Presence and properties of cellulase and hemicellulase enzymes of the gecarcinid land crabs *Gecarcoidea natalis* and *Discoplax hirtipes*

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Summary

Digestive juice from the herbivorous gecarcinid land crabs *Gecarcoidea natalis* and *Discoplax hirtipes* exhibited total cellulase activity and activities of two cellulase enzymes; endo-β-1,4-glucanase and β-1,4-glucosidase. These enzymes hydrolysed native cellulose to glucose. The digestive juice of both species also contained laminarinase, licheninase and xylanase, which hydrolysed laminarin, lichenin and xylan, respectively, to component sugars. The pH optima of β-1,4-glucosidase, endo-β-1,4-glucanase and total cellulase from *G. natalis* were 4–5.5, 5.5 and 5.5–7, respectively. In the digestive juice from *D. hirtipes*, the corresponding values were 4–7, 5.5–7 and 4–9, respectively. The pH of the digestive juice was 6.69±0.03 for *G. natalis* and 6.03±0.04 for *D. hirtipes* and it is likely that the cellulases operate near maximally *in vivo*. In *G. natalis,* total cellulase activity and endo-β-1,4-glucanase activity were higher than in *D. hirtipes*, and the former species can thus hydrolyse cellulose more rapidly. β-1,4-glucosidase from *G. natalis* was inhibited less by glucono-D-lactone (K_i=11.12 mmol l^{-1}) than was the β-1,4-glucosidase from *D. hirtipes* (K_i=4.53 mmol l^{-1}). The greater resistance to inhibition by the β-1,4-glucosidase from *G. natalis* may contribute to the efficiency of the cellulase system *in vivo* by counteracting the effects of product inhibition and possibly dietary tannins. The activity of β-1,4-glucosidase in the digestive juice of *D. hirtipes* was higher than that of *G. natalis*.

Key words: land crab, *Gecarcoidea natalis*, *Discoplax hirtipes*, cellulase, endo-β-1,4-glucanase, cellobiohydrolase, β-1,4-glucosidase, laminarinase, xylanase, licheninase, fibre digestion.

Introduction

Plant material contains significant amounts of structural polysaccharides, particularly cellulose and a range of hemicelluloses. These polymers are comprised of sugar units, usually glucose, joined by β-1,4 and β-1,3 glycosidic bonds. Whilst vertebrates generally lack the enzymes necessary to break these bonds, and can only access the sugars *via* the activity of microorganisms in the alimentary canal, cellulose digestion is common amongst invertebrates, and an increasing number have been shown to possess their own cellulases (Watanabe and Tokuda, 2001).

Crystalline or native cellulose is hydrolysed to its component glucose units by the combined activities of endo-β-1,4-glucanase (EG), cellobiohydrolase (CBH) and β-1,4-glucosidase. Endo-β,1-4-glucanase randomly hydrolyses internal β-1,4-glycosidic bonds of cellulose polymers of four or more glucose units (Scrivener and Slaytor, 1994; Tokuda et al., 1997; Wantanabe et al., 1997). It thus shortens cellulose chains, solubilizes cellulose polymers and provides a substrate for exo-β,1-4-glucosidase. However, it can also catalyse transglycosylation to rejoin the glucose oligomers (Lindner et al., 1983). Cellobiohydrolase attacks the non-reducing ends of cellulose chains and typically cleaves off the glucose-β-1,4

dimer, cellobiose. Cellobiohydrolase is also thought to disrupt the hydrogen bonding in crystalline cellulose, thus allowing the EG to endo-depolymerize. As this enzyme only attacks the ends of chains, its activity will be low unless endoglucanase is present to provide sufficient substrate. In the cockroach *Panesthia cribrata* and the termite *Reticulitermes speratus*, CBH activity is also catalyzed by the EG (Scrivener and Slaytor, 1994; Watanabe et al., 1997). The third enzyme involved in the complete hydrolysis of cellulose to glucose is β-1,4-glucosidase; this enzyme hydrolyses cello-oligosaccharides to glucose.

The term hemicellulose includes a range of alkali-soluble polysaccharides (Bacic et al., 1988). More specifically, they are defined as carbohydrate polymers of either xylose, glucose, mannose or mannose and glucose joined mainly by β-1,4 and β-1,3 glycosidic bonds (Bacic et al., 1988). Xylan, lichenin, β-D-glucan and laminarin are common hemicelluloses. Xylan, the next most abundant carbohydrate polymer after cellulose, is a β-1,4-linked polymer of xylose (Puls et al., 1988). Lichenin is a glucose polymer with the glucose units being joined with mainly β-1,4 glycosidic bonds and some β-1,3 glycosidic bonds in the chain (Bacic et al., 1988). Similar

polysaccharides, called mixed linkage β-glucan, are found in cereals and grasses (Terra and Ferreira, 1994). Like lichenin, laminarin (or callose) is a glucose polymer but with the sugars joined principally by β-1,3 glycosidic bonds (Bacic et al., 1988; Terra and Ferreira, 1994). Laminarin is present in the cell walls of fungi, in phloem and in plant wound tissue (Terra and Ferreira, 1994). Laminarin is the chief food reserve of algae (Vonk and Western, 1984). Xylanase, licheninase and laminarinase hydrolyse xylan, lichenin and laminarin, respectively (reviewed by Terra and Ferreira, 1994).

The diet of the gecarcinid land crabs *Gecarcoidea natalis* and *Discoplax hirtipes* mainly consists of green and brown leaves (Greenaway and Linton, 1995; Greenaway and Raghaven, 1998) of which cellulose and the hemicelluloses are major constituents. Cellulose makes up 12–18% of the dry matter of brown leaves of the fig (*Ficus macrophylla*) and 11% of the dry matter of green leaves (Greenaway and Linton, 1995; Greenaway and Raghaven, 1998), while hemicelluloses constitute 18–26% of the dry matter of brown fig leaves (Greenaway and Linton, 1995; Greenaway and Raghaven, 1998). Both *G. natalis* and *D. hirtipes* assimilate substantial amounts of cellulose and hemicellulose from their leaf litter diet (Greenaway and Linton, 1995; Greenaway and Raghaven, 1998); 43% and 49%, respectively, by *G. natalis* and 21% and 20%, respectively, by *D. hirtipes* fed brown leaf litter of *F. macrophylla* (Greenaway and Linton, 1995; Greenaway and Raghaven, 1998). As the crabs clearly hydrolyse these fibre components, it is probable that they possess cellulase and hemicellulase enzymes. In the present study, we investigated the presence and characteristics of cellulases and hemicellulases from *G. natalis* and *D. hirtipes*. Total cellulase activity and activities of β-1,4-glucosidase, EG, licheninase, laminarinase and xylanase were measured within the digestive juice of both species. Where possible, the kinetics (V_{max} and K_m) of the cellulase enzymes and the inhibitory constant (K_i) of glucono-D-lactone on β-1,4-glucosidase were also determined. It was envisaged that differences in the activities of the cellulase enzymes may explain reported differences in the assimilation of cellulose and hemicellulose between these two species.

