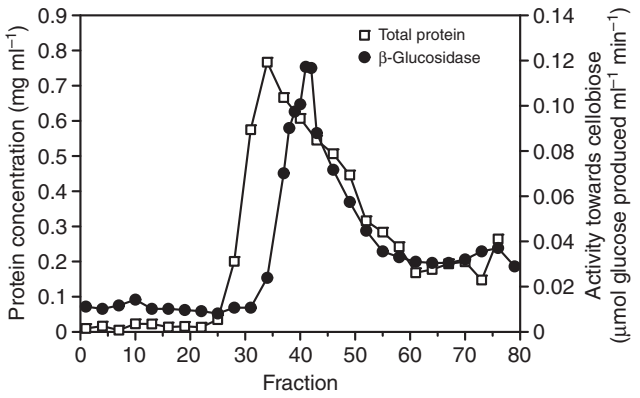


Fig. 3. Superdex gel-filtration chromatography of combined fractions 31–43 from the HIC purification step from *G. natalis*. Plot shows protein concentration (mg $ml^{-1}$) and β-glucosidase activity in the collected fractions. β-Glucosidase activity was measured as the rate of production of glucose from the hydrolysis of cellobiose (μmol glucose produced $ml^{-1}\,min^{-1}$).

### PAGE enzyme activity staining of midgut gland homogenate and partially purified enzyme

Activity staining of the midgut gland homogenate from *G. natalis* revealed three distinct β-glucosidase bands, with approximate sizes of 128.2±2.4, 31.0±0.1 and 27.1±0.1 kDa ($N$=4 homogenate samples; Fig. 4A). The 31 and 27 kDa bands corresponded to cellulase activity bands, as measured by hydrolysis of carboxymethyl cellulose on an agarose gel (Fig. 4B); the larger 128.2 kDa β-glucosidase band showed no activity towards carboxymethyl cellulose (Fig. 4B).

After purification, only a single 130.3±1.4 kDa band with β-glucosidase activity was detected (Fig. 4C); no activity was detected towards carboxymethyl cellulose (Fig. 4D). Thus, although the enzyme was not completely pure, it was successfully isolated away from the potentially contaminating cellulases found in the midgut gland homogenate.

### Characteristics of the partially purified enzyme

The partially purified β-glucosidase was capable of releasing glucose from cellobiose, cellotriose and cellotetraose (Fig. 5A–C). The enzyme released detectable levels of glucose within 5 min, regardless of the oligosaccharide substrate. Virtually none of the original substrates remained after 120 min, by which time only glucose and cellobiose were detected. The enzyme was able to release glucose units progressively from each substrate. Some cellotetraose was produced within the first 5 min from incubation with cellobiose, but this was hydrolysed again during the following 5 min (Fig. 5A). When incubated with cellotriose, both glucose and cellobiose were released within 5 min (Fig. 5B). As the incubation progressed, the cellobiose was hydrolysed subsequently to glucose (Fig. 5B). Low levels of cellotetraose were also produced during the first 30 min when the enzyme was incubated with cellotriose (Fig. 5B). This cellotetraose was again digested as the incubation continued. Cellotetraose was similarly hydrolysed to cellotriose and glucose (Fig. 5C). The cellotriose was subsequently hydrolysed to cellobiose, and the cellobiose to glucose, so that, after 120 min, virtually all of the substrate had been reduced to glucose (Fig. 5B,C).

Based on a one-way ANOVA of the Michaelis–Menten enzyme kinetics with a Tukey's post-hoc test, the relative affinity of the partially purified enzyme was: cellobiose<cellotriose=cellotetraose (ANOVA: $P$=0.06; Fig. 6). The $K_m$ value for cellobiose (1.72±0.22 mmol $l^{-1}$) was significantly higher than that for either cellotriose (0.69±0.17 mmol $l^{-1}$; $P$=0.011) or cellotetraose (0.63±0.09; $P$=0.009). The $K_m$ for cellotriose was statistically similar to that for cellotetraose ($P$=0.970). Cellobiose was hydrolysed fastest, with a $V_{max}$ of 0.188 μmol glucose produced $min^{-1}$ mg $protein^{-1}$. The $V_{max}$ values for cellotriose and cellotetraose were slightly lower than for cellobiose at 0.138 and 0.143 μmol glucose produced per minute per milligram of protein, respectively. The relative differences between the $V_{max}$ values for each substrate

