Biochemistry and Molecular Biology of Cellulases of Lower Termites

下等シロアリ類のセルラーゼに関する生化学・分子生物学的研究

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渡辺　裕文

CONTENTS

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GENERAL INTRODUCTION

Cellulose is the most abundant biomass on the earth (Tomme et al., 1995). It is a polymerized form of glucose molecules with β-1,4-linkages, consisting of composite forms of highly crystallized microfibrils among amorphous matrixes, thus refusing access to hydrolyzing enzymes (Tomme et al., 1995). As responses to the difficulty in cellulose digestion, cellulolytic fungi and bacteria have developed complex forms of cellulase systems which actively hydrolyze cellulose fibrils (Tomme et al., 1995). Plants are also known for cellulases, which hydrolyze cell walls according to their developmental stages (i.e., bean abscission, fruit ripening and abscission, and pedicellate abscission) (Bennett and Christoffersen, 1986; Brummell et al., 1994; Campillo and Bennett, 1996; Lashbrook et al., 1994; Milligan and Gasser, 1995; Trainotti et al., 1999a; Trainotti et al., 1999b; Tucker et al., 1986; Tucker and Milligan, 1991; Yung et al., 1999). However, higher animals have not been generally recognized to produce endogenous cellulases (Foley, 2001), despite that holding or acquisition of cellulose digestion ability means getting immense amount of energy source for animals.

The early report on animal cellulose digestion was found in 1920's and cellulase activity has been reported from Nematoda, Mollusca, Annelida, Crustacea, and Insecta (Nishizawa, 1974). In the beginning of study, it seemed that cellulases were considered as one of digestive enzymes produced by the digesting organs of the cellulose ingesting animals. The idea of endogenous cellulases was changed by a series of experiments of Cleveland, who eliminated protozoan fauna of the termite, *Reticulitermes flavipes* and the xylophagous cockroach, *Cryptocercus punctulatus*. The defaunated animals lost their viability on cellulose food, thus he contributed the origin of cellulose digesting enzymes to the hindgut protozoan fauna (Cleveland, 1924, 1934). Since then, the presence of symbiotic protozoa or bacteria has been used to explain cellulose digestion in higher animals (Chapman, 1998; Martin, 1991). Following Cleveland's experiments, much research was devoted to demonstrate

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symbiotic cellulase production by bacteria and fungi in addition to protozoa, which are harbored in invertebrate digestive systems. However, few reports were presented demonstrating symbiotic relationships between host invertebrates and intestinal microbes other than protozoa (Prins and Kreulen, 1991).

Discrepancies with theories based on symbiotic cellulose digestion arose from apparent contradictions between cellulolytic activity and the locations – or absence – of symbionts as enzyme sources. In the snail, *Helix pomatia*, cellulase and chitinase activities were detected in sterile hepatopancreas (Strasdine and Whitaker, 1963). In addition, both cellulase and chitinase activity in the digestive juices (luminal fluid of the gut) increased in proportion to body weight and the total protein of the hepatopancreas, but not in relation to increases in digestive juice bacterial counts, as when the snails emerge from hibernation and start eating. It was therefore concluded that these enzymes were produced endogenously (Strasdine and Whitaker, 1963). Similar arguments have also been put forth based on contradictions observed between the presence or absence of cellulolytic activities and gut microorganisms in earthworms (Marialigeti, 1979; Mishra and Dash, 1980; Nielsen, 1962; Parle, 1963; Tracey, 1951; Urbasek, 1990; Urbasek and Pizl, 1991; Vinceslas-Akpa and Loquet, 1996).

Slaytor and his colleagues demonstrated that the distribution of cellulase in the gut of the Australian wood-eating cockroach *Panesthia cribrata* was limited to the fore- and midgut, and was not found in protozoa inhabiting the hindgut (Scrivener et al., 1989). *P. cribrata* was maintained on filter paper with tetracycline (or crystalline cellulose) for over 12 weeks without reduction of cellulase activity (Scrivener et al., 1989). During this experiment, the respiratory quotient value (produced CO_2/consumed O_2) stayed between 0.98 and 1.03 (0.7 for starved individuals) (Scrivener et al., 1989), showing that the *P. cribrata* cockroaches lived on the cellulose, but not on the stored energy, similar to the other Australian wood-eating cockroaches *Calolampra elegans* and *Geoscapheus dilatatus* (Zhang et al., 1993). The possible role of acquired fungal cellulases has also been proposed in *P.*

cribrata (Martin, 1991), but quantification of the fungal cellulase in food material did not support this (Scrivener and Slaytor, 1994a). Cockroaches are a group of insects including fossil species from the Paleozoic Period. They are taxonomically cross-related to termites (Cornwell, 1968) and join a monophyletic clade which includes termites (Lo et al., 2000). It is estimated that there are about 3,500 cockroach species, but less than one percent of them are domestic omnivorous pests. Most others live in the ground, or commensally in the nests of ants, wasps or termites of tropical rainforests, and a few species bore into and inhabit wood (Cornwell, 1968). Elucidation of cellulose digestion in such cockroaches may help to clarify the origin of that in termites.