Materials and methods

Materials

Gecarcoidea natalis (Pocock 1888) and *Discoplax hirtipes* (Dana 1851) were collected from rainforest in the Australian Territory of Christmas Island, Indian Ocean and airfreighted to Sydney where they were maintained at 25°C on a 12 h:12 h light:dark cycle. Tapwater was provided for drinking and the crabs were fed with fallen leaves of *Ficus macrophylla* Desf. ex Pers. subspecies *macrophylla*.

Methods

Digestive juice used in measurements was taken from the foreguts of experimental animals as follows. The crabs were held ventral side up on a polystyrene board, and a fine polythene tube was inserted into the cardiac stomach *via* the mouth and oesophagus. A small plastic wedge was used to prevent the mandibles from cutting the tube. Up to 2 ml of dark brown digestive juice could be collected by gentle suction with a 2-ml syringe attached to the tubing. The procedure did not harm the crabs. Fluid was centrifuged at 10 000 *g* for 5 min, to remove food debris, and the supernatant was used for analyses. Fluid could be stored at 4°C for several days without loss of enzyme activity.

Measurement of enzyme activities

Cellulase activities

Total cellulase activity and activities of β-1,4-glucosidase (cellobiase; EC 3.2.1.21) and EG (EC 3.2.1.4) were measured in digestive juice taken from the cardiac stomach of *Gecarcoidea natalis* and *Discoplax hirtipes* using modified versions of the methods of Schulz et al. (1986) and Hogan et al. (1988). Reactions and incubations were carried out at 40°C in 1.5-ml Eppendorf centrifuge tubes in an Eppendorf thermomixer. Measurement at 40°C allowed direct comparison with data for cellulase activities of other invertebrates. Absorption values of the samples were measured using an LKB Ultraspec II spectrophotometer. Activities of the enzymes are presented per ml of digestive juice. Expression per mg of protein is not meaningful in this situation where crude juice is used since the bulk of the protein does not represent the enzyme of interest, is highly variable and may even be dietary in origin. It is likely that the volume of fluid in the foregut remains relatively constant. Hanes plots derived from the data on cellulase activity at different substrate concentrations were used to determine if enzyme activity followed Michaelis-Menten kinetics. Where this was established, the kinetic parameters (K_m, V_{max}) were then calculated from the plots.

β-1,4-glucosidase. Activity was measured as the rate of production of glucose from cellobiose (Cat. No. C-7252; Sigma Chemical Corp., St Louis, MO, USA). Digestive juice (25 µl) was mixed with 25 µl of 0.1 mol l^{-1} acetate buffer (pH 5.5) and 50 µl of either 2.92, 14.61, 29.21 or 58.4 mmol l^{-1} cellobiose in the same buffer, and the mixture was incubated at 40°C for 30 min. The reaction was stopped by the addition of 25 µl of 0.3 mol l^{-1} tri-chloro acetic acid, and excess acid was neutralized with 5 µl of 2.5 mol l^{-1} K$_2$CO$_3$. Precipitated protein was pelleted by centrifugation at 10 000 *g* for 10 min. A blank (75 µl buffer plus 25 µl digestive juice) and a standard (50 µl of 7 mol l^{-1} glucose in buffer + 25 µl digestive juice + 25 µl buffer) were prepared for each sample analysed. This enabled correction for the background absorption due to the digestive juice at the wavelength measured.

Glucose concentration was measured in 50 µl (*G. natalis*) or 25 µl (*D. hirtipes*) samples of the incubation mixture using a commercial glucose assay kit (Sigma Cat No. 510-A). The 50 µl or 25 µl samples were diluted to a total of 100 µl with water in a 1.5-ml micro test tube. 1 ml of the colour reagent supplied with the kit was then added, and the mixture was vortexed and incubated at 37°C for 30 min. After incubation, the absorbance of the samples was read at 445 nm.

Glucono-D-lactone competitively inhibits β-1,4-glucosidase (Scrivener and Slaytor, 1994; Shewale and Sadana, 1981; Santos and Terra, 1985). The inhibitory constant of glucono-D-lactone on β-1,4-glucosidase was also determined by measuring β-1,4-glucosidase activity in the presence of 20 mmol l^{-1} glucono-D-lactone and 0–58.43 mmol l^{-1} cellobiose.

Endo-β-1,4-glucanase. Activity was measured as the rate of production of reducing sugars from the substrate, carboxymethyl cellulose (Sigma Cat. No. C-5678). Digestive juice (20 µl) was mixed with 80 µl buffer and 100 µl of 0.1, 0.5, 1 and 2% (w/v) carboxymethyl cellulose in the same buffer. Blanks contained 20 µl of digestive juice and 180 µl of buffer, while standards contained 20 µl of digestive juice plus 100 µl glucose (13 mmol l^{-1}) in buffer and 80 µl buffer. The buffer was 0.1 mol l^{-1} acetate buffer, pH 5.5, containing 30 mmol l^{-1} of the β-1,4-glucosidase inhibitor glucono-D-lactone. Samples, standards and blanks were incubated at 40°C for 10 min and the reaction stopped by the addition of 25 µl of 0.3 mol l^{-1} HCl. Excess acid was then neutralized by the addition of 5 µl of 2.5 mol l^{-1} K$_2$CO$_3$. The reducing sugar produced during the incubation was measured as glucose equivalents by the tetrazolium blue method of Jue and Lipke (1985) using 5 mmol l^{-1} glucose as a standard. Absorption of samples, standards and blanks was read at 660 nm.