Table 1. Specific activity, purification factor, units of enzyme and percentage yield for each step in the partial purification of β-1,4-glucosidase from *G. natalis* by using liquid chromatography

| Fraction | β-1,4-Glucosidase specific activity (μmol $min^{-1}$ mg $protein^{-1}$) | Purification factor | Units of β-1,4-glucosidase recovered | Percentage yield |
|---|---|---|---|---|
| Homogenate | 0.067 | 1 | 27.7 | |
| Redissolved ammonium sulphate precipitate | 0.042 | 0.6 | 17.5 | 63.2 |
| Combined fractions 38–48 after anion-exchange chromatography | 0.100 | 1.5 | 3.4 | 12.1 |
| Combined fractions 31–43 after HIC | 0.114 | 1.7 | 1.7 | 6.1 |
| Combined fractions after Superdex gel-filtration chromatography | 0.228 | 3.4 | 0.2 | 0.7 |

Purification factors and percentage yields are expressed, respectively, as a proportion and percentage of that in the initial homogenate. Units of enzyme are given as μmol glucose produced per minute.

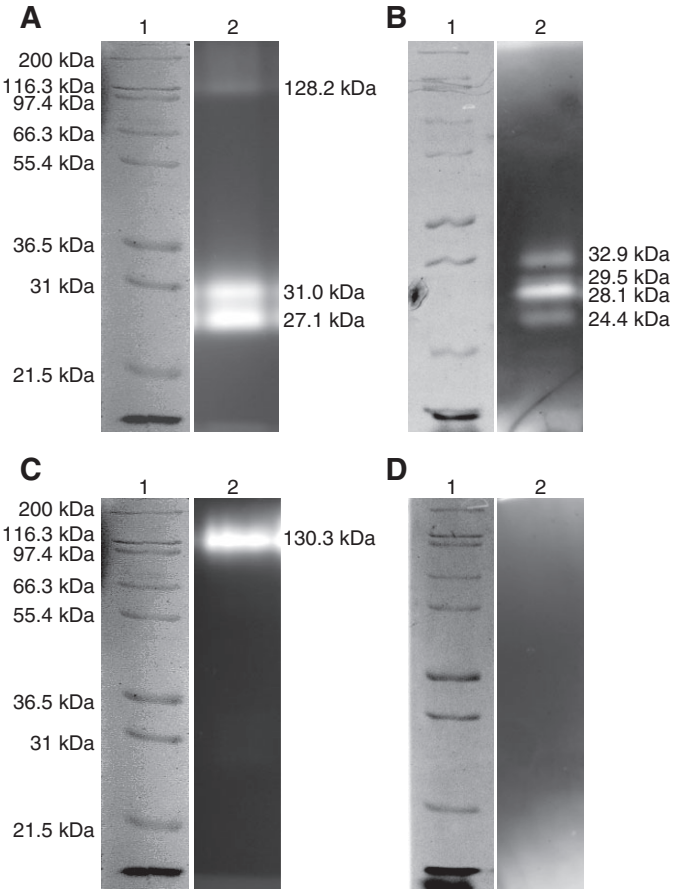


Fig. 4. Activity-stained polyacrylamide gels of midgut gland homogenate (A,B) and partially purified β-glucosidase (C,D) from *G. natalis*. Samples were stained for either β-glucosidase activity (A,C) or endo-β-1,4-glucanase activity (B,D). (A,C) Lane 1, molecular mass standards stained with Coomassie blue. Lane 2, midgut gland homogenate (A) or partially purified β-glucosidase (C) stained with 4-methylumbelliferyl β-D-glucopyranoside to identify β-glucosidase activity. (B,D) Separate gels showing ladder stained with Coomassie blue (Lane 1) and midgut gland homogenate (B) or partially purified β-glucosidase (D) when incubated with 0.2% carboxymethyl cellulose (CMC) and stained with 2% Congo red (Lane 2).

