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# Purification and characterisation of endo-β-1,4-glucanase and laminarinase enzymes from the gecarcinid land crab *Gecarcoidea natalis* and the aquatic crayfish *Cherax destructor*

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## SUMMARY

**Laminarinase and endo-β-1,4-glucanase were purified and characterised from the midgut gland of the herbivorous land crab *Gecarcoidea natalis* and the crayfish *Cherax destructor*. The laminarinase isolated from *G. natalis* was estimated to have a molecular mass of 41 kDa by SDS-PAGE and 71 kDa by gel filtration chromatography. A similar discrepancy was noted for *C. destructor.* Possible reasons for this are discussed. Laminarinase (EC 3.2.1.6) from *G. natalis* had a $V_{max}$ of 42.0 μmol reducing sugars produced $min^{-1}$ mg $protein^{-1}$, a $K_m$ of 0.126% (w/v) and an optimum pH range of 5.5–7, and hydrolysed mainly β-1,3-glycosidic bonds. In addition to the hydrolysis of β-1,3-glycosidic bonds, laminarinase (EC 3.2.1.39) from *C. destructor* was capable of significant hydrolysis of β-1,4-glycosidic bonds. It had a $V_{max}$ of 19.6 μmol reducing sugars produced $min^{-1}$ mg $protein^{-1}$, a $K_m$ of 0.059% (w/v) and an optimum pH of 5.5. Laminarinase from both species produced glucose and other short oligomers from the hydrolysis of laminarin. Endo-β-1,4-glucanase (EC 3.2.1.4) from *G. natalis* had a molecular mass of 52 kDa and an optimum pH of 4–7. It mainly hydrolysed β-1,4-glycosidic bonds, but was also capable of significant hydrolysis of β-1,3-glycosidic bonds. Two endo-β-1,4-glucanases, termed 1 and 2, with respective molecular masses of 53±3 and 52 kDa, were purified from *C. destructor.* Endo-β-1,4-glucanase 1 was only capable of hydrolysing β-1,4-glycosidic bonds and had an optimum pH of 5.5. Endo-β-1,4-glucanases from both species produced some glucose, cellobiose and other short oligomers from the hydrolysis of carboxymethyl cellulose.**

Key words: land crab, *Gecarcoidea natalis*, *Cherax destructor*, laminarinase, endo-β-1,4-glucanase.

## INTRODUCTION

A wide variety of crustacean species ranging from specialised herbivorous gecarcinid and sesarmid crabs to detritivorous freshwater crayfish, consume plant material (Linton and Greenaway, 2007). These animals are capable of significant cellulose and hemicellulose assimilation; these compounds represent a significant source of energy (Linton and Greenaway, 2007). For example, gecarcinid land crabs such as the Christmas Island red crab, *Gecarcoidea natalis*, and the Christmas Island blue crab, *Discoplax hirtipes*, are able to assimilate 26–43% of cellulose and 14–49% of hemicellulose from a diet of brown leaf litter (Greenaway and Linton, 1995; Greenaway and Raghaven, 1998). This can be attributed to the activities of the enzymes cellulase (e.g. endo-β-1,4-glucanase and β-1,4-glucosidase) and hemicellulase (e.g. xylanase, lichenase and laminarinase), which hydrolyse cellulose and hemicellulose, respectively, to their component sugars (Linton and Greenaway, 2007).

Cellulose hydrolysis involves the combined action of endo-β-1,4-glucanase, cellobiohydrase and β-1,4-glucosidase (Watanabe and Tokuda, 2001). Endo-β-1,4-glucanase randomly cleaves β-1,4-glycosidic bonds within cellulose chains, releasing smaller fragments of random length (Watanabe and Tokuda, 2001). These fragments are then processed by cellobiohydrase, progressively releasing cellobiose units from the non-reducing end of the molecule (Watanabe and Tokuda, 2001). Finally, the cellobiose disaccharides are hydrolysed by β-1,4-glucosidase to release two free glucose molecules (Genta et al., 2003; Wang et al., 2004; Watanabe and Tokuda, 2001). Crustaceans, like other arthropods, are purported to lack cellobiohydrase and the combined action of endo-β-1,4-glucanase and β-1,4-glucosidase may hydrolyse cellulose completely in arthropods (Scrivener and Slaytor, 1994). Endo-β-1,4-glucanase (EC 3.2.1.4) is a key enzyme involved in cellulose hydrolysis since it is capable of hydrolysing β-1,4-glycosidic bonds and in arthropods it may function as a cellobiohydrase (Scrivener and Slaytor, 1994).

Crustaceans, like arthropods generally, are able to synthesise endo-β-1,4-glucanase endogenously (Byrne et al., 1999; Davison and Blaxter, 2005; Linton et al., 2006); the enzyme is a GHF9 glycosyl hydrolase, which in crustaceans is produced by F cells of the midgut gland (Byrne et al., 1999). Zymograms suggest that it consists of a 30 or 40 kDa protein and is capable of hydrolysing both β-1,3- and β-1,4-glycosidic bonds (Xue et al., 1999). While endo-β-1,4-glucanase activity has been readily measured in crustaceans, the enzyme is yet to be purified and characterised in terms of molecular mass, substrate specificity, reaction catalysed and kinetic parameters. Such characterisation would allow a better understanding of the enzymatic mechanism of cellulose hydrolysis in crustaceans and more generally in other invertebrates that consume plant material.

In addition to cellulose, crustaceans consuming plant material and algae will also encounter large amounts of hemicellulose (Linton and Greenaway, 2007). Laminarin, the major storage polysaccharide of algae, is one such hemicellulose consisting of glucose monomers joined together by mainly β-1,3-glycosidic bonds with some β-1,6-glycosidic bonds (Bacic et al., 1988). The molecule, also known by

its alternative name callose, is also present in the cell walls of fungi, plant wound tissue and transient structures such as pollen mother walls, sieve plates and cotton seed hairs (Bacic et al., 1988; Ruiz-Herrera, 1992; Terra and Ferreira, 1994). Laminarinase is a hemicellulase enzyme that is capable of hydrolysing laminarin or callose. To date, two laminarinases have been identified, endo-β-1,3(4)-glucanase (EC 3.2.1.6) and endo-β-1,3-glucanase (EC 3.2.1.39); endo-β-1,3(4)-glucanase (EC 3.2.1.6) is capable of hydrolysing both β-1,3- and β-1,4-glycosidic bonds, while endo-β-1,3-glucanase (EC 3.2.1.39) is capable of hydrolysing mainly β-1,3-glycosidic bonds (Boeckmann et al., 2003; Terra and Ferreira, 1994). Laminarinase may also work synergistically with cellulases such as endo-β-1,4-glucanase to hydrolyse structural polysaccharides within the plant cell wall (Mansfield et al., 1999). Although laminarinase activity has been detected in a range of herbivorous crustacean species (Linton and Greenaway, 2007), it is unclear which laminarinase is present, what the catalytic properties of the enzyme are, and its corresponding function.

Gecarcinid land crabs such as *G. natalis* and *D. hirtipes* are specialist herbivores, whose ancestors adopted a mainly leaf litter diet during the colonisation of land (Greenaway and Linton, 1995; Greenaway and Raghaven, 1998). During this adoption, cellulase and hemicellulase enzymes, which appear to be present in their aquatic ancestors, must have become adapted to efficiently hydrolyse the increasing amounts of cellulose and hemicellulose associated with the intake of terrestrial plant material. The nature of these adaptations is unknown, but they may have included alteration of substrate specificity and kinetic parameters ($K_m$ and $V_{max}$). The substrate specificity may have broadened to allow the utilisation of various plant structural compounds. Conversely it may have become more specific, increasing the efficiency of cellulose hydrolysis. Purifying and characterising cellulase and hemicellulase enzymes from a herbivorous land crab and a distant aquatic relative such as a freshwater crayfish may help to elucidate these adaptations.

In this study we have purified and characterised the key enzymes endo-β-1,4-glucanase and laminarinase from the gecarcinid land crab *G. natalis*, and the freshwater crayfish *Cherax destructor*. To elucidate possible adaptations towards the hydrolysis of cellulose in a terrestrial plant diet, the characteristics of the purified enzymes from *G. natalis* and *C. destructor* were compared.

## MATERIALS AND METHODS

### Animal collection and housing

*G. natalis* Pocock 1888 were collected from the rainforest of Christmas Island, Indian Ocean (10°30′S, 106°E) and air freighted to Deakin University, Geelong, Victoria, Australia. Animals were maintained at 25°C on a 12 h:12 h light:dark cycle in a round communal tub (1.15 m diameter, 0.45 m height). Crabs were fed brown leaf litter collected from local deciduous trees and had *ad libitum* access to water. *C. destructor* Clark 1936 were collected from ponds of the Geelong campus of Deakin University and maintained in plastic aquaria containing dechlorinated tap water and PVC pipes for shelter. Crayfish were fed weekly with commercial cat biscuits, supplemented with lucerne hay.

### Preparation of midgut gland homogenate

Prior to dissection, animals were anaesthetised on ice for at least 30 min, and killed by removing the carapace and heart. Midgut glands from three *G. natalis* and between five and eight *C. destructor* were removed, pooled and homogenised using a Tissumizer homogeniser (Tekmar, Cincinnati, OH, USA) in two volumes of homogenisation buffer (100 mmol $l^{-1}$ sodium acetate, pH 5.5, containing 1 mmol $l^{-1}$ dithioerythritol and 0.2 mmol $l^{-1}$ phenylmethylsulphonyl fluoride). The homogenate was filtered through two layers of wet cheesecloth and spun at 2060 ***g*** for 30 min. The supernatant was then removed, its volume measured, and kept for ammonium sulphate precipitation.

### Protein separation

#### Ammonium sulphate precipitation

Ammonium sulphate was slowly added to the supernatant until its concentration reached 30% of saturation. This solution was then incubated with stirring for 3.5 h at 4°C to allow protein precipitation. The suspension was centrifuged at 2060 ***g*** for 30 min at 4°C using a Beckman Allegra™ 21R centrifuge (Beckman Coulter, Fullerton, CA, USA), the supernatant removed, its volume measured and the protein pellet kept. This procedure was then repeated with an ammonium sulphate concentration of 60% and 80% of saturation.