Total cellulase activity. Total cellulase activity was measured as the rate of production of glucose from microcrystalline cellulose (Sigmacell 20). Digestive juice (50·µl) was mixed with 100 µl of either 0.1, 0.5, 1 or 2% (w/v) Sigmacell 20 (Sigma Cat. No. S3504) made up in buffer. Suspension of the cellulose was ensured by vortexing the stock cellulose immediately before pipetting. Blanks contained digestive juice and buffer while standards contained digestive juice, buffer and 7 mmol l^{-1} glucose. The buffer used was 0.1 mol l^{-1} acetate, pH 5.5. The mixture was incubated and agitated for 60 min at 40°C in an Eppendorf thermomixer before the reaction was stopped by the addition of 25 µl of 0.3 mol l^{-1} tri-chloro acetic acid. The excess acid was neutralized with 5 µl of 2.5 mol l^{-1} K$_2$CO$_3$ before assay of glucose. The incubation mixture was centrifuged (10 000 *g* for 10 min) and the glucose concentration determined in a 25 µl or 50 µl aliquot of the supernatant as described for β-1,4-glucosidase.

The K_m values for EG and CBH are given as mg substrate ml^{-1} reaction mixture since the substrates (carboxymethyl cellulose and cellulose) consist of carbohydrate polymers of varying length and do not have a uniform molecular mass.

Hemicellulase activities

Activities of the hemicellulase enzymes laminarinase [endo-β-1,3-glucanase (EC 3.2.1.39)], licheninase [endo-β-1,3; 1,4 glucanase (EC 3.2.1.73)], xylanase [endo-β-1,4-xylanase (EC 3.2.1.8) and 1,4-β-D-xylan xylanhydrolase (EC 3.2.1.37)] were measured in the digestive juice from *D. hirtipes* and *G. natalis*.

Laminarinase. Laminarinase activity was measured as the production of reducing sugars from the hydrolysis of laminarin (from *Laminaria digitata*; Sigma Cat. No. L-9634). Digestive juice (20 µl) was mixed with 50 µl of 1% (w/v) laminarin and 130 µl of 0.1 mol l^{-1} Na acetate buffer, pH 5.5. Blanks and standards were run at the same time. Blanks consisted of 20 µl of digestive juice and 180 µl of assay buffer, while standards consisted of 20 µl of digestive juice, 100 µl of 13 mmol l^{-1} glucose and 80 µl of assay buffer. Samples, blanks and standards were incubated with agitation at 40°C for 10 min. The reaction was stopped by the addition of 50 µl of 0.3 mol l^{-1} HCl and neutralized with 10 µl of 2.5 mol l^{-1} K$_2$CO$_3$. Reducing sugars were measured in a 10 µl aliquot as described above.

Licheninase. The activity of licheninase was measured by the production of reducing sugars from the hydrolysis of lichenin (from *Cetraria islandica*; Sigma Cat. No. L-6133). Digestive juice (20 µl) was mixed with 100 µl of 0.1% (w/v) lichenin and 80 µl of 0.1 mol l^{-1} Na acetate buffer, pH 5.5. To correct for the background absorbance of the digestive juice, blanks and standards were run at the same time. Digestive juice (20 µl) and 180 µl of assay buffer constituted the blank while 20 µl of digestive juice plus 100 µl of 13 mmol l^{-1} glucose and 80 µl of assay buffer constituted the standard. Samples, blanks and standards were incubated with agitation at 40°C for 10 min. The reaction was stopped with 50 µl of 0.3 mol l^{-1} HCl and the mixture was then neutralized with 10 µl of 2.5 mol l^{-1} K$_2$CO$_3$. Reducing sugars were measured in a 10 µl sample as described above.

Xylanase. Xylanase activity was measured as the production of reducing sugars from the hydrolysis of xylan (from birchwood, *Betula*; Sigma Cat. No. X-0502). Digestive juice (20·µl) was incubated with 100 µl of 1% (w/v) xylan and 80 µl of 0.1 mol l^{-1} Na acetate buffer, pH 5.5. Blanks (20 µl of digestive juice and 180 µl of buffer) and standards (20 µl of digestive juice, 100 µl of 13 mmol l^{-1} glucose and 80 µl of assay buffer) were run at the same time. Samples, blanks and standards were incubated with agitation at 40°C for 60 min. After this period, the reaction was stopped by precipitating the protein with 50 µl of 0.3 mol l^{-1} HCl and was neutralized by the addition of 10 µl of 2.5 mol l^{-1} K$_2$CO$_3$. Reducing sugars were measured in a 10 µl sample of this reaction mixture as described above.

pH

The activities of β-1,4-glucosidase, EG and total cellulase were measured over a pH range of 4–9. Acetate buffer was used to maintain pH values of 4 and 5.5, and Tris buffer was used for the higher pH values of 7–9. The pH of gut fluid was measured anaerobically at 25°C using freshly drawn samples of fluid from the foregut and a Radiometer G299a capillary pH electrode and Radiometer PHM 73 meter (Radiometer, Copenhagen, Denmark).

Protein

The concentrations of protein in samples of digestive juice were measured using a BioRad protein assay kit and bovine γ-globulin standard (BioRad, Hercules, CA, USA).

Statistics

Statistical comparisons (ANCOVA and one- and two-way ANOVA with Tukey's HSD *post hoc* tests) were made using the statistical computing package Systat 7 (Systat Software Inc., Richmond, CA, USA) to calculate the statistical probabilities.

Results

Cellulase activity in digestive juice

Incubation of crystalline cellulose with digestive juice from the foregut of *G. natalis* and *D. hirtipes* yielded glucose and thus established the presence of total cellulase activity (Fig. 1). As such activity is likely to result from the combined activity of CBH, EG and β-1,4-glucosidase, assays were then conducted to test for activity of each enzyme (Figs 1–3).