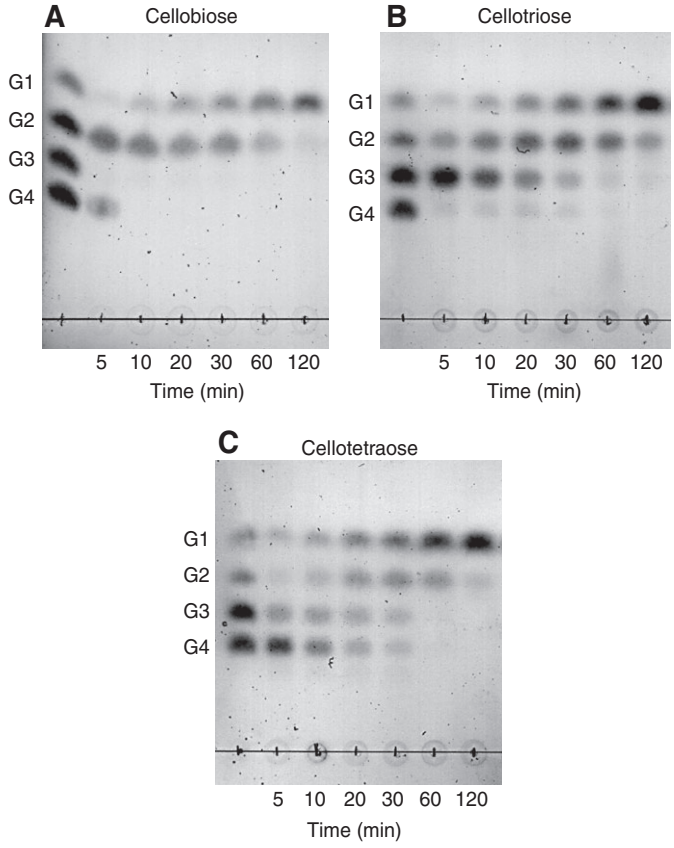


Fig. 5. Thin-layer chromatography separation of the oligosaccharides released when incubating partially purified β-glucosidase with cellobiose (A), cellotriose (B) and cellotetraose (C). All samples were run against the standards glucose (G1), cellobiose (G2), cellotriose (G3) and cellotetraose (G4). Separate reactions with each substrate were stopped after 5, 10, 20, 30, 60 and 120 min.

were confirmed in three replicate enzyme samples. However, as each sample had a different overall purification factor, the curve for each sample differed by an order of magnitude on the *Y*-scale. For this reason, data for only a single representative sample were included.

## DISCUSSION

Separation of the midgut gland homogenate from *G. natalis* using liquid chromatography yielded a partially purified β-glucosidase with activity towards cellobiose. The activity peak of this enzyme was estimated to be 169 kDa when separated by gel-filtration chromatography. This peak corresponded to a single fluorescent activity band of approximately 130 kDa when incubated with 4-methylumbelliferyl β-D-glucopyranoside under non-denaturing conditions (Fig. 4). Although the partially purified enzyme was active towards both 4-methylumbelliferyl β-D-glucopyranoside and cellobiose, it cannot be classified as a true β-glucosidase (EC 3.2.1.21) as these usually have higher affinity for cellobiose and decreasing affinity for longer substrates (Hrmova et al., 1998). As the enzyme described here had a higher affinity for cellotriose and cellotetraose than for cellobiose, it is suggested that it is a glucohydrolase (EC 3.2.1.74). Glucohydrolases are exoglucanases that release glucose from small polysaccharides (cellobiose and larger), with a higher affinity for longer substrates (Yernool et al., 2000). As the enzyme was purified from the midgut gland, which produces other cellulases endogenously (Allardyce and Linton, 2008), it is likely that glucohydrolase is also an endogenous enzyme of *G. natalis*. Although the digestive tract of decapod crustaceans does harbour bacteria that might synthesise the glucohydrolase, the crustacean midgut gland appears to contain an order of magnitude fewer bacteria than either the hindgut or foregut (Gulmann, 2004). Anaerobic fermentation is also absent from the foregut of the gecarcinid crab *Cardisoma guahumi*, suggesting that decapod crabs are not dependent on symbiotic cellulases (Wolcott and Wolcott, 1987).

Although classified as a glucohydrolase, the enzyme purified from *G. natalis* was similar in size to β-glucosidases found in other arthropods, such as the larvae of the moth *Erinnyis ello* (Santos and Terra, 1985) and the rice weevil *Sitophilus oryzae* (Baker and Woo, 1992). The β-glucosidase from *S. oryzae*, like the glucohydrolase from *G. natalis*, was active towards both cellobiose and 4-methylumbelliferyl β-D-glucopyranoside. It is therefore possible that the enzyme found in *S. oryzae* is similar to that from *G. natalis* and,

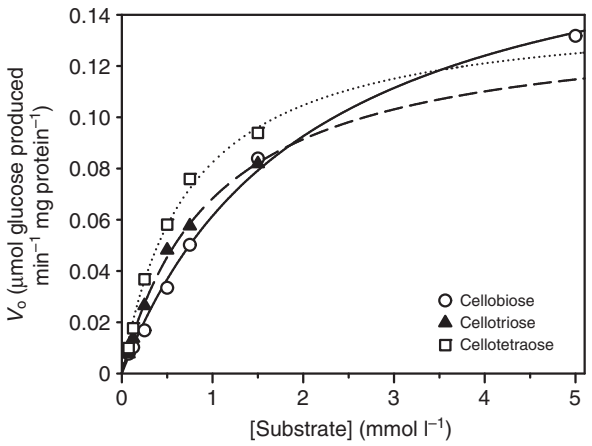


Fig. 6. Glucose released by partially purified β-glucosidase when incubated with increasing concentrations of cellobiose, cellotriose and cellotetraose. Lines indicate Michaelis–Menten regressions.