The precipitated protein in the 0–30%, 30–60% and 60–80% ammonium sulphate precipitates was re-dissolved in one volume of 100 mmol $l^{-1}$ sodium acetate buffer (pH 5.5). These re-dissolved precipitates and the supernatant containing ammonium sulphate at 80% of saturation were assayed for protein content and endo-β-1,4-glucanase, laminarinase and β-1,4-glucosidase activities. Enzymes of interest were purified from the re-dissolved 30–60% precipitate using a combination of anion exchange, hydrophobic interaction and gel filtration chromatography. All chromatography was carried out at 4°C.

### Liquid chromatography steps

#### Anion exchange chromatography

Before anion exchange chromatography, the ammonium sulphate was removed and the buffer changed in the re-dissolved 30–60% ammonium sulphate precipitate by dialysing it against two changes of 20 mmol $l^{-1}$ PIPES buffer (pH 5.5). The dialysate was centrifuged at 3300 ***g*** for 30 min to remove any fine particles and then applied to a 2.5 cm×60 cm Macro-Prep DEAE column (Bio-Rad, Hercules, CA, USA) for anion exchange chromatography. Proteins were eluted from the DEAE column using an Econo Gradient pump (Bio-Rad) at a flow rate of 0.3 ml $min^{-1}$ and 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 to 1000 mmol $l^{-1}$; 860 to 1060 min, sodium chloride concentration was held at 1000 mmol $l^{-1}$; 1060–1260 min, sodium chloride concentration was decreased linearly from 1000 to 0 mmol $l^{-1}$; 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 using a model 2110 fraction collector (Bio-Rad). Collected fractions were stored at –20°C until analysis. Every third fraction was analysed for protein content and endo-β-1,4-glucanase and laminarinase 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 weight limit (NMWL) Ultra-15, Ultracel-10K filter (Amicon, Houston, TX, USA).

#### Hydrophobic interaction chromatography

Prior to hydrophobic interaction chromatography (HIC), the buffer in the protein concentrate was changed by adding four volumes of 20 mmol $l^{-1}$ PIPES buffer (pH 5.5) to the concentrate and re-concentrating by centrifugation in a 10 000 NMWL ultrafilter as described above.

Before chromatography a Macro-Prep methyl HIC (Bio-Rad) column (1.5 cm×50 cm) was equilibrated with 20 mmol $l^{-1}$ PIPES

buffer (pH 5.5), containing 1.5 mol $l^{-1}$ ammonium sulphate at a flow rate of 0.3 ml $min^{-1}$. Samples were then loaded and the protein eluted using a linear gradient of 1.5 to 0 mol $l^{-1}$ ammonium sulphate at a flow rate of 0.3 ml $min^{-1}$ as follows: 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 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 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 analysed for protein and endo-β-1,4-glucanase and laminarinase activity. Fractions containing activity of the enzyme of interest were combined and concentrated by centrifugation in a 10 000 NMWL ultrafilter as described above.

#### Gel filtration chromatography

Protein concentrates from either the anion exchange chromatography step or the HIC step were applied to a 1.5 cm×50 cm P-100 medium bead size gel filtration column (Bio-Rad). Isocratic elution of proteins was achieved with 0.1 mol $l^{-1}$ sodium acetate buffer (pH 5.5) at a flow rate of 0.1 ml $min^{-1}$; 1 ml fractions were collected every 10 min from 120 to 920 min.

#### Strong anion exchange chromatography

Protein concentrates were applied to a 1.0 cm×30 cm Mono-Q column (Bio-Rad) for strong anion exchange chromatography. Prior to strong anion exchange chromatography, the buffer of the protein concentrate was changed to the starting buffer by adding 4 volumes of 20 mmol $l^{-1}$ PIPES buffer (pH 5.5) and reconcentrated in a 10 000 NMWL ultrafilter as described above. The sample was then applied to the column and the proteins eluted with a 0–1000 mmol $l^{-1}$ sodium chloride gradient at a flow rate of 0.4 ml $min^{-1}$ as follows: 0–40 min, isocratic elution with 20 mmol $l^{-1}$ PIPES buffer (pH 5.5); 40–160 min, sodium chloride concentration in the buffer was increased linearly from 0 to 1000 mmol $l^{-1}$; 160–190 min, isocratic elution with 20 mmol $l^{-1}$ PIPES buffer (pH 5.5) containing 1000 mmol $l^{-1}$ sodium chloride; 190–210 min, sodium chloride concentration in the PIPES buffer was reduced from 1000 to 0 mmol $l^{-1}$; 210–240 min, column was re-equilibrated with isocratic flow of the starting buffer, 20 mmol $l^{-1}$ PIPES buffer (pH 5.5); 1.25 ml fractions eluted from the Mono-Q column were collected every 3 min for the entire 240 min of the chromatography run and stored at –20°C until analysis. Protein and activity of the enzyme of interest were measured in every third fraction.

### Protein size determination using gel filtration chromatography

The molecular mass of the purified enzymes was calculated by eluting a 250 μl aliquot on a 1.0 cm×30 cm Superdex 200 gel filtration column (GE Healthcare, Chalfon St Giles, Bucks, UK) and eluted isocratically using 0.1 mmol $l^{-1}$ sodium acetate buffer (pH 5.5) containing 0.05 mmol $l^{-1}$ sodium chloride at a flow rate of 0.3 ml $min^{-1}$. Protein eluting from the column was detected with an EM-1 Econo UV monitor (Bio-Rad). The molecular mass of the enzyme was estimated by comparing its retention time with that of the log of the molecular mass of the standards cytochrome *c* (12.4 kDa), carbonic anhydrase (29 kDa), albumin (66 kDa), alcohol dehydrogenase (150 kDa) and β-amylase (200 kDa; Sigma, St Louis, MO, USA). Enzyme activity was measured in every third fraction in order to confirm that the protein peak corresponded to the elution of the purified enzyme.

### Analysis of fractions and protein concentrates arising form the various chromatography steps

Eluted fractions and the concentrates arising from the various chromatography steps were analysed for total protein and the enzymes endo-β-1,4-glucanase or laminarinase. Endo-β-1,4-glucanase and laminarinase activities were measured as the rate of production of reducing sugars from the respective hydrolysis of carboxymethyl cellulose (Sigma, catalogue no. C-5678) and laminarin (from *Laminaria digitata*; Sigma, catalogue no. L-9634). Protocols used for the enzyme assays have been described previously (Linton and Greenaway, 2004). Protein concentrations were determined using the Bradford protein assay (Bio-Rad), as per the manufacturer's instructions, and bovine serum albumin was used as the standard (MP Biomedicals, Aurora, OH, USA).

#### SDS-PAGE

The purity of endo-β-1,4-glucanase and laminarinase after liquid chromatography was analysed by SDS-PAGE electrophoresis using a Mini Protean system (Bio-Rad), and run as previously described (Laemmli, 1970). Samples and HiMark™ unstained high molecular mass protein standards (Invitrogen, Carlsbad, CA, USA) were run on pre-cast 10% polyacrylamide gels (Bio-Rad) at 200 V. Samples were prepared for SDS-PAGE using methods described previously (Deutscher, 1990). Protein bands were visualised using a silver staining kit (Bio-Rad). Protein size was determined by plotting log molecular mass against relative migration distance of the standards with a similar mass range to the proteins of interest. All molecular mass are expressed as means ± s.d.

### Enzyme characterisation

#### Substrate specificity

Purified enzymes were incubated with carboxymethyl cellulose, laminarin, lichenan and cellobiose and the specific activity of the enzyme was expressed as a percentage of the activity of the enzyme using its native substrate. Endo-β-1,4-glucanase, laminarinase, lichenase and β-1,4-glucosidase activities were measured as previously described (Linton and Greenaway, 2004).

#### Enzyme kinetics and pH optimum

The activity of the purified enzymes was determined at substrate concentration ranges of 1–7% w/v for endo-β-1,4-glucanase and 0.125–3.6% w/v for laminarinase. The kinetic properties $V_{max}$ and $K_m$ were then calculated from the resulting Michaelis–Menten plot using the graphing software Prism® v. 5 (Graphpad Software, San Diego, CA, USA). The optimum pH was determined by measuring enzyme activity in the following buffers: acetate buffer (pH 4.0), sodium acetate buffer (pH 5.5), phosphate buffer (pH 7.0), Tris buffer (pH 8.0) and glycine buffer (pH 9.0). All endo-β-1,4-glucanase reactions were carried out with a final substrate concentration of 1% w/v, while the final concentration of substrate in laminarinase reactions was 0.25% w/v. The means at each pH were compared using a one-way ANOVA followed by least significant difference (LSD) analysis. Statistical probabilities were determined using SPSS v.15 (SPSS Inc., Chicago, IL, USA).

#### Thin layer chromatography

A 100 μl aliquot of purified endo-β-1,4-glucanase from either *G. natalis* or *C. destructor* was incubated overnight at 40°C with

carboxymethyl cellulose at a final concentration of 2% w/v (pH 5.5). Purified laminarinase was similarly incubated overnight with laminarin (final concentration 1% w/v, pH 5.5). Incubated samples were separated on a silica thin layer chromatography (TLC) plate and stained as previously described (Nishida et al., 2007).

## RESULTS

### Purification of laminarinases and endo-β-1,4-glucanases from *G. natalis*

#### DEAE chromatography

DEAE chromatography of the re-dissolved 30–60% ammonium sulphate precipitate revealed two major endo-β-1,4-glucanase activity peaks and one major laminarinase peak (Fig. 1). Enzyme activity for the first endo-β-1,4-glucanase activity peak was eluted in fractions 37–55 with peak activity occurring in fraction 46. Enzyme activity for the second endo-β-1,4-glucanase peak was contained in fractions 56–70 with peak activity being in fraction 61 (Fig. 1). Fractions 37–55 also contained laminarinase activity with fraction 46 containing the highest laminarinase activity. Given this, fractions 37–55 were combined, concentrated and then applied to a HIC column. Fractions 56–70 containing the second endo-β-1,4-glucanase peak were combined, concentrated and applied to a Bio-Rad P-100 gel filtration column for gel filtration chromatography.