The presence of EG was confirmed by the production of reducing sugars during the incubation of carboxymethyl cellulose with digestive juice (Fig. 2). Carboxymethyl cellulose is not a substrate for either CBH or β-1,4-glucosidase. In similar fashion, the presence of β-1,4-glucosidase in digestive juice was confirmed by the production of significant quantities of glucose when cellobiose was incubated with digestive juice (Fig. 3). This enzyme is generally ineffective against crystalline or carboxymethyl cellulose. The activities of total cellulase, EG and β-1,4-glucosidase, from both *G. natalis* and *D. hirtipes*, initially increased with substrate concentration but levelled out at high concentrations (Figs 1–3), consistent with saturation of these enzymes at the higher substrate concentrations.

pH and cellulase activities

Foregut fluid from both study species was slightly acidic, with that of *D. hirtipes* (6.03±0.04, N=7) significantly more acid than the juice from *G. natalis* (6.69±0.03, N=4) (*t*-test; P<0.001). The activities of β-1,4-glucosidase, EG and total cellulase from both *G. natalis* and *D. hirtipes* were affected by the pH of the incubation buffer (Table 1) and shared similar pH maxima and hence pH ranges for optimal activity. For total cellulase from *G. natalis*, optimal activity occurred at pH 5.5, but for *D. hirtipes* total cellulase activities were low with no obvious pH optimum (Table 1). For β-1,4-glucosidase, optimal activity occurred between pH 4 and 7 (Table 1); high levels of activity were measured at both pH 4 and pH 5.5 for *G. natalis* and at pH 4, 5.5 and 7 for *D. hirtipes* (Table 1). Endo-β-1,4-glucanase activities were maximal at pH 5.5, 7 and 9 and lower at pH 4 and 8 in digestive juice from both *D. hirtipes* and *G. natalis* (Table 1).

Enzyme activities

Total cellulase activity

The total cellulase activity on crystalline cellulose was higher in the digestive juice from *G. natalis* than in that from *D. hirtipes* (Fig. 1); 2.8× higher at the highest substrate concentration used of 13.3 mg ml^{-1} cellulose (Table 2). Total cellulase activity from *G. natalis* followed Michaelis-Menten kinetics since the Hanes plot of its activity was linear (r^2=0.624, P=0.001; Fig. 4). Total cellulase activity had a K_m of 2.43 mg ml^{-1} cellulose incubation mixture and a V_{max} of 0.117 μmol min^{-1} ml^{-1} digestive juice (Table 2). By contrast, the Hanes plot of total cellulase activity from *D. hirtipes* was
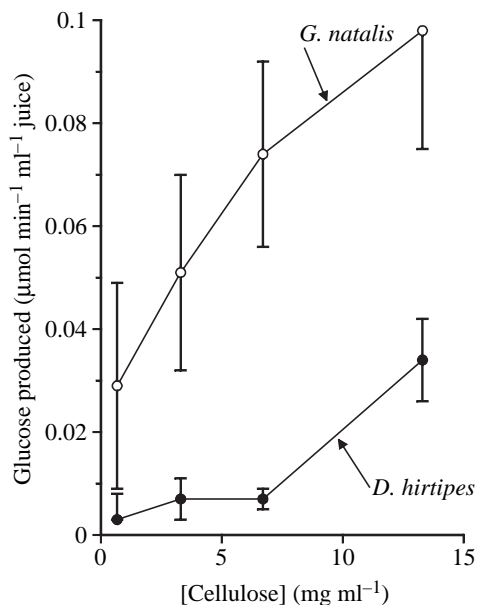

Fig. 1. Total cellulase activity in digestive juice from *G. natalis* and *D. hirtipes*. Activity differed significantly among cellulose concentrations (P<0.05) and between *G. natalis* and *D. hirtipes* (two-way ANOVA).
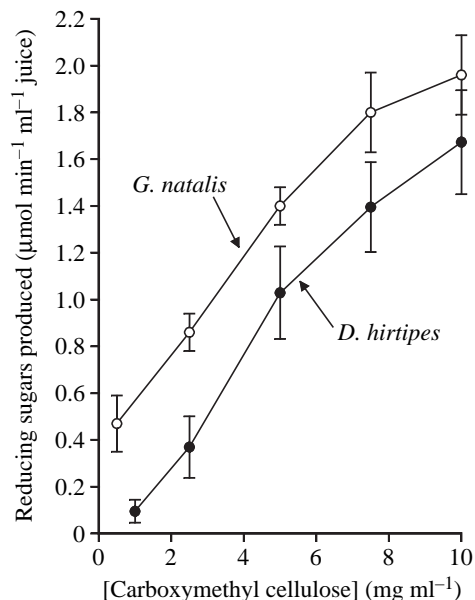

Fig. 2. Activities of endo-β-1,4-glucanase in digestive juice of *G. natalis* and *D. hirtipes*. Enzyme activities were significantly different (P<0.05) between concentrations of carboxymethyl cellulose and between species (two-way ANOVA).
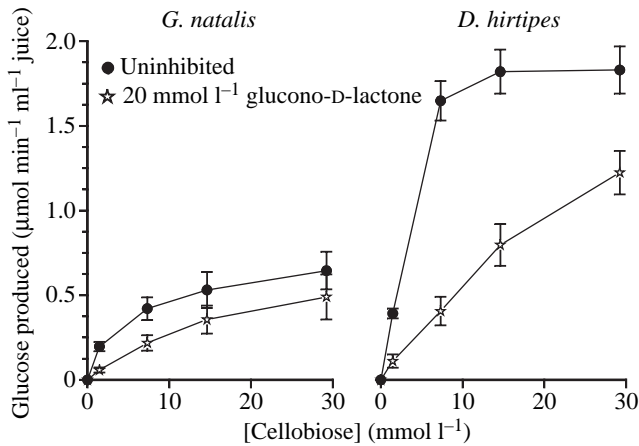
Fig. 3. Activities of β-1,4-glucosidase activities in digestive juice of *G. natalis* and *D. hirtipes* in the presence (stars) and absence (circles) of 20 mmol l^{-1} glucono-D-lactone. For both *G. natalis* and *D. hirtipes*, the β-1,4-glucosidase activity increased with increasing cellobiose concentrations and was higher in the absence of glucono-D-lactone (*P*<0.05; two-way ANOVA). For β-1,4-glucosidase activity from *D. hirtipes*, there was a significant interaction (*P*<0.05) between cellobiose concentration and treatment (two-way ANOVA). Uninhibited β-1,4-glucosidase activities from *D. hirtipes* were higher than uninhibited β-glucosidase activities from *G. natalis* (*P*<0.05; two-way ANOVA).

not linear (r^2=0.003, *P*=0.774; Fig. 4) and did not follow Michaelis-Menten kinetics (Fig. 4), so its kinetic parameters could not be calculated.