In termites, the first possible endogenous cellulase was demonstrated in the Japanese subterranean termite *Luecotermes (Reticulitermes) speratus* (Yokoe, 1964). A drastic reduction of hindgut protozoa by heat exposure of these termites did not affect cellulase activity as measured by decrease of the viscosity of carboxymethylcellulose (CMC) solution. Thus cellulase activity was attributed to the host (Yokoe, 1964). Later, both salivary gland cellulase activity against CMC, and that in hindgut protozoan fauna against filter-paper and cotton, were separated by Yamaoka and Nagatani (1975), who proposed that these two cellulases synergistically break down native cellulose in an analogy to the fungal cellulase system (Reese et al., 1950). Undoubtedly, ingested cellulose will come into contact with both the salivary and protozoan cellulases, but whether both cellulases act simultaneously in a synergistic action (Reese et al., 1950) has yet to be clarified.

Termites belonging to the family Termitidae, which are called "higher termites", which make up three-quarters of known termite species, generally do not contain protozoan fauna, but many of them ingest cellulosic material (Krishna, 1970). To explain the cellulose digestion of the higher termites, the source of cellulases in higher termites has been interpreted as due to the acquisition of cellulolytic bacteria in the hindgut (Martin, 1991), isolation of cellulolytic bacteria from the gut has been attempted, but only cellulolytic facultative-heterotrophs and inherent non-cellulolytic

spirochetes were found and the enrollment of intestinal bacteria in cellulose digestion has not been confirmed (O'Brien and Slaytor, 1982; Slaytor, 1992). A comparative study of the Australian arboreal higher termite *N. walkeri* with the mound-building lower (=protozoa-inhabiting) termite *Coptotermes lacteus* showed that the intestinal fauna and flora play no (*N. walkeri*) or relatively little (*C. lacteus*) role in the cellulase production of these species (O'Brien et al., 1979). Removal of intestinal bacteria and protozoa by tetracycline treatments (assumed to eliminate protozoa indirectly by the reduction of bacteria) did not affect cellulase activities in the guts of both species except for a 20% decrease seen in the hindgut of *C. lacteus* (O'Brien et al., 1979). In *N. walkeri* and the mound-building higher species *N. exitiosus* the majority of cellulase activity was located in the epithelia and the luminal contents of the midgut (Hogan et al., 1988b).The function of the intestinal bacteria was explained as to produce acetate and fix nitrogen in order to digest cellulose consumed by the host (O'Brien and Slaytor, 1982; Prins and Kreulen, 1991).

Following these researches on the origin of cellulases in animals, the description about animal cellulose digestion has been changing in some textbooks as we see in "Biology" (2nd edition, edited by Knox, Ladiges, Evans and Saint), "It used to be thought that no animals produced their own cellulases, but we now know that several Australian insects, including some termites and the wood cockroach, and some land snails can produce cellulase. Nonetheless, it is likely that all animals (certainly all vertebrates) that digest cellulose rely primarily on cellulases produced by symbiotic microorganisms (Foley, 2001)." It seemed that simultaneous presence of host and symbionts-origin cellulases in termites has been recognized finally, although it was accepted as exceptional cases.

Recent advancement on molecular-based biology changed the method in cellulase study, which elucidates the amino-acid sequences encoded on the cellulase genes without amino-acid analysis. The sequence information also allowed researchers to study their phylogenic origins and gene-regulation, as well as to predict three-dimensional structures and catalytic mechanisms and to study kinetics based on

recombinant proteins. Based on the molecular information, cellulases have been classified based on their amino-acid (nucleotide) sequences into 14 families reflecting their phylogenic origins (Henrissat, 1991; Henrissat and Bairoch, 1993, 1996; Henrissat and Davies, 1997). In addition to the first structures (amino-acid sequences), three-dimensional structures and catalytic mechanisms have been elucidated in many bacterial and fungal cellulases. Whilst in animals, there had been a long history of animal cellulase study since early 20th century but no studies on the molecular level. In this thesis, the endogenous and symbiotic (protozoan) cellulases (and their responsible genes) of termites were isolated and characterized to clarify entities of the cellulases of both origins, thus, to elucidate the aspects of cellulose digestion of protozoa-inhabited termites. Moreover, origins and distributions of animal cellulases were discussed in detail.