### HIC of combined fractions 37–55 from DEAE containing laminarinase and endo-β-1,4-glucanase

#### Endo-β-1,4-glucanase

Four (two major and two minor) endo-β-1,4-glucanase activity peaks were eluted from the HIC column. The two major endo-β-1,4-glucanase peaks had their peak activity in fractions 43 and 49 while the two minor peaks had their peak activity in fractions 13 and 37 (Fig. 2).

#### Laminarinase

One large laminarinase peak was eluted late from the HIC column (Fig. 2). Fractions 52–58 contained laminarinase activity with peak activity being contained in fraction 55 (Fig. 2). Protein containing laminarinase activity was well separated from the majority of the protein and thus substantial purification of laminarinase was achieved by this chromatography step (Fig. 2). Fractions 52–58 containing laminarinase activity were combined, concentrated and run on a Bio-Rad P-100 gel filtration column for size exclusion chromatography.

#### Final step in the purification of laminarinase – Bio-Rad P-100 gel filtration column

Size exclusion chromatography of the combined and concentrated fractions 52–58 from HIC revealed one early eluting laminarinase activity peak (Fig. 3). Laminarinase activity was detected in fractions 10–19 with fraction 13 containing the largest enzyme activity (Fig. 3). This laminarinase activity peak corresponded to a single well-resolved protein peak (Fig. 3). Fractions 7–21 containing laminarinase activity were combined and concentrated. This concentrate had a laminarinase specific activity of 7.80 μmol reducing sugars produced $\text{min}^{-1}$ mg $\text{protein}^{-1}$ (Table 1). When run on an SDS-PAGE gel, this concentrate contained a single protein band with an estimated molecular mass of 41 kDa (Fig. 4). However, the molecular mass estimated from the calibration of the Superdex 200 gel filtration column was 71 kDa (data not shown). Since a single protein band was demonstrated by SDS-PAGE, the laminarinase was deemed to be purified to homogeneity. After this

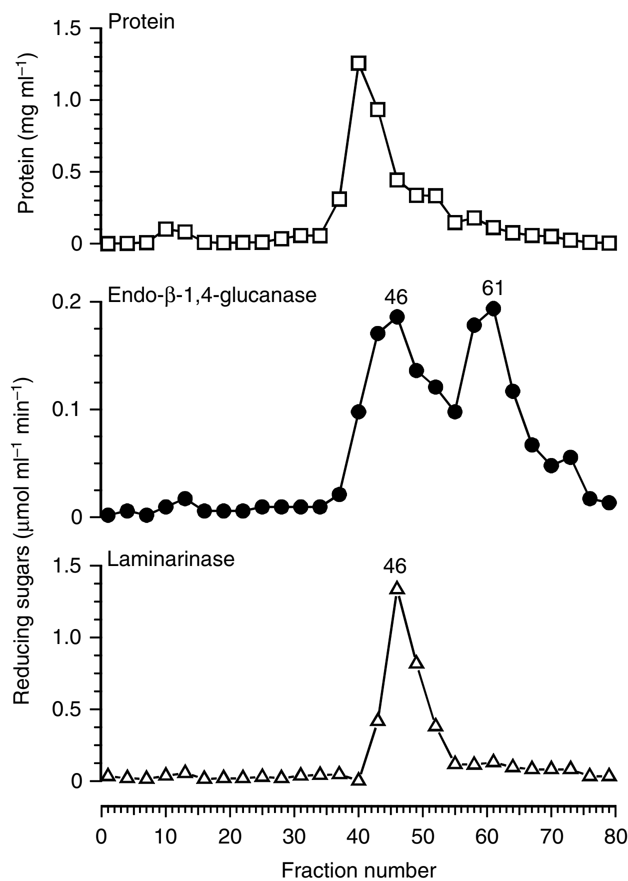



Fig. 1. DEAE anion exchange chromatography of the dialysed protein fraction derived from the midgut gland of *G. natalis* that was precipitated by ammonium sulphate at concentrations between 30 and 60% saturation. Protein content (□; mg protein $\text{ml}^{-1}$) and endo-β-1,4-glucanase (●) and laminarinase (Δ) activities (μmol reducing sugars produced $\text{min}^{-1}$ $\text{ml}^{-1}$) in the collected fractions.

chromatography step laminarinase had been purified 80.9 times with a yield of 4.96% (Table 1).

#### Final step in the purification of endo-β-1,4-glucanase – Bio-Rad P-100 gel filtration chromatography

The second endo-β-1,4-glucanase peak resolved by anion exchange chromatography (fractions 56–70) was applied to a P-100 gel filtration column (Fig. 5). Endo-β-1,4-glucanase activity was present in fractions 22–43 with fraction 34 containing the highest enzyme activity (Fig. 5). Combined and concentrated fractions 22–43 had an endo-β-1,4-glucanase specific activity of 12.74 μmol reducing sugars produced $\text{min}^{-1}$ mg $\text{protein}^{-1}$ and contained a single 52 kDa protein (Fig. 4A; Table 2). Hence endo-β-1,4-glucanase was purified to homogeneity; a purification factor of 552 times had been achieved with a yield of 10.2% (Table 2).

### Purification of laminarinases and endo-β-1,4-glucanases from the aquatic crayfish *C. destructor*

#### DEAE chromatography

DEAE anion exchange chromatography of the re-dissolved 30–60% ammonium sulphate precipitate revealed five large endo-β-1,4-glucanase activity peaks. Fractions 1, 19, 34, 40 and 52 contained the highest endo-β-1,4-glucanase activity and were respectively designated as endo-β-1,4-glucanase peaks 1, 2, 3, 4

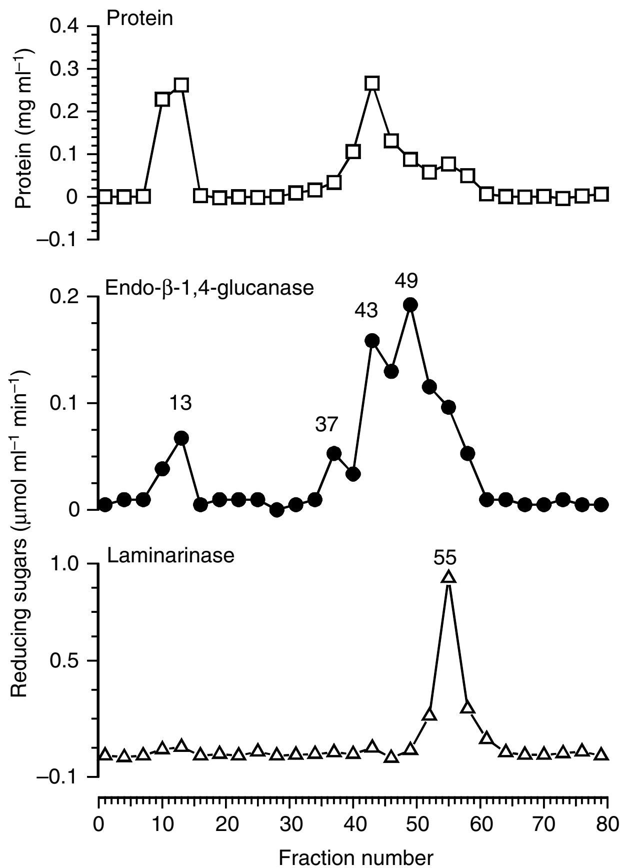



Fig. 2. Hydrophobic interaction chromatography (HIC) of the combined and concentrated fractions 37–55 from anion exchange chromatography described in Fig. 1. The concentrate contained endo-β-1,4-glucanase and laminarinase activities and was derived from the midgut gland of *G. natalis.* Protein content (□, mg protein $ml^{-1}$) and endo-β-1,4-glucanase (●) and laminarinase (Δ; μmol reducing sugars or glucose produced $min^{-1}$ $ml^{-1}$) activities of the collected fractions.

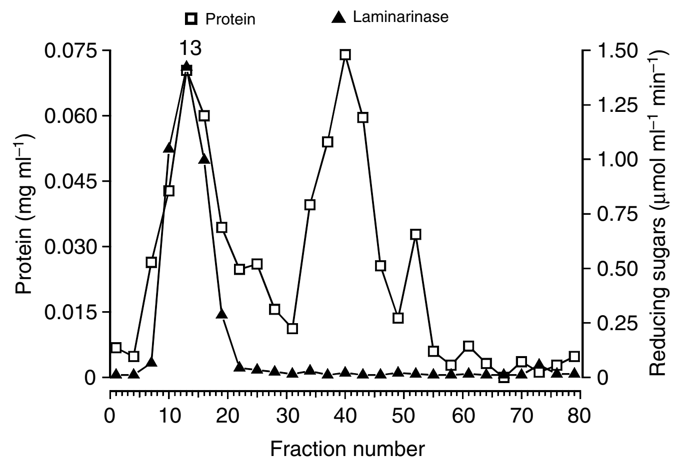



Fig. 3. Elution profile of protein (□; mg protein $ml^{-1}$) and laminarinase activity (▲; μmol reducing sugars or glucose produced $min^{-1}$ $ml^{-1}$) for fractions from a Bio-Rad P-100 gel filtration column. Fractions 52–58 from the HIC described in Fig. 2 were combined, concentrated and loaded onto the Bio-Rad P-100 column. This concentrate was derived from the midgut gland of *G. natalis.*

and 5 (Fig. 6). Endo-β-1,4-glucanase 1 and 2 were clearly resolved from laminarinase and were purified separately (Fig. 6). Endo-β-1,4-glucanase 3 co-eluted with laminarinase. Given that laminarinase possesses some endo-β-1,4-glucanase activity (see below) it was thought that endo-β-1,4-glucanase peak 3 may represent this residual activity. Endo-β-1,4-glucanase 4 and 5 were not purified further.

A large laminarinase peak was eluted from the DEAE column (Fig. 6). It was eluted in fractions 27–36 with fraction 34 containing the highest laminarinase activity (Fig. 6). Fractions 27–36 containing laminarinase were combined, concentrated and then applied to a HIC column.