Endo-β-1,4-glucanase

Endo-β-1,4-glucanase activities in digestive juice were slightly, but consistently, higher in *G. natalis* than in *D. hirtipes* (*P*<0.05; two-way ANOVA; Fig. 2). The differences in activities between species were maintained across the range

of substrate concentration used. Endo-β-1,4-glucanase activity from *G. natalis* was 1.17× the EG activity from *D. hirtipes* at a substrate concentration of 10 mg ml^{-1} CMC (Table 2). The Hanes plot of the EG activity from *G. natalis* was linear (r^2=0.514, *P*=0.0001) while that from *D. hirtipes* was not (r^2=0.041, *P*=0.2; Fig. 5). Hence, the EG from *G. natalis* followed Michaelis-Menten kinetics (Fig. 5) with a K_m of 3.03 mg ml^{-1} CMC incubation mixture and a V_{max} of 6.11 μmol min^{-1} ml^{-1} digestive juice (Table 2). Equivalent values could not be calculated for EG from *D. hirtipes*.

β-1,4-glucosidase

The activity of β-1,4-glucosidase from *D. hirtipes* was higher than that from *G. natalis* (*P*<0.05; two-way ANOVA; Fig. 3). At a cellobiose concentration of 29.22 mmol l^{-1}, the activities differed by a factor of 2.8 (Fig. 3; Table 2). β-1,4-Glucosidase from both species followed Michaelis-Menten kinetics since Hanes plots of β-glucosidase activities were linear (Fig. 6). The V_{max} of β-1,4-glucosidase from *D. hirtipes* was 3.4× higher than that from *G. natalis* (*P*<0.005; ANCOVA; Table 2) and the K_m value for β-1,4-glucosidase from *D. hirtipes* was lower than that from *G. natalis* (*P*<0.001; ANCOVA; Table 2). Hence, β-1,4-glucosidase activity from *D. hirtipes* saturated at lower concentrations of cellobiose and was higher at all substrate concentrations than β-1,4-glucosidase from *G. natalis*.

Effect of inhibitor on the activity of β-1,4-glucosidase

V_{max} values for inhibited and uninhibited β-glucosidase were similar in both *G. natalis* and *D. hirtipes* (Figs 3, 6; Table 2). The K_m of β-1,4-glucosidase in the presence of 20 mmol l^{-1} glucono-D-lactone was much higher than in its absence (Figs 3, 6; Table 2).

This situation (similar V_{max} but different K_m values in the presence and absence of an inhibitor) is indicative of competitive inhibition of β-1,4-glucosidase by glucono-D-

Table 1. *The effect of pH on the activity of cellulase enzymes from the foregut of the gecarcinid crabs* Gecarcoidea natalis *and* Discoplax hirtipes

Enzyme	Activity (μmol min^{-1} ml^{-1} digestive juice)				
	pH 4	pH 5.5	pH 7	pH 8	pH 9
Total cellulase					
G. natalis	0.018±0.016[a]	0.043±0.016[b]	0.017±0.010[a]	0.021±0.020[a]	0.011±0.011[a]
D. hirtipes	0.014±0.005[a]	0.020±0.005[a]	0.023±0.006[a]	0.005±0.002[a]	0.010±0.003[a]
β-1,4-glucosidase					
G. natalis	0.397±0.253[a,b]	0.521±0.253[a]	0.254±0.131[b,c]	0.075±0.032[c,d]	0.031±0.019[d]
D. hirtipes	1.779±0.083[a]	1.729±0.066[a]	1.961±0.074[a]	0.214±0.046[b]	0.121±0.028[b]
Endo-β-1,4-glucanase					
G. natalis	0.297±0.174[a]	2.076±0.104[b]	2.304±0.116[b]	0.945±0.189[c]	1.981±0.106[b]
D. hirtipes	0.627±0.092[a]	1.534±0.206[b]	1.434±0.101[b]	0.710±0.169[a]	1.041±0.151[a,b]

Total cellulase and β-1,4-glucosidase activities were measured as the rate of glucose production from the hydrolysis of either crystalline cellulose or cellobiose, respectively. Endo-β-1,4-glucanase activities were measured as the rate of reducing sugars produced from the hydrolysis of carboxymethyl cellulose. Within a row, similar superscript letters indicate similar means (*P*>0.05). Values are means ± S.E.M. (*N*=10).

Table 2. *Kinetic parameters (K$_m$, V$_{max}$) and activities of total cellulose, endo-β-1,4-glucanase and β-1,4-glucosidase from the foregut fluid of* Gecarcoidea natalis *and* Discoplax hirtipes

Enzyme	Gecarcoidea natalis			Discoplax hirtipes		
	Activity (μmol min^{-1} ml^{-1})	K_m	V_{max} (μmol min^{-1} ml^{-1})	Activity (μmol min^{-1} ml^{-1})	K_m	V_{max} (μmol min^{-1} ml^{-1})
Total cellulase	0.01±0.23	2.43 mg ml^{-1} cellulose	0.117	0.03±0.01		
Endo-β-1,4-glucanase	1.96±0.17	3.03 mg ml^{-1} CMC	6.11	1.67±0.22		
β-1,4-glucosidase						
Uninhibited	0.655±0.11	5.84 mmol l^{-1} cellobiose[c]	0.606[a]	1.83±0.14	4.23 mmol l^{-1} cellobiose[f]	2.08[e]
Inhibited (20 mmol l^{-1} glucono-D-lactone)	0.49±0.13	16.28 mmol l^{-1} cellobiose[d]	0.721[a]	1.22±0.13	22.87 mmol l^{-1} cellobiose[g]	2.16[e]

Activities of total cellulase, endo-β-1,4-glucanase (EG) and β-1,4-glucosidase were measured at substrate concentrations of 13.3 mg ml^{-1} cellulose, 10 mg ml^{-1} carboxymethyl cellulose (CMC) and 29.22 mmol l^{-1} cellobiose, respectively. Enzyme activities are expressed as μmol glucose min^{-1} ml^{-1} digestive juice (total cellulase and β-1,4-glucosidase) or as μmol reducing sugars min^{-1} ml^{-1} (EG). Inhibited β-glucosidase from both *G. natalis* and *D. hirtipes* had a similar V_{max} and a significantly higher K_m than the uninhibited enzyme. Within a column, significant differences between values for β-glucosidase are indicated by different superscript letters. There were also significant differences in K_m and V_{max} between species for uninhibited β-1,4-glucosidase. Values are means ± S.E.M.

lactone in both species. Glucono-D-lactone has also been identified as a competitive inhibitor of β-1,4-glucosidase in previous studies (Scrivener and Slaytor, 1994; Shewale and Sadana, 1981; Santos and Terra, 1985). The inhibitory constant of glucono-D-lactone on β-1,4-glucosidase (K_i) was calculated using K_m values from Table 2 and the equation:

$$K_i = (K_m[I]) / (K_{app} - K_m) ,$$

where K_m is the K_m for β-1,4-glucosidase in the absence of glucono-D-lactone, K_{app} is the apparent K_m for β-1,4-glucosidase in the presence of 20 mmol l^{-1} glucono-D-lactone, and [I] is the concentration of glucono-D-lactone used (20 mmol l^{-1}).