therefore, might also be a glucohydrolase. Glucohydrolase activity has been noted in other insects, such as the larvae of the cardinal beetle *Pyrochroa coccinea* (Chararas et al., 1983) and the termite *Coptotermes lacteus* (Hogan et al., 1988), but was typically considered to be a minor component of cellulose digestion (Scrivener and Slaytor, 1994). These enzymes have therefore largely been ignored when discussing cellulolytic systems in invertebrates.

The enzymatic digestion of cellulose by *G. natalis* presumably occurs through the interaction between the endo-β-1,4-glucanases described previously (Allardyce and Linton, 2008) and the glucohydrolase described here. The endo-β-1,4-glucanases would initially attack cellulose, randomly cleaving amorphous regions to release chain ends and reduce the degree of polymerisation (Fig. 7). The endo-β-1,4-glucanases might also be capable of releasing small oligomers of random length from these regions; such activity is believed to improve the digestion of native cellulose in insect systems (Watanabe and Tokuda, 2001). The activity of these endo-β-1,4-glucanases towards native cellulose would be complemented by that of the glucohydrolase described here (Fig. 7). Small oligomers released by the endo-β-1,4-glucanase would be directly hydrolysed by the glucohydrolase (Fig. 7). Glucohydrolases, like cellobiohydrolases, are also known to be active towards crystalline cellulose (Hogan et al., 1988). Therefore, the glucohydrolase identified from *G. natalis* might be able to release glucose directly from the chain ends of crystalline regions of native cellulose. The synergistic activity of the endo-β-1,4-glucanases and glucohydrolase from *G. natalis* would therefore be similar to the interactions between endo-β-1,4-glucanases and cellobiohydrolases in fungal systems (Lynd et al., 2002), except that the product would be glucose rather than cellobiose.

Although relatively few invertebrate cellulase systems have been fully characterised, it appears that other systems share the ability to digest cellulose without a cellobiohydrolase. Cellobiohydrolases appear to play no role in the initial digestion of cellulose by termites and wood-eating cockroaches (Watanabe and Tokuda, 2010); cellulolytic systems from these insects consist of multiple endo-β-1,4-glucanases and β-glucosidases (Genta et al., 2003; Schulz et al., 1986; Scrivener and Slaytor, 1994; Slaytor, 1992; Tokuda et al., 1999). Other invertebrate endo-β-1,4-glucanases are known to produce cellobiose, cellotriose and cellotetraose (Watanabe and Tokuda, 2010; Xu and Distel, 2004), whereas partially purified β-glucosidases from other species are able to hydrolyse cellotriose, cellotetraose and cellopentaose (Ferreira et al., 2001; Ferreira et al., 2003; Marana et al., 2000). It is possible that the cellulase system from these animals employs a similar mechanism to that of *G. natalis*. In addition to insects, cellulose hydrolysis in the mollusc *Corbicula japonica* occurs as a result of an endo-β-1,4-glucanase, with some activity towards crystalline cellulose, and a β-glucosidase (Sakamoto et al., 2007). This further strengthens the argument that cellobiohydrolases are absent from a broad range of invertebrate animal phyla.

Although the presence of an efficient cellulase enzyme system is important for the digestion of cellulose, other factors might affect the efficiency of hydrolysis. Mechanical fragmentation using the mandibles and gastric mill is an important component of overall cellulose degradation (Watanabe and Tokuda, 2010). The gastric mill of *G. natalis* consists of sharp, robust features that are well suited to shredding plant material, thus allowing access to cellulose in the cell walls (Allardyce and Linton, 2010). This process increases the surface area of cellulose exposed to the enzymes within the digestive juice, therefore increasing the efficiency of enzymatic breakdown (Jeoh et al., 2007). The mechanical disruption of cellulose increases the efficiency of enzymatic breakdown by increasing the surface area of cellulose exposed to the enzymes within the digestive juice (Jeoh et al., 2007) and might further reduce the need for a cellobiohydrolase by improving access to glucose residues normally buried within crystalline regions of cellulose