For purification, characterization of the termite endogenous cellulase and cloning of the responsible gene (Chapter I and II), a species from the lower termites *Reticulitermes speratus* was used because of its abundance and easy supply for experiments, although it is not ideal for elucidation of protozoan cellulases because of its puzzling composition of the hindgut protozoan fauna, which consists of 11 species (Kitade and Matsumoto, 1998b). For elucidation of protozoan cellulases, another lower species *Coptotermes formosanus* was chosen because of its simple composition of the hindgut protozoan fauna consisting of three main species (*Pseudotrichonympha grassii*, *Holomastigotoides mirabile (hartmanni)* and *Spirotrichonympha leidyi*) and two other minor species in the population (Kitade and Matsumoto, 1998b). Following the current study, it has been elucidated that *C. formosanus* has endogenous cellulase genes encoding almost identical cellulases to that of *R. speratus* (Nakashima et al., 2002a). Thus, the basic information about the endogenous cellulase of *R. speratus* would be adaptable for that of *C. formosanus* without causing inconsistence between two species.

CHAPTER 1

Site of Secretion and Properties of Endogenous Endo-β-1,4-glucanase Components from *Reticulitermes speratus* (Kolbe), a Japanese Subterranean Termite

Abstract

Two endo-β-1,4-glucanase components (YEG1 and YEG2) of the endogenous cellulase from the Japanese subterranean termite, *Reticulitermes speratus*, were purified to homogeneity using gel filtration and hydroxylapatite chromatography, and their enzymatic properties investigated. Using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), YEG1 and YEG2 had M_r of 42kDa and 41kDa, respectively. Both components had an optimal pH of 6.0, an optimal temperature of 50°C and were stable at 40°C for at least 30 min. Both components showed high activity on sodium carboxymethylcellulose (CMC), 73.6 U/mg protein for YEG1 and 83.4 U/mg protein for YEG2. The K_m values of YEG1 and YEG2 on CMC were 1.83 mg/ml and 1.48 mg/ml, respectively. YEG1 did not hydrolyze cellotetraose or cellotriose, whereas YEG2 hydrolyzed cellotetraose to cellobiose and cellotriose to cellobiose and glucose. Both YEG1 and YEG2 hydrolyzed cellopentaose to cellotriose and cellobiose. Neither component hydrolyzed cellobiose. The hydrolytic products from crystalline cellulose (Sigmacell Type 20) by YEG1 and YEG2 were cellobiose and a trace amount of glucose. Polyclonal mouse anti-serum raised against YEG2 cross-reacted with YEG1, suggesting a common origin for both components. Using this anti-serum, Western blotting and immunohistochemistry showed the presence of YEG1 and YEG2 in the salivary glands, but not in the midgut epithelium. The data suggest that the salivary glands are the site of secretion of endo-β-1,4-glucanase in *R. speratus*.

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Introduction

The digestion of cellulose by xylophagous insects has interested biologists since the beginning of the 20th century. In the early years of the study, the ability to digest cellulose was thought to consist of single enzyme "cellulase" originated from the symbiotic organisms like intestinal protozoa. Later, it was demonstrated that the cellulase includes several different cellulolytic activities presumably consisting of various components, and insects themselves also produced some activities (Breznak and Brune, 1994; Martin, 1991; Slaytor, 1992).

Materials and methods

Termites

Pine logs inhabited by *Reticulitermes speratus* (Kolbe) were collected in Tsukuba, Ibaraki prefecture, Japan and termites were maintained in the laboratory in plastic containers with pine logs at room temperature.

Preparation of enzyme extracts

Sodium acetate buffer (0.1 M, pH 5.5)(O'Brien et al., 1979) was used in the preparation of all extracts and in all assays, unless otherwise indicated. Worker and soldier termites (10 g) were homogenized in 100 ml of buffer and centrifuged at 4,000 *g* for 10 min. The supernatant (crude extract) was filtered through filter paper and precipitated using 400 ml of cold (-20°C) acetone. After centrifugation at 4,000 *g* for 5 min and vacuum-drying, the sample was dissolved in 50 ml of ammonium acetate buffer (0.3 M, pH 5.0). The solution was filtered using a polysulphone membrane (pore size 0.8 μm; Advantec Toyo). The filtrate was concentrated to 5 ml using a polysulphone ultrafilter (UK-10, molecular weight cut off: 10,000; Advantec Toyo) in an ultrafiltration cell (8050; Amicon). This concentrated extract was used for the purification of endo-β-1,4-glucanase components.