The K_i of glucono-D-lactone on β-1,4-glucosidase was 11.12 mmol l^{-1} for β-1,4-glucosidase from *G. natalis* and 4.53 mmol l^{-1} for β-1,4-glucosidase from *D. hirtipes*.

Hemicellulase activities in the digestive juice

Incubation of laminarin, lichenin or xylan with digestive juice from *G. natalis* or *D. hirtipes* yielded reducing sugars (Table 3), an outcome consistent with the presence of laminarinase, licheninase and xylanase in the digestive juice. The activities of the enzymes in the digestive juice of both *G. natalis* and *D. hirtipes* were in the order laminarinase > licheninase > xylanase (Table 3). Licheninase activity in the digestive juice from *D. hirtipes* was 1.73× that of *G. natalis* (Table 3) whilst the activities of laminarinase were similar between species and this was also the case for xylanase (Table 3).

Activities of the individual hemicellulases (licheninase, laminarinase and xylanase) measured were higher than those for total cellulase activity in the two species (Tables 2, 3). Laminarinase activity was much higher than the activity of EG and β-1,4-glucosidase (Tables 2, 3). For *G. natalis*, licheninase activity was similar to EG activity but higher than β-1,4-glucosidase activity (Tables 2, 3). Licheninase activity from *D. hirtipes* was higher than activities of both EG and β-1,4-glucosidase (Tables 2, 3). Xylanase activities for both *G. natalis* and *D. hirtipes* were lower than the respective EG and β-glucosidase activities (Tables 2, 3).

Discussion

Both *Gecarcoidea natalis* and *Discoplax hirtipes* digest significant amounts of the cellulose and hemicellulose present in their diets (Greenaway and
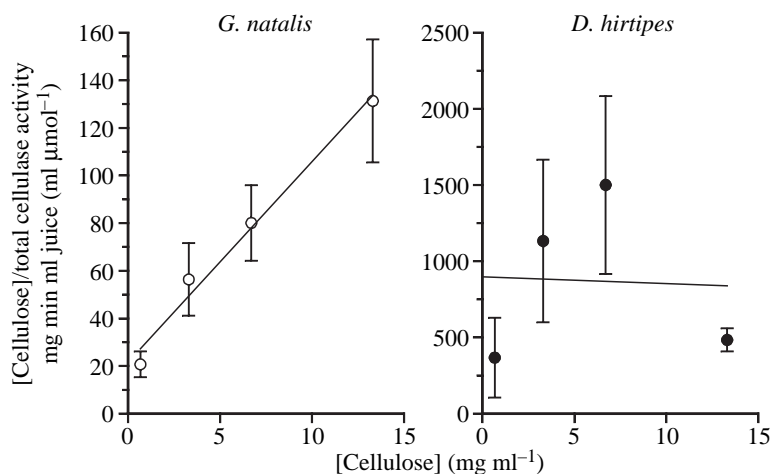

Fig. 4. Hanes plots of the activity of total cellulase activity from digestive juice of *G. natalis* and *D. hirtipes*. The plot was linear for *G. natalis* {[Cellulose]/total cellulase activity=8.52×[cellulose]+20.82 (r^2=0.624, P=0.001, N=7)} but not for *D. hirtipes* {[Cellulose]/total cellulase activity=952×[cellulose]–12.659 (r^2=0.003, P=0.774, N=10)}.
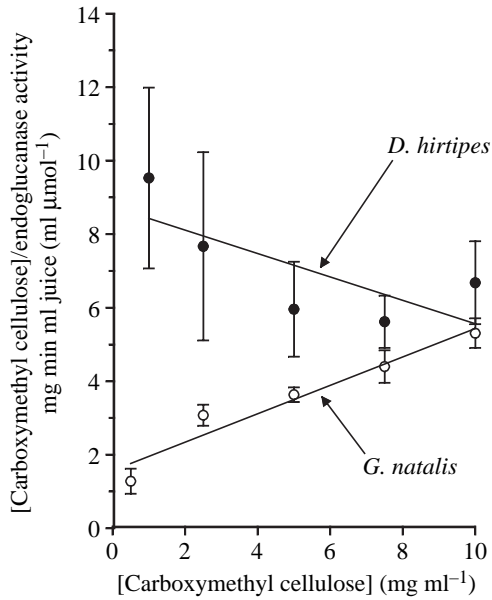
Fig. 5. Hanes plots of the activity of endo-β-1,4-glucanase activity from the digestive juice of *G. natalis* and *D. hirtipes*. The plot was linear for *G. natalis* {[CMC]/endo-β-1,4-glucanase activity= 0.329×[CMC]+2.018 (r^2=0.514, *P*=0.0001, *N*=7)} but not for *D. hirtipes* {[CMC]/endo-β-1,4-glucanase activity=−0.298×[CMC]+ 8.588 (r^2=0.041, *P*=0.2, *N*=10)}.

Linton, 1995; Greenaway and Raghaven, 1998) and in this study it has been demonstrated that this capacity is conferred by activity of cellulolytic and hemicellulolytic enzymes. The confirmed cellulase enzymes present were EG and β-1,4-glucosidase and the hemicellulases were laminarinase, licheninase and xylanase. Several invertebrates lack a separate CBH, and the CBH reaction is instead catalysed by EG (Scrivener and Slaytor, 1994; Watanabe et al., 1997). The presence of a separate CBH in the digestive juice of gecarcinid crabs remains to be established but, as in other animals, it may be absent.
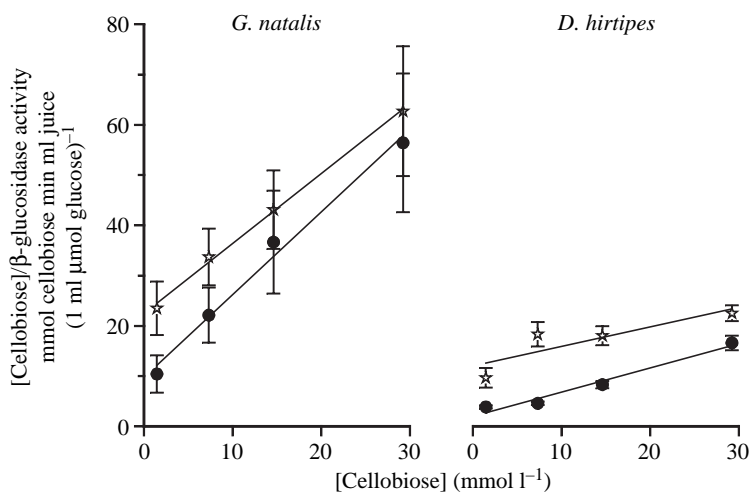