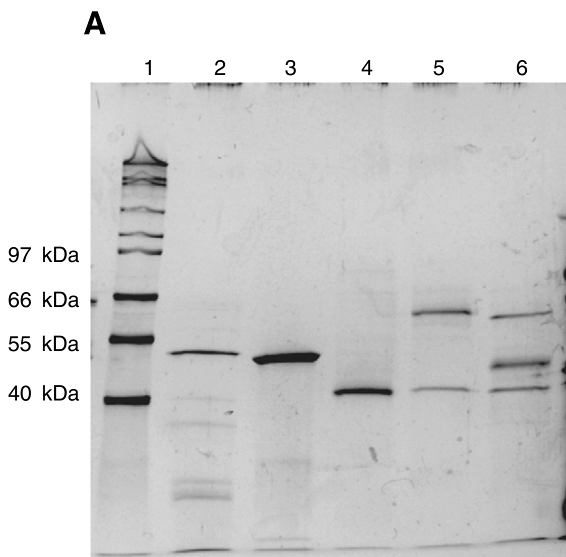
### Comparison with DEAE chromatography of the 30–60% ammonium sulphate fractions from *C. destructor* and *G. natalis*

The elution profiles of laminarinase from the DEAE column were similar for *C. destructor* and *G. natalis*. For both species there was one large late-eluting laminarinase peak (Figs 1, 6). The elution profile of endo-β-1,4-glucanase from the DEAE column differed between species. For *C. destructor* there were six endo-β-1,4-glucanase peaks while there were only two endo-β-1,4-glucanase peaks for *G. natalis* (Figs 1, 6). An early eluting endo-β-1,4-glucanase peak was observed for *C. destructor*; however, no such peak was observed for *G. natalis*. For both *G. natalis* and *C. destructor* there was a large endo-β-1,4-glucanase peak that co-eluted with laminarinase (Figs 1, 6).

Table 1. Specific activity, purification factor, units of enzyme and percentage yield for each step in the purification of laminarinase from *G. natalis*

| Fraction | Laminarinase specific activity (μmol $min^{-1}$ mg $protein^{-1}$) | Purification factor | Units of laminarinase recovered after each chromatography step | Percentage yield |
|---|---|---|---|---|
| Homogenate | 0.096 | 1 | 190 | |
| Re-dissolved 30–60% ammonium sulphate precipitate | 0.077 | 0.8 | 37 | 19.5 |
| Combined fractions 37–55 after DEAE anion exchange chromatography | 0.252 | 2.6 | 22 | 11.7 |
| Combined fractions 52–56 after HIC chromatography | 0.945 | 9.8 | 4.0 | 2.1 |
| Combined fractions 7–21 after P-100 gel filtration chromatography | 7.80 | 80.9 | 9.43 | 4.96 |

Purification factors and percentage yields are respectively expressed as a proportion and percentage of that in the initial homogenate. Units of enzyme are given as μmol $min^{-1}$.



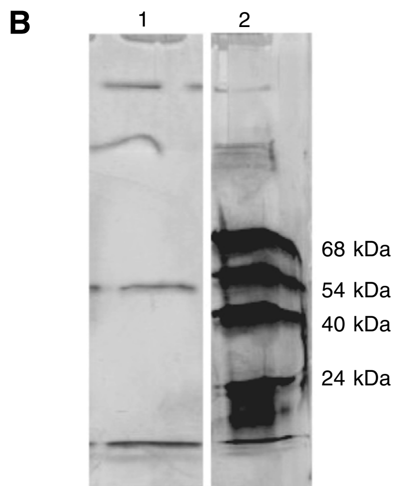




Fig. 4. SDS polyacrylamide gel electrophoresis of purified and partially purified endo-β-1,4-glucanase and laminarinase from the midgut gland of *C. destructor* and *G. natalis*. Gels were silver stained. (A) Lane 1, molecular mass standards with their sizes indicated in kDa. Lane 2, endo-β-1,4-glucanase purified from the midgut gland of *G. natalis.* Lane 3, endo-β-1,4-glucanase 2 purified from the midgut gland of C. *destructor*. Lane 4, laminarinase purified from the midgut gland of G. *natalis*. Lanes 5 and 6, laminarinases partially purified from midgut gland of *C. destructor* using either Bio-Rad P-100 gel filtration chromatography (Lane 5) or Mono-Q strong anion chromatography (Lane 6) as the third purification step. (B) Lane 1, endo-β-1,4-glucanase 1 purified from the midgut gland of *C. destructor*. Lane 2, molecular mass standards with their masses indicated in kDa.

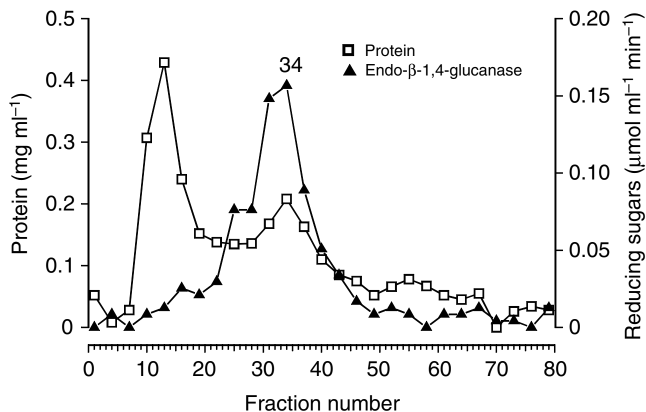



Fig. 5. Size exclusion chromatography of endo-β-1,4-glucanase derived from the midgut gland of *G. natalis*. Combined and concentrated fractions 56–70 containing endo-β-1,4-glucanase activity from the DEAE anion exchange chromatography described in Fig. 1 were loaded onto a Bio-Rad P-100 column. Protein (□, mg protein ml$^{-1}$) and endo-β-1,4-glucanase activity (▲, μmol reducing sugars produced min$^{-1}$ ml$^{-1}$) were measured in the fractions collected.

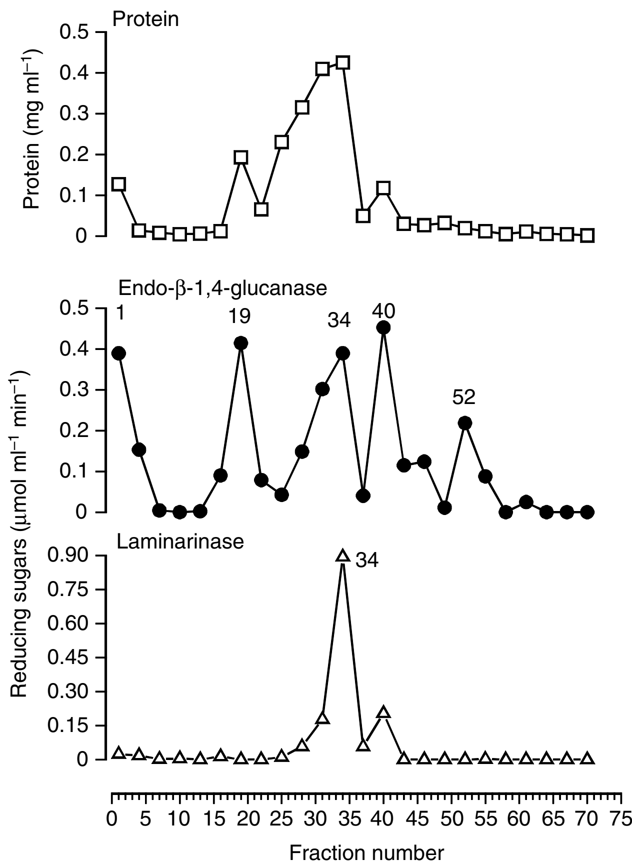



Fig. 6. Anion exchange chromatography of the dialysed protein fraction from the midgut gland homogenate of *C. destructor* that was precipitated by ammonium sulphate at concentrations between 30 and 60% of saturation. Protein sample was loaded onto a Bio-Rad DEAE column and protein concentration (mg protein ml$^{-1}$, □) and endo-β-1,4-glucanase (●) and laminarinase (Δ) activities (μmol reducing sugars produced min$^{-1}$ ml$^{-1}$) were measured in the eluted fractions.

## Purification of laminarinase

### Hydrophobic interaction chromatography

HIC of the laminarinase concentrate from the anion exchange chromatography step described above revealed one large, late-eluting laminarinase activity peak (Fig. 7). Laminarinase activity was contained in fractions 60–62 with fraction 61 containing the highest enzyme activity (Fig. 7). Fractions 60–62 were combined, concentrated and then loaded onto a Bio-Rad Mono-Q column for strong anion chromatography. The elution profile from the HIC column was similar to the elution profile of laminarinase from *G. natalis* eluted from the HIC column, i.e. a single late-eluting laminarinase peak (Figs 2, 7).

Table 2. Specific activity, purification factor, units of enzyme and percentage yield for each step in the purification of endo-β-1,4-glucanase from the midgut gland of *G. natalis*

| Fraction | Endo-β-1,4-glucanase specific activity (μmol $min^{-1}$ mg $protein^{-1}$) | Purification factor | Units of endo-β-1,4-glucanase recovered after each chromatography step | Percentage yield |
|---|---|---|---|---|
| Homogenate | 0.023 | 1 | 45.5 | |
| Re-dissolved 30–60% ammonium sulphate precipitate | 0.022 | 1 | 10.6 | 23.4 |
| Combined fractions 56–70 from DEAE anion exchange chromatography | 0.166 | 7.2 | 8.13 | 17.9 |
| Combined fractions 28–43 from P-100 gel filtration chromatography | 12.74 | 552 | 4.64 | 10.2 |

Purification factors and percentage yields are respectively expressed as a proportion and percentage of that in the initial homogenate. Units of enzyme are given as μmol $min^{-1}$.