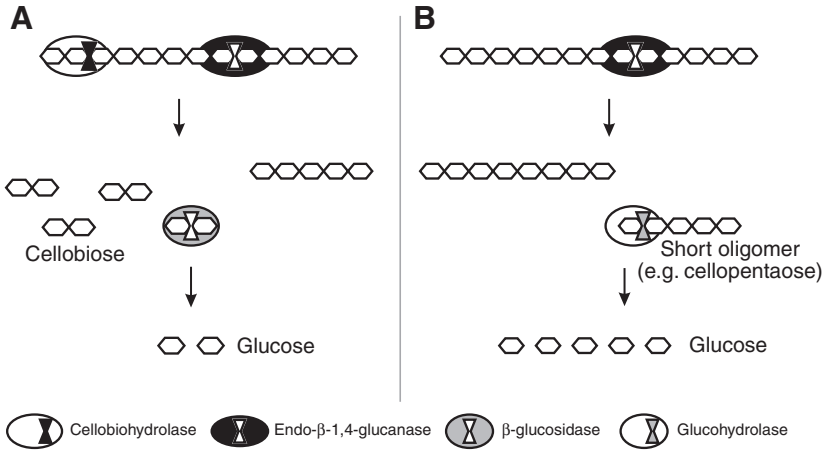


Fig. 7. (A) Traditional model for the hydrolysis of cellulose, based on the system employed by some fungi. Cellobiohydrolases act on chain ends, progressively releasing cellobiose units into solution. Endo-β-1,4-glucanase randomly cleaves cellulose, exposing new chain ends. β-Glucosidase subsequently cleaves cellobiose units, releasing glucose. Diagram adapted from Watanabe and Tokuda (Watanabe and Tokuda, 2010). (B) Proposed model for cellulose hydrolysis in *G. natalis*. Endo-β-1,4-glucanase randomly cleaves cellulose, releasing small oligomers (two or more residues) into solution. Glucohydrolase progressively releases glucose directly from these oligomers. Enzymes and substrates are not drawn to scale.

fibres. This is in contrast to fungal and bacterial systems, which lack a mechanical mill, relying instead on the presence of a cellobiohydrolase and its synergistic activity with the endo-β-1,4-glucanase to digest crystalline cellulose efficiently (Watanabe and Tokuda, 2010).

Another important factor affecting cellulose digestion is the accessibility of cellulose as a result of crosslinked hemicellulose molecules; cellulose digestibility can be improved by any pre-treatment that removes hemicellulose (Jeoh et al., 2007). The digestive juice of *G. natalis* contains laminarinase, lichenase and xylanase (Linton and Greenaway, 2004) activity, suggesting that it is able to digest hemicellulose, thereby increasing the access of cellulases to the cellulose. The laminarinase from *G. natalis* was also able to release a significant amount of glucose from laminarin (Allardyce and Linton, 2008), indicating that hemicellulose can also be utilised as an energy source.

The glucohydrolase from *G. natalis* was able to produce some cellotetraose when incubated with both cellobiose and cellotriose. Transglycosylation reactions have been noted in fungal β-glucosidases such as the 110 kDa enzyme from *Fusarium oxysporum*. It was capable of producing oligomers, including cellotetraose, when incubated with cellobiose (Christakopoulos et al., 1994). Unlike the β-glucosidase from *F. oxysporum*, the enzyme from *G. natalis* transiently produced cellotetraose without producing any cellotriose. This suggests the ligation of two cellobiose units by transcellobiosylation rather than the progressive transglycosylation of cellobiose to cellotriose, and finally to cellotetraose. Transcellobiosylation reactions are a feature of fungal exocellulases (Hayashi et al., 2003; Okada and Nisizawa, 1975). A similar transient ligation step has been observed for β-glucosidase from *P. cribrata* and the mealworm *Tenebrio molitor* (Ferreira et al., 2001; Scrivener and Slaytor, 1994). Transglycosylation reactions are typical of glucohydrolases; it is believed that such reactions play a role in activating the enzyme through facilitating the production of certain oligomeric substrates (Yernool et al., 2000), as well as in the induction of cellulase gene expression (Mach et al., 1995).

## CONCLUSIONS

Cellulose digestion in *G. natalis* appears to involve four key steps. First, the specialised gastric mill of *G. natalis* shreds fibrous plant material, tearing open cellulose-rich cell walls and exposing them to the digestive enzymes. Second, hemicellulases such as laminarinase, lichenase and xylanase hydrolyse hemicellulose fibres, further increasing access by the cellulases and releasing some glucose. Third, the endo-β-1,4-glucanases attack cellulose, increasing the number of chain ends available for subsequent hydrolysis and releasing small oligomers into solution. Finally, the glucohydrolase from *G. natalis* hydrolyses not only the small oligomers released by the endo-β-1,4-glucanases but possibly also the chain ends of cellulose directly. The end-result of these interactions would be the release and subsequent absorption of sufficient glucose to meet the energy demands of the animal.

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