Table 3. *Activities of laminarinase, licheninase and xylanase in the digestive juice from* G. natalis *and* D. hirtipes *(μmol reducing sugars min⁻¹ ml⁻¹ digestive juice)*

	G. natalis	*D. hirtipes*
Laminarinase	12.09±0.49 (8)[a]	12.98±0.97 (8)[a]
Licheninase	1.76±0.19 (9)[a]	3.04±0.52 (7)[b]
Xylanase	0.33±0.05 (7)[a]	0.22±0.03 (8)[a]

Values are means ± S.E.M. (*N*). Similar superscript letters within a row indicate similar means (*P*>0.05).

Cellulase activity

Total cellulase and EG activities from *G. natalis* were higher than those from *D. hirtipes* (Figs 1, 2) and this should allow more rapid hydrolysis of cellulose by *G. natalis*, which is consistent with the earlier reports of higher cellulose assimilation efficiency in the latter species (Greenaway and Linton, 1995; Greenaway and Raghaven, 1998).

In *G. natalis*, the K_m for EG was similar to values reported for other invertebrates (Tables 2, 4). The EG activity reported here may well exceed the *in vivo* value since the experimental substrate (carboxymethyl cellulose), unlike the natural substrate (crystalline cellulose), is in solution and more available for enzymatic hydrolysis.

β-1,4-glucosidase

β-1,4-glucosidase activity in the digestive juice exhibited a higher V_{max} and substrate affinity in *D. hirtipes* than in *G. natalis* (Fig. 3; Table 2). This contrasted with the findings for the other cellulase enzymes – EG and total cellulase activity – where activity was higher in *G. natalis*. This difference may be explained by the affinity of β-1,4-glucosidase for the inhibitor glucono-D-lactone and consequent substrate and product affinity (discussed below).

Competitive inhibition of β-1,4-glucosidase with glucono-D-lactone

The inhibition constant (K_i) for β-1,4-glucosidase was higher in *G. natalis* (11.12 mmol l⁻¹) than in *D. hirtipes* (4.53 mmol l⁻¹), and hence the β-1,4-glucosidase from *G. natalis* had a lower affinity for the inhibitor than that from *D. hirtipes*. This is consistent with the β-1,4-glucosidase from *G. natalis* being resistant to inhibition.

Fig. 6. Hanes plots of the activity data for β-1,4-glucosidase from the digestive juice of *G. natalis* and *D. hirtipes*. All plots were linear. *G. natalis*, uninhibited (circles) {[cellobiose]/β-1,4-glucosidase activity=1.651×[cellobiose]+9.650 (r^2=0.410, *P*=0.00, *N*=7)}; *G. natalis*, inhibited (stars) {[cellobiose]/β-1,4-glucosidase activity=1.387×[cellobiose]+22.58 (r^2=0.387, *P*=0.002, *N*=7)}. *D. hirtipes*, uninhibited (circles) {[cellobiose]/β-1,4-glucosidase activity=0.480×[cellobiose]+ 2.033 (r^2=0.832, *P*=0.00, *N*=7)}. *D. hirtipes*, inhibited (stars) {[cellobiose]/β-1,4-glucosidase activity=0.464×[cellobiose]+ 10.587 (r^2=0.450, *P*=0.000, *N*=7)}.

The high K_i of β-glucosidase from both gecarcinid species relative to K_i values for insects (Table 4) indicates that this enzyme is not particularly sensitive to inhibition by glucono-D-lactone in gecarcinids.

Glucono-D-lactone inhibits activity of β-1,4-glucosidase by mimicking the transitional form involved in the hydrolysis of the β-glycosidic bond (Terra and Ferreira, 1994). A lower affinity for the transitional form, and hence glucono-D-lactone, means that this form is not stabilized as well and the reaction would proceed at a slower rate, and this may explain why the digestive juice of *G. natalis* had a lower β-glucosidase activity than that from *D. hirtipes* (Fig. 3). Reduced β-1,4-glucosidase activity would not affect the overall rate of cellulose hydrolysis unless its activity was lower than that of the slowest step, probably the solubilization and initial depolymerization of the cellulose chains. A lower affinity for the transitional form would also mean that the affinity of the enzyme for its substrate and product would be reduced. This could account for lower substrate affinity exhibited by the β-1,4-glucosidase from *G. natalis* (cf. *D. hirtipes*; Fig. 3). In comparison with insects, the affinity of β-1,4-glucosidase from *G. natalis* was low since its K_m was in the upper range of the K_m for these animals (Tables 2, 4).

The inhibition resistance of β-1,4-glucosidase may counteract product inhibition. This means that the cellulase enzymes of *G. natalis* can hydrolyse cellulose despite the presence of the end product, glucose. Such a property would contribute to the overall efficiency of the cellulase system of *G. natalis.* The cellulase enzymes of *G. natalis* may also be able to hydrolyse cellulose even in the presence of high levels of sugars from other dietary items such as fruits and seeds. High concentrations of glucose may be present in the stomach given that the stomach is the site of mastication and some enzymatic digestion while the midgut gland is the site of absorption.