Salivary glands, midguts and hindguts were dissected out from 100 workers in phosphate buffered saline (PBS; 150 mM NaCl in 20 mM Na^+K^+ phosphate buffer, pH 7.4). The gut contents and peritrophic membranes of the midguts were removed in sodium acetate buffer. The salivary glands, midguts and hindguts were homogenized separately in micro-centrifuge tubes (1.5 ml) with 0.1 ml of sodium acetate buffer, and centrifuged at 21,600 *g* for 20 min. The supernatants were used for chromatography and electrophoresis.

Column chromatography

All column chromatography was carried out at 4°C. Fractions containing enzyme activity were desalted and concentrated using an ultrafiltration cell (Model 8010 or 8050; Amicon) and a UK-10 membrane (Advantec Toyo) with distilled water. Protein was monitored at 280 nm using a UV-1 monitor (Pharmacia).

Extracts were chromatographed on a HiLoad 16/60 Superdex 75 prep grade column (Pharmacia), equilibrated with ammonium acetate buffer (0.3 M, pH 5.0), at a flow rate of 0.5 ml/min. Fractions (1.2 ml) were assayed for endo-β-1,4-glucanase and β-glucosidase activities. Those fractions containing endo-β-1,4-glucanase were pooled and adsorbed on a hydroxylapatite (DNA grade, Bio-Rad) column (60 x ø10 mm). The column was washed with 60 ml of starting buffer (2 mM potassium phosphate, pH 5.3) and eluted with a 50 ml linear gradient to 500 mM potassium phosphate (pH 5.3), at a flow rate of 0.2 ml/min, with the collection of 0.5 ml fractions. The salivary gland extract was similarly chromatographed on a hydroxylapatite column (100 x 10 mm).

Electrophoresis

Purified enzymes and crude extracts were examined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of (Laemmli, 1970). The slab gel consisted of a 10% (w/v) resolving gel and a 5% (w/v)

stacking gel. Samples were boiled for 60 s with one third volume of treatment buffer (16 g SDS, 80 g glycerol, 16 mg bromophenol blue in 100 ml Tris HCl buffer, pH 6.8, and 8.7 ml 2-mercaptoethanol). Proteins and a M_r standard mixture (GIBCO BRL) were stained using Coomassie Brilliant Blue R250 (Fluka Chemie AG).

Enzyme assays

β-Glucosidase. Samples (10 µl) were incubated with 200 µl of a 2% (w/v) solution of cellobiose (Nacalai Tesque) in sodium acetate buffer at 37°C for 5 min. The reaction was stopped by the addition of 2 ml of Glucose CII Test reagent (Wako Pure Chemical). Glucose was measured spectrophotometrically at 505 nm. One unit is defined as the amount of activity which produced 1 µmol of glucose/min.

Endo-β-1,4-glucanase. Samples (10 µl) were incubated with 200 µl of a 2% (w/v) solution of sodium carboxymethylcellulose (CMC) (degree of polymerization: 500-700, degree of carboxymethyl substitution: 55-65%; Kokusan Chemical) in sodium acetate buffer at 37°C for 5 min. The incubation time was lengthened to 100 min to measure the activity in hindgut fractions from Superdex 75. The reaction was stopped by the addition of 500 µl of copper carbonate/tartrate reagent (Somogyi, 1952) and reducing sugars in the reaction mixture were measured spectrophotometrically at 500 nm according to the method of Nelson (1944) as modified by Somogyi (1952), with glucose as a standard. The lower limit of detection was 0.2 µmol/ml with glucose in the reaction mixture. One unit is defined as the amount of activity which produced 1 µmol of reducing sugar/min.

Optimal temperature and pH

The activity of pure endo-β-1,4-glucanase components was measured quantitatively over the range of 20-70°C, with 5°C intervals, to determine the optimal temperature. To evaluate thermal stability, enzyme samples were preincubated at a constant temperature (20-70°C; 5°C intervals) for 30 min and then assayed at 37°C.

In order to measure optimal pH, 1% (w/v) CMC solutions in citrate-phosphate wide range buffer (pH 3.5-9.0; 0.5 pH intervals; McIlvaine, 1921) were used.