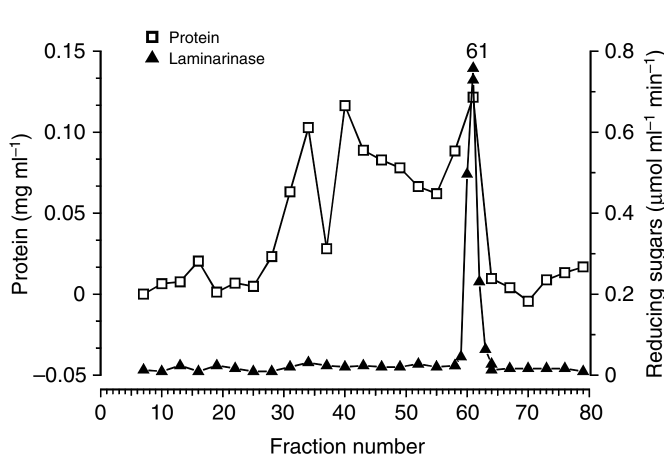



Fig. 7. Hydrophobic interaction chromatography of combined and concentrated fractions 27–36 from the DEAE anion exchange chromatography described in Fig. 5. This concentrate contained laminarinase 1 activity and was derived from the midgut gland of *C. destructor.* Protein concentration (mg $ml^{-1}$, □) and laminarinase activity (μmol reducing sugars produced $min^{-1}$ $ml^{-1}$, ▲) in the collected fractions.

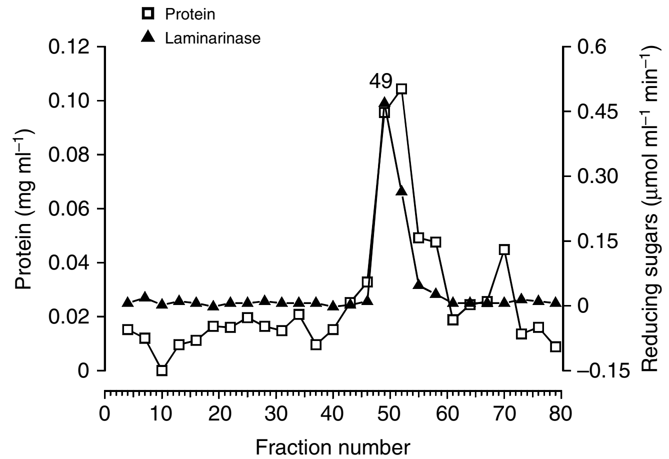



Fig. 8. Strong anion exchange chromatography of the laminarinase from *C. destructor* on a Bio-Rad Mono-Q column. Combined and concentrated fractions 60–62 from the HIC described in Fig. 6 and containing laminarinase 1 derived from the midgut gland of *C. destructor* were loaded onto the Mono-Q column. Protein concentration (mg protein $ml^{-1}$, □) and laminarinase activity (μmol reducing sugars produced $min^{-1}$ $ml^{-1}$, ▲) in the collected fractions.

### Mono-Q chromatography

Mono-Q chromatography of the combined and concentrated fractions 60–62 from HIC revealed a large late-eluting laminarinase activity peak (Fig. 8). Laminarinase was eluted in fractions 49–52 with fraction 49 containing the highest enzyme activity (Fig. 8). This laminarinase peak corresponded to a single protein peak indicating substantial protein purification had been achieved (Fig. 8). Fractions 47–53 containing the purified laminarinase were combined and concentrated to a final volume of 2 ml. This concentrate contained 4.47 units of laminarinase and three proteins, 41, 49 and 62 kDa in size (Table 3, Fig. 4). After this chromatography step the laminarinase was purified 104.94 times and a 14.82% yield had been achieved (Table 3). Hence laminarinase has been partially purified from *C. destructor*. Given that a laminarinase of 44 kDa has been purified from *G. natalis* the 41 kDa protein from *C. destructor* is probably laminarinase 1.

### Gel filtration chromatography

During another purification run, fractions from HIC containing laminarinase activity from *C. destructor* were combined, concentrated and applied to a Bio-Rad P-100 gel filtration column for size exclusion chromatography. This ensured that laminarinase was purified from *G. natalis* and *C. destructor* using a consistent purification method. One large laminarinase activity peak was eluted from this column (Fig. 9). Fractions 7–25 contained laminarinase activity with fraction 16 containing the highest activity (Fig. 9). By comparison with the elution volumes of protein molecular mass standards from the gel filtration column, this laminarinase was estimated to be 62 kDa in size. This is likely to be an underestimate given the laminarinase eluted very near the void volume and more protein was loaded onto the column compared with that of protein molecular mass standards (elution volume:void volume=1.21). However the concentrate of combined and concentrated fractions 7–25 contained two proteins of 41 and 62 kDa in size when it was run on an SDS-PAGE gel (Fig. 4).

### Gel filtration chromatography of endo-β-1,4-glucanase 1 and endo-β-1,4-glucanase 2

Combined and concentrated fractions containing endo-β-1,4-glucanase 1 (combined fractions 1–6) and endo-β-1,4-glucanase 2 (combined fractions 16–22) from DEAE anion exchange chromatography were loaded onto a Bio-Rad P-100 gel filtration column for size exclusion chromatography. Separate runs of these endo-β-1,4-glucanase 1 and 2 concentrates revealed that both endo-β-1,4-glucanases were eluted as a single activity peak (Fig. 10). Endo-β-1,4-glucanase 1 was eluted in fractions 22–31 with fraction

Table 3. Specific activity, purification factor, units of enzyme and percentage yield for each step in the purification of laminarinase 1 from *C. destructor*

| Fraction | Laminarinase specific activity (μmol min$^{-1}$ mg protein$^{-1}$) | Purification factor | Units of laminarinase recovered after each chromatography step | Percentage yield |
|---|---|---|---|---|
| Homogenate | 0.17 | – | 30.17 | – |
| Re-dissolved 30–60% ammonium sulphate precipitate | 0.23 | 1.3 | 23.08 | 76.48 |
| Combined fractions 27–36 after DEAE chromatography | 1.32 | 7.55 | 10.74 | 35.58 |
| Combined fractions 60–61 after HIC chromatography | 6.37 | 36.52 | 6.07 | 20.12 |
| Combined fractions 47–53 after Mono-Q | 18.31 | 104.94 | 4.47 | 14.82 |

Purification factors and percentage yields are respectively expressed as a proportion and percentage of that in the initial homogenate. Units of enzyme are given as μmol min$^{-1}$.

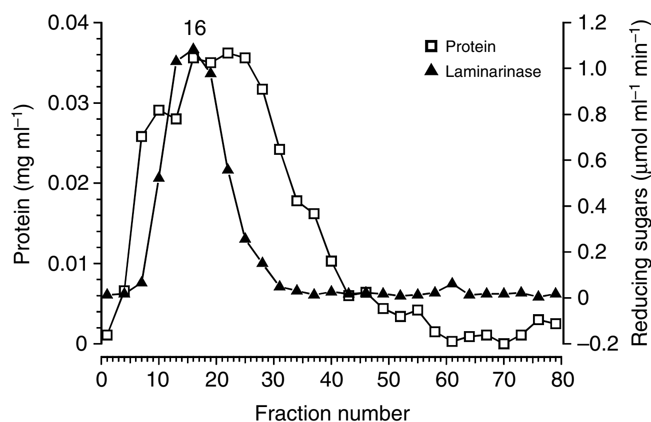



Fig. 9. Gel filtration chromatography of laminarinase derived from *C. destructor* and after sequential anion exchange chromatography and HIC. Elution profiles of protein (mg protein ml$^{-1}$, □) and laminarinase activity (μmol reducing sugars produced min$^{-1}$ ml$^{-1}$, ▲) when combined and concentrated fractions containing laminarinase activity were applied to a Bio-Rad P-100 gel filtration column.

28 containing the highest enzyme activity (Fig. 10A) while endo-β-1,4-glucanase 2 was eluted in fractions 13–28 with the largest activity being present in fraction 19 (Fig. 10B). Accordingly, fractions 22–31 eluted from the P-100 column and containing endo-β-1,4-glucanase 1 were combined and concentrated. Similarly, fractions 13–28 containing endo-β-1,4-glucanase 2 were combined and concentrated. The concentrates of both endo-β-1,4-glucanase 1 and 2 contained a single protein (Fig. 4); hence endo-β-1,4-glucanase 1 and 2 were purified to homogeneity. The molecular mass of endo-β-1,4-glucanase 1 was estimated to be 53±2 kDa ($N$=3 purification runs) while the molecular mass of endo-β-1,4-glucanase 2 was estimated to be 51±0 kDa ($N$=2 purification runs). Endo-β-1,4-glucanase 1 had a specific activity of 19.09 μmol min$^{-1}$ mg protein$^{-1}$ with a purification factor of 83.18 times and a percentage yield of 13.24% (Table 4). Endo-β-1,4-glucanase 2 had a specific activity of 7.43 μmol min$^{-1}$ mg protein$^{-1}$ with a purification factor of 32.36 times and a yield of 10.23% (Table 5).

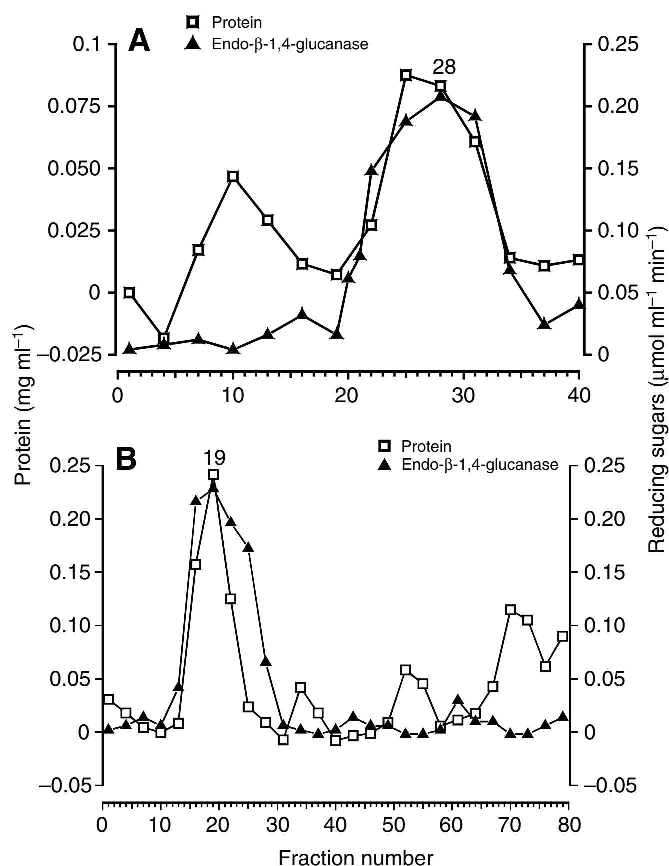



Fig. 10. Gel filtration chromatography of endo-β-1,4-glucanases derived from the midgut gland of *C. destructor*. Samples were loaded onto a Bio-Rad P-100 gel filtration column and the protein concentration (mg protein ml$^{-1}$, □) and endo-β-1,4-glucanase activity (μmol reducing sugars produced min$^{-1}$ ml$^{-1}$, ▲) measured. (A) Chromatography of combined and concentrated fractions 1–6 from DEAE anion exchange chromatography described in Fig. 5 containing endo-β-1,4-glucanase 1. (B) Size exclusion chromatography of combined and concentrated fractions 16–22 from DEAE chromatography described in Fig. 5 containing endo-β-1,4-glucanase 2.