By contrast, the cellulases of *D. hirtipes* had low activity and were susceptible to product inhibition, and overall cellulose hydrolysis in this species was not as efficient as that in *G. natalis*. Thus, *D. hirtipes* may require a diet that contains a greater amount of more readily digestible material to satisfy its energy requirements. This could explain why *D. hirtipes* prefers green to brown leaves while *G. natalis* shows no preference (Greenaway and Raghaven, 1998). Green leaves contain more digestible components such as cellular proteins, storage carbohydrates and lipids and less cellulose than brown leaves (Greenaway and Raghaven, 1998).

pH optima for cellulase activity

The activities of the cellulases examined were maintained across quite a broad range of pH (Table 1) but with maximal activities in the pH range 5.5–7. As the measured pH of the digestive juices of the study species (6.69±0.03 for *G. natalis* and 6.03±0.04 for *D. hirtipes*) fell within this range, the cellulase enzymes will operate at or near their maximal activity. EG and β-1,4-glucosidase from insects and the crustacean *Cherax quadricarinatus* had similar pH maxima, ranging between pH 4 and 6 (Watanabe et al., 1997; Tokuda et al.,1997; Table 4). The pH values of the digestive juices of *G. natalis* and *D. hirtipes* were similar to the pH of the gut of the herbivorous isopod *Porcellio scaber* (pH 5.5–6.5; Zimmer and Topp, 1997). Endo-β-1,4-glucanase from both *G. natalis* and *D. hirtipes* exhibited additional activity peaks at pH 9 (Table 1), however this is considered to be an artefact of the assay and would not occur *in vivo*.

Tannins inhibit digestive enzymes by binding to the protein and precipitating it. They precipitate more protein under acid than under alkaline conditions, and certain insects [e.g. the cricket *Telleogryllus commodus*, the New Zealand grass grub (*Costelytra zealandica*) and the gypsy moth (*Lymantria dispar*)] have highly alkaline guts (pH range 8–11) to counteract the precipitation of enzymes by dietary tannins (Cooper and Vulcano, 1997; Briggs and McGregor, 1996; Schultz and Lechowicz, 1986). The foregut fluid from *G. natalis* was slightly more alkaline than that of *D. hirtipes*, but the small difference is unlikely to significantly alter the effects of dietary tannins on the digestive enzymes of the two species as both pH values fell within the acid range.

Table 4. *Properties of endo-β-1,4-glucanases and β-1,4-glucosidases from various invertebrates*

| Species | Endo-β-1,4-glucanase | | β-1,4-glucosidase | | | Reference |
	pH optimum	K_m (mg ml^{-1} CMC)	pH optimum	K_m (mmol l^{-1} cellobiose)	K_i (mmol l^{-1})	
Reticulitermes speratus (termite)	6	1.83, 1.48				Watanabe et al. (1997)
Nasutitermes takasagoensis (termite)	5.8	8.7				Tokuda et al. (1997)
Panesthia cribrata (cockroach)		9.4, 6.8		2.4, 13.8	0.33	Scrivener and Slaytor (1994)
Geoscapheus dilatatus (cockroach)		8.7, 4.6, 7.6				Scrivener et al. (1997)
Erinnyis ello (cassava hornworm)			6.5	2±0.2	0.0009	Santos and Terra (1985)
Locusta migratoria (locust)			5.5	1.95		Morgan (1975)
Cherax quadricarinatus (decapod crustacean)	4–5					Xue et al. (1999)
Gecarcoidea natalis (decapod)	5.5–7	3.03	4–7	5.84	11.12	Present study
Discoplax hirtipes (decapod)	5.5–7		4–7	4.23	4.53	Present study

CMC, carboxymethyl cellulose. K_i is the inhibitory constant of glucono-D-lactone on β-1,4-glucosidase.

Hemicellulase activities

Digestive juices from the gecarcinid land crabs *G. natalis* and *D. hirtipes* hydrolyse laminarin, lichenin and xylan and so contain activities of the hemicellulases laminarinase, licheninase and xylanase. Given that cellulose, laminarin, lichenin and xylose are carbohydrate polymers with sugar units joined by β-glycosidic bonds, it is possible that only one or two multicatalytic enzymes may catalyse all the reactions rather than multiple enzymes each catalysing one reaction. Exactly how many β-glycohydrolases are present in the digestive juice of the gecarcinid crabs is unknown and requires further investigation.

The respective activities of β-glycohydrolases in the study species were laminarinase > licheninase > xylanase > total cellulase. Consequently, assimilation of the different hemicelluloses and cellulose would be expected to follow a similar rank order, notably laminarin > lichenin > xylan > cellulose (Tables 2, 3). Thus, in diets containing equal amounts of hemicellulose and cellulose, both *G. natalis* and *D. hirtipes* would be expected to gain more carbohydrate from hemicellulose than cellulose. The high activities of laminarinase in the gut fluid of *G. natalis* and *D. hirtipes* indicate that they are particularly efficient in the hydrolysis of laminarin (Table 3). Laminarin is the chief food reserve of algae (Vonk and Western, 1984), and both species have been observed to scrape algae from rocks, soil and tree root buttresses (S.M.L., personal observation). Mixed β-D-glucans are a major component of cereals and grasses (Terra and Ferreira, 1994; McCleary, 1988). Gecarcinid crabs are able to utilise β-D-glucans since both *G. natalis* and *D. hirtipes* possess moderate licheninase activity (Table 3). *D. hirtipes* is better able to assimilate lichenin than *G. natalis* since its digestive juice has a higher licheninase activity (Table 3). *G. natalis* and *D. hirtipes* possess low xylanase activities and will only be able to hydrolyse xylan slowly.

Differences in assimilation coefficients for hemicellulose reported previously for the study species (Greenaway and Linton, 1995; Greenaway and Raghaven, 1998) cannot be interpreted in terms of hemicellulase activities measured in this study, as the types of hemicellulose present in *Ficus* and *Erythrina* leaves are not known.

Differences in cellulase activities and properties may explain differences in cellulase assimilation and dietary preferences

G. natalis had a higher cellulose assimilation efficiency than *D. hirtipes* when fed a diet of brown fig leaves (43% and 21%, respectively; Greenaway and Linton, 1995; Greenaway and Raghaven, 1998), and the current study confirms that *G. natalis* hydrolyses cellulose more effectively than *D. hirtipes* since it has higher total cellulase activity. In addition, the β-1,4-glucosidase from *G. natalis* is resistant to product inhibition and consequently it can hydrolyse cellulose despite the presence of high levels of glucose from cellulose hydrolysis or simple sugars from other dietary items such as fruit. This property may also be indicative of resistance of the enzyme to the effects of dietary tannins.

Origin of the cellulase and hemicellulase enzymes

Cellulase and hemicellulase enzymes may be either produced endogenously or by microorganisms within the gut. Endogenous production is possible in the study species, given that other invertebrate groups such as insects, molluscs and nematodes possess a gene for EG and synthesize this enzyme endogenously (reviewed by Watanabe and Tokuda, 2001). The origin of cellulases in gecarcinid crabs is investigated in a subsequent manuscript.

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