### Characterisation of the purified enzymes from *G. natalis*

#### Laminarinase

Out of all of the substrates tested, laminarinase purified from the midgut gland of *G. natalis* hydrolysed laminarin the fastest (Table 6). This laminarinase was also capable of limited hydrolysis of carboxymethyl cellulose and hence possessed a low endo-β-1,4-glucanase activity (Table 6). Hydrolysis of lichenan and cellobiose was negligible (Table 6). Thus laminarinase from *G. natalis* is capable of hydrolysing mainly β-1,3-glycosidic bonds with very limited ability to hydrolyse β-1,4-glycosidic bonds; it is not capable of hydrolysing mixed β-D-glucans such as lichenan (Table 6), so

can be assigned the EC number 3.2.1.39 (Boeckmann et al., 2003). Laminarinase purified from *G. natalis* had a $V_{max}$ of 42.0 μmol reducing sugars produced min$^{-1}$ mg protein$^{-1}$ and a $K_m$ of 0.126% (w/v) (Fig. 11). The enzyme was active over a broad pH range of 4–8, but was highest at pH 5.5–7 and lower at pH 4 and 9 (Fig. 12A). Incubation of purified laminarinase with 1% (final concentration) laminarin for 24 h produced glucose, laminaribose and short oligomers of the laminarin polymer (Fig. 13A).

### Endo-β-1,4-glucanase

Endo-β-1,4-glucanase purified from the midgut gland of *G. natalis* hydrolysed carboxymethyl cellulose the fastest (Table 6). It also possessed significant activity of laminarinase (20.4% of endo-β-1,4-glucanase activity) and very low activities of lichenase (4.6% of endo-β-1,4-glucanase activity) and β-1,4-glucosidase (1.6% of endo-β-1,4-glucanase activity; Table 6). Given that the endo-β-1,4-glucanase was mainly active against carboxymethyl

Table 4. Specific activity, purification factor, units of enzyme and percentage yield for each step in the purification of endo-β-1,4-glucanase 1 from *C. destructor*

| Fraction | Endo-β-1,4-glucanase specific activity (μmol min$^{-1}$ mg protein$^{-1}$) | Purification factor | Units of endo-β-1,4-glucanase recovered after each chromatography step | Percentage yield |
|---|---|---|---|---|
| Homogenate | 0.23 | – | 39.7 | – |
| Re-dissolved 30–60% ammonium sulphate precipitate | 0.17 | 0.75 | 17.5 | 44.09 |
| Combined fractions 1–6 after DEAE anion exchange chromatography | 11.61 | 50.57 | 6.01 | 15.14 |
| Combined fractions 21–34 after P-100 gel filtration chromatography | 19.09 | 83.18 | 5.26 | 13.24 |

Purification factors and percentage yields are respectively expressed as a proportion and percentage of that in the initial homogenate. Units of enzyme are given as μmol min$^{-1}$.

Table 5. Specific activity, purification factor, units of enzyme and percentage yield for each step in the purification of endo-β-1,4-glucanase 2 from the midgut gland of *C. destructor*

| Fraction | Endo-β-1,4-glucanase specific activity (μmol min$^{-1}$ mg protein$^{-1}$) | Purification factor | Units of endo-β-1,4-glucanase recovered after each chromatography step | Percentage yield |
|---|---|---|---|---|
| Homogenate | 0.23 | – | 39.7 | – |
| Re-dissolved 30–60% ammonium sulphate precipitate | 0.17 | 0.75 | 17.5 | 44.09 |
| Combined fractions 1–6 after DEAE anion exchange chromatography | 5.13 | 22.36 | 11.15 | 28.08 |
| Combined fractions 21–34 after P-100 gel filtration chromatography | 7.43 | 32.36 | 4.06 | 10.23 |

Purification factors and percentage yields are respectively expressed as a proportion and percentage of that in the initial homogenate. Units of enzyme are given as μmol min$^{-1}$.

Table 6. The ability of laminarinase and endo-β-1,4-glucanase purified from the midgut gland of *G. natalis* and *C. destructor* to hydrolyse carboxymethyl cellulose (endo-β-1,4-glucanase activity), laminarin (laminarinase activity), lichenan (lichenase activity) and cellobiose (β-1,4-glucosidase activity)

| | Endo-β-1,4-glucanase activity (μmol min$^{-1}$ mg protein$^{-1}$) | Laminarinase activity (μmol min$^{-1}$ mg protein$^{-1}$) | Lichenase activity (μmol min$^{-1}$ mg protein$^{-1}$) | β-1,4-Glucosidase activity (μmol min$^{-1}$ mg protein$^{-1}$) |
|---|---|---|---|---|
| *Gecarcoidea natalis* | | | | |
| Laminarinase | 0.93±0.77 | 15.21±7.41 | 0.07±0.01 | 0.14±0.02 |
| Enzyme activity as a percentage of laminarinase activity | 6.1 | 100 | 0.4 | 0.9 |
| Endo-β-1,4-glucanase | 11.43±1.30 | 2.34±0.37 | 0.52±0.49 | 0.18±0.15 |
| Enzyme activity as a percentage of endo-β-1,4-glucanase activity | 100 | 20.4 | 4.6 | 1.6 |
| *Cherax destructor* | | | | |
| Laminarinase | 5.29 | 18.31 | 1.37 | 0.09 |
| Enzyme activity as a percentage of laminarinase activity | 28.9 | 100 | 7.49 | 0.5 |
| Endo-β-1,4-glucanase | 19.09 | 0.47 | – | 0.14 |
| Enzyme activity as a percentage of endo-β-1,4-glucanase activity | 100 | 2.46 | – | 0.71 |

Specific activities of endo-β-1,4-glucanase, laminarinase and lichenase are expressed as μmol of reducing sugars produced min$^{-1}$ mg protein$^{-1}$. Specific activity of β-1,4-glucosidase is expressed as μmol of glucose produced min$^{-1}$ mg protein$^{-1}$. Enzyme activities are also expressed as a percentage of the highest activity.

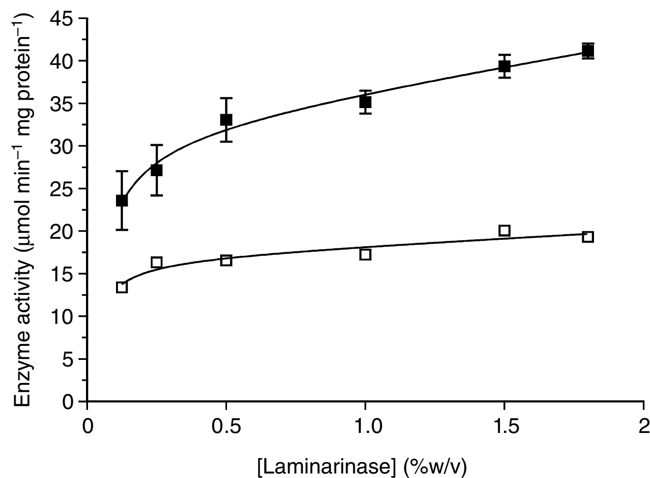



Fig. 11. Activity of laminarinase (μmol reducing sugars produced $\min^{-1}$ $ml^{-1}$) purified from the midgut glands of *G. natalis* (■) and *C. destructor* (□) with increasing concentrations of laminarin. Lines running through the data have been created after the data were fitted to the Michaelis–Menten enzyme kinetics model. Laminarinase from *G. natalis* had a $V_{max}$ of 42.04 μmol reducing sugars produced $\min^{-1}$ mg $protein^{-1}$ and a $K_m$ of 0.1259% (w/v). Laminarinase from *C. destructor* had a $V_{max}$ of 19.6 μmol reducing sugars produced $\min^{-1}$ mg $protein^{-1}$ and a $K_m$ of 0.059% (w/v).

cellulose, it was allocated the EC number 3.2.1.4 (Boeckmann et al., 2003).

Endo-β-1,4-glucanase purified from the midgut gland of *G. natalis* had the highest activity at pH 4–7. At more alkaline pH values (8 and 9) its activity was lower (Fig. 12B). The kinetic parameters for endo-β-1,4-glucanase could not be determined given the enzyme activity did not saturate at the highest workable carboxymethyl cellulose concentrations tested in this study. Carboxymethyl cellulose solutions at concentrations greater than 8% w/v are too viscous to work with. Incubation of the purified endo-β-1,4-glucanase with carboxymethyl cellulose produced cellobiose and short oligomers of glucose, most likely three and four residues long. A very small of amount of glucose was also produced (Fig. 13C).

### Characterisation of the purified enzymes from *C. destructor*

#### Laminarinase

In addition to laminarin, laminarinase purified form *C. destructor* was able to hydrolyse carboxymethyl cellulose and lichenan (Table 6). Hence it possessed substantial endo-β-1,4-glucanase activity (28.9% of laminarinase activity) and significant amounts of lichenase activity (7.49% of laminarinase activity). Cellobiose was hydrolysed at very low levels by the laminarinase (Table 6); thus it did not possess significant β-1,4-glucosidase activity. Given that it can hydrolyse both β-1,3- and β-1,4-glycosidic bonds it can be assigned the EC number 3.2.1.6 (Boeckmann et al., 2003). The purified enzyme had a $V_{max}$ of 19.6 μmol reducing sugars produced $\min^{-1}$ mg $protein^{-1}$ and a $K_m$ of 0.0593% (w/v) (Fig. 11). Laminarinase activity was maximal at pH 5.5 (Fig. 12C). Incubation of the purified enzyme with laminarin produced mainly glucose, and while other short oligomers were again present, their production appeared to be negligible compared with the amount of glucose formed (Fig. 13B).

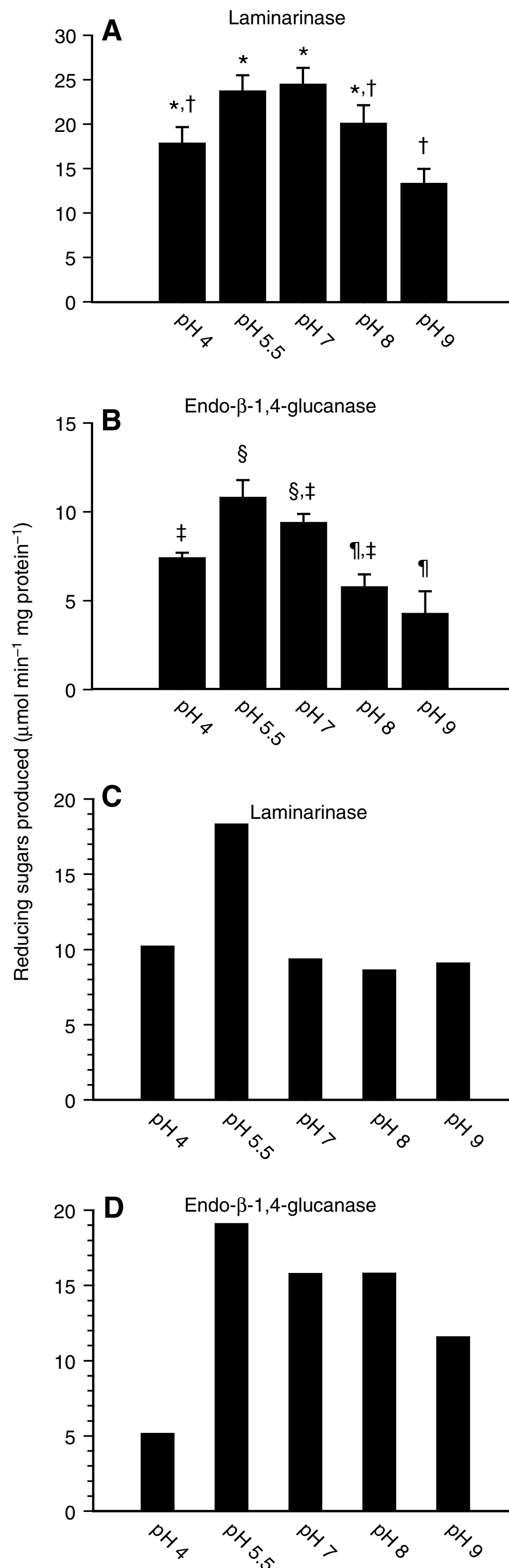



Fig. 12. Activity (μmol reducing sugars produced $\min^{-1}$ mg $protein^{-1}$) of laminarinase (A,C) and endo-β-1,4-glucanase (B,D) purified from the midgut glands of *G. natalis* (A,B) and *C. destructor* (C,D) at different pH values. Similar symbols indicate statistically similar means.

### Endo-β-1,4-glucanase

Endo-β-1,4-glucanase purified from *C. destructor* was able to hydrolyse mainly carboxymethyl cellulose, but possessed very low activities of laminarinase (2.46% of endo-β-1,4-glucanase activity) and β-1,4-glucosidase (0.71% of endo-β-1,4-glucanase activity) (Table 6). Thus, endo-β-1,4-glucanase hydrolyses mainly β-1,4-glycosidic bonds and can be assigned the cellulase EC number 3.2.1.4 (Boeckmann et al., 2003). Endo-β-1,4-glucanase activity was maximal at pH 5.5 and decreased moderately at higher pH values. At pH 4 endo-β-1,4-glucanase activity was low compared with the other pH values (Fig. 12D). From the hydrolysis of carboxymethyl cellulose, endo-β-1,4-glucanase from *C. destructor* is capable of producing cellobiose, small amounts of glucose and other short oligomers. Hence it possesses both endo-β-1,4-glucanase and cellobiohydrase activity (Fig. 13D).

## DISCUSSION

### Laminarinase characteristics

The laminarinase purified from the gecarcinid land crab *G. natalis* was capable of hydrolysing mainly β-1,3-glycosidic bonds, with negligible activity towards β-1,4-glycosidic bonds (Table 6); therefore it was assigned the EC number 3.2.1.39. It followed Michaelis–Menten kinetics, with a $V_{max}$ of 42.0 μmol reducing sugars produced $min^{-1}$ mg $protein^{-1}$ and a $K_m$ of 0.126% (w/v). In contrast, the laminarinase isolated from *C. destructor* was capable of hydrolysing both β-1,3- and β-1,4-glycosidic bonds; hence, it was allocated the EC number 3.2.1.6. This laminarinase also followed Michaelis–Menten kinetics with a $V_{max}$ of 19.6 μmol reducing sugars produced $min^{-1}$ mg $protein^{-1}$ and a $K_m$ of 0.0593% (w/v).

Laminarinase from *G. natalis* produced glucose and oligomers of two and three glucose residues from the hydrolysis of laminarin, confirming it is capable of significant glucose production and suggesting that it may also work in conjunction with β-1,4-glucosidase to hydrolyse laminarin completely. Laminarin is the main storage polysaccharide in algae; it is also produced in plants in response to damage or infection and is the main structural component in fungal cell walls (Bacic et al., 1988). Large activities of substrate-specific laminarinase within the midgut gland of *G. natalis* suggest it is an important energy source and would allow *G. natalis* to utilise fungi and algae growing on detritus within the rainforest. Corroborating evidence comes from observations that *G. natalis* scrape off and consume algae from rocks and tree buttresses (Linton and Greenaway, 2007). In contrast to *G. natalis*, the laminarinase from *C. destructor* produced predominantly glucose, with only small amounts of short oligomers being detected. This suggests that sources of laminarin such as algae may be a more important component of the diet of *C. destructor* when compared with *G. natalis*.

While the laminarinase from *G. natalis* is specialised to hydrolyse only β-1,3-glycosidic bonds, the enzyme from *C. destructor* is more generalised since it was also capable of limited hydrolysis of β-1,4-glycosidic bonds. The difference between these two distantly related species is unclear. However, the specialisation of the enzyme in *G. natalis* may be a result of the development of a more efficient enzyme, or it may indicate that the laminarinase from *C. destructor* aids the endo-β-1,4-glucanase in hydrolysing cellulose, thus making the cellulase system more efficient.

The molecular mass of the laminarinase isolated from the midgut gland of *G. natalis* was estimated to be 41 kDa by SDS-PAGE and 71 kDa from gel filtration chromatography (Figs 3, 4). This discrepancy suggests that laminarinase may exist as a dimer, joined by either a disulphide bond or van der Waals forces. Given that β-1,6-glycosidic bonds within laminarin cause the molecule to be branched (Lépagnol-Descamps et al., 1998), the laminarinase dimer may fit in between these branches. Simultaneous hydrolysis of the two branches may make the laminarinase more efficient. Alternatively, the laminarinase may contain a laminarin-binding domain, which is a similar size to the catalytic component. A similar discrepancy was observed for *C. destructor*; a 41 kDa band as well as a second, larger band of 62 kDa was detected by SDS-PAGE (Fig. 4). The molecular mass of the laminarinase was estimated to be 62 kDa from gel filtration chromatography. The 41 kDa band corresponds to the 41 kDa laminarinase from *G. natalis*, while the larger band may either be a minor impurity or represent a native laminarinase dimer.

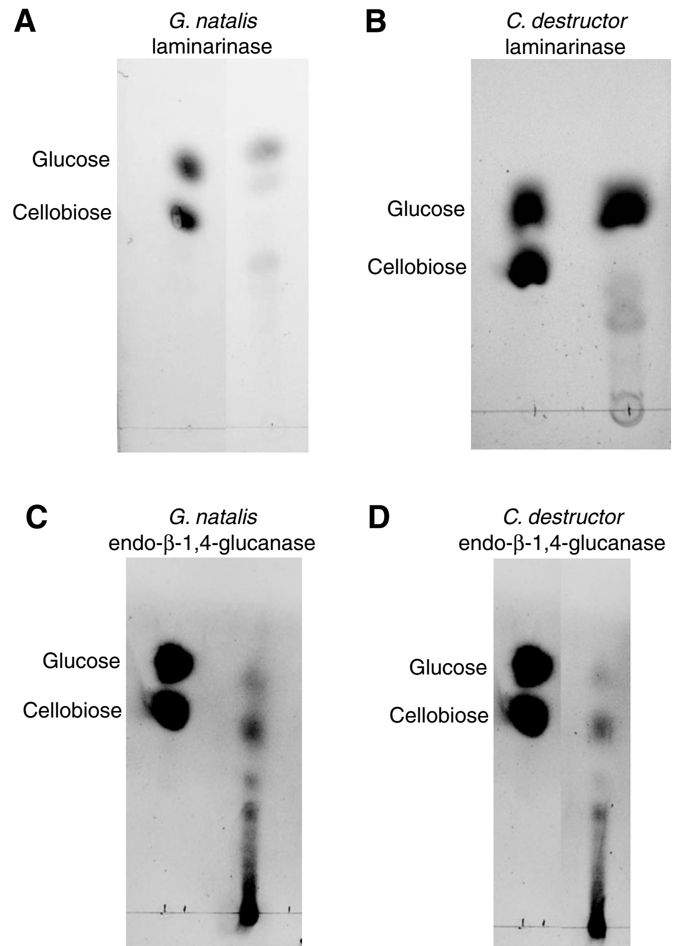



Fig. 13. Carbohydrates produced by the hydrolysis of laminarin when incubated with laminarinase from *G. natalis* (A) and *C. destructor* (B), and by the hydrolysis of carboxymethyl cellulose when incubated with endo-β-1,4-glucanase purified from *G. natalis* (C) and *C. destructor* (D). Hydrolysate and standards (glucose and cellobiose) were run on a silica thin layer chromatography (TLC) plate with a mobile phase of *n*-butanol/acetic acid/water (2:1:1, v/v/v) (Nishida et al., 2007). Carbohydrate products were visualised by spraying the TLC plate with 10% (v/v) sulphuric acid in ethanol and incubating at 120°C for 15 min (Nishida et al., 2007).

The size range of laminarinases isolated from various invertebrates is 22–146 kDa (Table 7). The 41 kDa laminarinase isolated from *G. natalis* and *C. destructor* is similar in size to that of the cockroach *Periplaneta americana* (Genta et al., 2003), suggesting that the enzyme may be inherited from a common ancestral arthropod.

Table 7. Comparison of known laminarinases and endo-β-1,4-glucanases from various species of invertebrates

| Species | Size (kDa) | Optimum pH | Source |
|---|---|---|---|
| Laminarinase | | | |
| *Periplaneta americana* (insect) | 46 | 6 | (Genta et al., 2003) |
| *Abracris flavolineata* (insect) | 146 | 5.7 | (Ferreira et al., 1999) |
| *Rhagium inquisitor* (insect) | 95–100 | 5–6 | (Chipoulet and Chararas, 1984) |
| *Haliotis tuberculata* (mollusc) | 60 | 7 | (Lépagnol–Descamps et al., 1998) |
| *Spisula sachalinensis* (mollusc) | 22 | – | (Sova et al., 1970) |
| *Euphausia superba* (crustacean) | 65–70 | 4.3–5 | (Suzuki et al., 1987) |
| *C. destructor* (crustacean) | 41 | 5.5 | Present study |
| *G. natalis* (crustacean) | 41 | 7 | Present study |
| Endo-β-1,4-glucanase | | | |
| *Reticulitermes speratus* (insect) | 41 and 42 | – | (Watanabe et al., 1998) |
| *Nasutitermes takasagoenis* (insect) | 47 | 5.8 | (Tokuda et al., 1997) |
| *Psacothea hilaris* (insect) | 47 | 5.5 | (Sugimura et al., 2003) |
| *Panesthia cribrate* (insect) | 49 and 54 | – | (Scrivener and Slaytor, 1994) |
| *Apriona germari* (insect) | 36 | 6 | (Lee et al., 2005) |
| *Lyrodus pedicellatus* (mollusc) | 40 | 6.8 | (Xu and Distel, 2004) |
| *Haliotis discus hannai* (mollusc) | 66 | 6.3 | (Suzuki et al., 2003) |
| *Strongylocentrotus nudus* (echinoderm) | 54 | 6.5 | (Nishida et al., 2007) |
| *Cherax quadricarinatus* (crustacean) | 48 and 50 | – | (Crawford et al., 2004) |
| *C. destructor* (crustacean) | 53 | 5.5 | Present study |
| *G. natalis* (crustacean) | 52 | 5.5 | Present study |

Interestingly, the molecular mass of laminarinase estimated from gel filtration chromatography is similar in size to that of abalone *Haliotis tuberculata* (Lépagnol-Descamps et al., 1998), krill *Euphausia superba* (Suzuki et al., 1987) and termite *Rhagium inquisitor* (Chipoulet and Chararas, 1984) (Table 7). The variation in the size estimation of laminarinase may be due to the laminarinase monomer being observed in some studies and the dimer in others.

The laminarinase is most likely to be endogenously produced given its high activity within the midgut gland of both *G. natalis* and *C. destructor*, and the endogenous production of endo-β-1,4-glucanase by arthropods (Byrne et al., 1999; Crawford et al., 2004; Davison and Blaxter, 2005; Linton and Greenaway, 2004; Linton et al., 2006). However, the gene for the crustacean laminarinase remains to be sequenced and the midgut gland would be the most logical tissue in which to look for expression of this gene.

### Endo-β-1,4-glucanase characteristics

The major endo-β-1,4-glucanase purified from *G. natalis* had a broader substrate specificity than that from *C. destructor*. The endo-β-1,4-glucanase from *G. natalis* hydrolysed mainly β-1,4-glycosidic bonds but was also capable of significant hydrolysis of β-1,3-glycosidic bonds. In contrast the endo-β-1,4-glucanase from *C. destructor* only hydrolysed β-1,4-glycosidic bonds (Table 6). The broader substrate specificity of endo-β-1,4-glucanase from *G. natalis* suggests that this species encounters β-1,3- and β-1,4-glycosidic bonds within its diet and requires an enzyme to break these bonds. This is in contrast to *C. destructor*, which has a typical arthropod endo-β-1,4-glucanase that is only capable of hydrolysing β-1,4-glycosidic bonds. Broader substrate specificity suggests that the enzyme from *G. natalis* should be allocated a different EC number that reflects this. However, given this glycosyl hydrolase has a preference for β-1,4-glycosidic bonds and is most likely to be a GHF9 glycosyl hydrolase, it should retain the classic endo-β-1,4-glucanase EC number of 3.2.1.4 (Linton et al., 2006). This example highlights the need for caution when trying to classify glycosyl hydrolases neatly into EC groups.

Endo-β-1,4-glucanase from *G. natalis* hydrolysed internal β-1,4-glycosidic bonds within the glucose polymer carboxymethyl cellulose to produce short glucose oligomers, cellobiose and a very small amount of glucose. Production of small oligomers of three and four glucose residues is characteristic of endo-β-1,4-glucanase enzymes and production of cellobiose is indicative of cellobiohydrase activity (Linton et al., 2006). Thus, the combined action of endo-β-1,4-glucanase and β-1,4-glucosidase may be sufficient to hydrolyse cellulose completely to glucose. Like other arthropods, *G. natalis* may lack a cellobiohydrase (Scrivener and Slaytor, 1994); however, it remains to be tested whether the combined action of endo-β-1,4-glucanase and β-1,4-glucosidase in an *in vitro* system can explain the rate of cellulose hydrolysis observed *in vivo*. The endo-β-1,4-glucanase from *C. destructor* also produced short oligomers as well as glucose from carboxymethyl cellulose. The retention of this similar activity between two distantly related species of crustaceans further reinforces the importance of cellulose as a source of glucose in herbivorous crustaceans.

The endo-β-1,4-glucanases purified from *G. natalis* and *C. destructor* are most likely to be GHF9 endo-β-1,4-glucanases given they have similar molecular masses of 52 and 53±2 kDa, respectively, when compared with other GHF9 cellulases from arthropod species (Table 7). This combined with the large activity within the midgut gland suggests they are likely to be the product of the endogenous GHF9 gene present in crustaceans, and arthropods in general, and hence are synthesised endogenously within this tissue (Byrne et al., 1999; Crawford et al., 2004; Linton et al., 2006). Endo-β-1,4-glucanases from both *G. natalis* and *C. destructor* are also most probably glycosylated given that glycosylation is a common feature of GHF9 endo-β-1,4-glucanases from other arthropods (Table 7) (Lee et al., 2005; Nishida et al., 2007; Zhou et al., 2007). Glycosylation may prevent proteolytic degradation and thus may explain why the endo-β-1,4-glucanases are relatively stable (Langsford et al., 1987).

### Enzyme activity at different pH

Both the laminarinase and the endo-β-1,4-glucanase from *G. natalis* had substantial activity over a pH range of 5.5 to 8 (Fig. 12). While both enzymes had high activity at both pH 5.5 and pH 7, the

laminarinase had maximal activity at pH 7, while endo-β-1,4-glucanase activity was slightly higher at pH 5.5. Since the pH of the digestive juice of *G. natalis* is 6.69±0.3 (Linton and Greenaway, 2004), both enzymes would be working maximally *in vivo*. This is also true of the enzymes from *C. destructor*, both of which had maximal activity over a pH range of 5.5 to 8. The pH of the gastric juice of *C. destructor* is assumed to be similar to that of the closely related *C. quadricarinatus*, which is pH 5.8 (Figueiredo et al., 2001).

### Other endo-β-1,4-glucanase activity peaks

Four other endo-β-1,4-glucanase peaks were resolved from *G. natalis* from HIC (Fig. 2). The elution profile for two of these endo-β-1,4-glucanases from the subsequent gel filtration chromatography suggests that the enzymes had a similar molecular mass to that already purified (52 kDa). Similarly, in *C. destructor* two endo-β-1,4-glucanases of similar molecular mass were purified to homogeneity. The most likely explanation for this is that both species produce more than one endo-β-1,4-glucanase. Given the similar molecular masses observed, they are most likely to be isomers. Two endo-β-1,4-glucanases have been purified from the termite *Reticulitermes speratus* (Watanabe et al., 1998) and the cockroach *Panesthia cribrata* (Lo et al., 2003; Scrivener and Slaytor, 1994). There may be more than one copy of the GHF9 gene in crustaceans since two separate studies (Byrne et al., 1999; Crawford et al., 2004) contain discrepancies in the sites of introns identified for the endo-β-1,4-glucanase gene in *C. quadricarinatus*.

### Function of the endo-β-1,4-glucanase and laminarinase within *G. natalis* and *C. destructor*

Herbivorous crustaceans such as *G. natalis* possess a range of cellulase and hemicellulase enzymes such as the endo-β-1,4-glucanase and laminarinase examined in this study. Similar enzymes are also present in distantly related aquatic species such as *C. destructor*. This suggests that the aquatic ancestors of the herbivorous gecarcinid land crabs may have possessed these enzymes that have then evolved to efficiently cope with the adoption of a leaf litter diet. Given the complex nature of the structural compounds present within the cell walls of plants, carbohydrate polymers with mixed β-1,4-, β-1,3- and β-1,6-glycosidic bonds, hemicellulase and cellulase enzymes may work synergistically to achieve total hydrolysis of carbohydrate polymers. The possible role of synergism in cellulase systems remains largely unknown; however, the isolation and purification of the enzymes described here will enable this to be investigated in an *in vitro* system. The enzymes may also interact with the function of the gastric mill to achieve efficient cellulose hydrolysis.

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