Activity of purified components on crystalline cellulose

Solutions of pure endo-β-1,4-glucanase components (10 μl) and 40 μl of 2% (w/v) Sigmacell Type 20 suspension in buffer were incubated in microfuge tubes with shaking (200 strokes/min) at 37°C for 6 h. The reaction was stopped either by the addition of 100 μl of copper carbonate/tartrate reagent for estimation of reducing sugars or by boiling for 10 min for thin layer chromatography (TLC) analysis. TLC was performed according to the method of (Damonte et al., 1971), as modified by (Scrivener and Slaytor, 1994b). Developed TLC plates were scanned by a GT-6000 image scanner (EPSON) and the density of each spot was analysed using NIH Image software on a Macintosh computer.

Analysis of hydrolytic products from cellodextrins

Samples (5 μl) were incubated with 20 μl of 60 mM solutions of cellotriose, cellotetraose and cellopentaose (Sigma) in sodium acetate buffer at 37°C. Aliquots (5 μl) were collected at 0 time and 5 min intervals for 15 min, placed on ice to stop the reaction, and then analysed by TLC.

Estimation of kinetic constants

For the evaluation of K_m and V_{max} values, solutions of cellotriose, cellotetraose, cellopentaose and CMC in buffer were used as substrates. Pure endo-β-1,4-glucanase components (5 μl) were incubated with substrate solutions (95 μl) at 37°C for 5 min and reducing sugars measured. The concentrations were 1.9 to 7.5 mM for cellotriose, 1.4 to 14 mM for cellotetraose, 0.1 to 5.7 mM for cellopentaose, and 1 to 19 mg/ml for CMC.

Protein

The protein concentration of samples was measured by a direct spectrophotometric method. Absorbance at 260 nm and 280 nm was measured with a spectrophotometer (UV-1200, Shimadzu) and the protein concentration calculated by the following equation (Layne, 1957):

Protein concentration (mg/ml) = $1.45A_{280}-0.74A_{260}$

The values obtained using this method were comparable to those derived from the Lowry method (Lowry *et al.*, 1951) using bovine serum albumin as the standard.

Production of polyclonal antibodies

Pure YEG2 (100 ng in 200 μl) was injected under the epithelium of a mouse (BALB/c, female) with the same volume of Freund's complete adjuvant (Nacalai Tesque) as the first antigen. Two and three weeks after the first injection, 100 ng of the protein without adjuvant was again injected into a tail vein as a booster. One week later, blood was collected by cutting the tip of the tail. The serum was separated from the blood cells using a serum separator tube (Microtainer, Becton Dickinson) and stored with 0.02% (w/v) sodium azide at –70°C.

Western blotting

Samples were run on SDS-PAGE. Proteins on the gel were transferred to a PVDF membrane (Trans Blot Transfer Medium, 0.2 μm, Bio-Rad) using a transfer cell (TransBlott SD, Bio-Rad) at 15 V for 30 min. The membrane was blocked with skim milk solution [4% (w/v) in TPBS (0.02 M phosphate buffered saline with 0.15 M NaCl, pH 7.4, 0.02% (v/v) Tween 20)] for 30 min at room temperature. The membrane was incubated with polyclonal mouse serum solution [0.5% (v/v) anti-YEG2 mouse serum, 2% (w/v) skim milk and 0.02% (w/v) sodium azide in

TPBS] overnight at 37°C. Antibody bands on the membrane were detected using a Vectastain ABC AP KIT and the alkaline phosphatase substrate, SK-5400 (both from Vector Laboratories).

Immunohistochemistry

Salivary glands and midguts dissected out in PBS (0.02 M phosphate buffered saline with 0.15 M NaCl, pH 7.4) were fixed in 5% (w/v) trichloroacetic acid solution in PBS with microwave radiation (500 W) 3 times for 3 s each to accelerate the impregnation of the fixative (Boon *et al.*, 1986). The fixed tissues were dehydrated in an ethanol series [60 - 100% (v/v)] and xylene, then embedded in wax and sectioned into 5-8 μm thickness by a microtome. They were dewaxed using xylene and rehydrated in the same ethanol series in reverse order. After incubation with anti-YEG2 mouse serum [0.2% (v/v) in PBS], the sectioned tissues were stained as described for Western blotting.

Results

Purification

Two endo-β-1,4-glucanase components and one major β-glucosidase component were present in whole body extracts of *R. speratus*, and, in addition, three minor β-glucosidase components were present in hindgut extracts.

The major β-glucosidase component was eluted at 53 ml from Superdex 75 from the whole body extract (Fig. 1a), but it was not present as a prominent peak in the hindgut extract (Fig. 1b). The minor β-glucosidase components were eluted from Superdex 75 at 44, 48 and 58 ml (Fig. 1b) from the hindgut extract, but they were not detected in whole body extracts (Fig. 1a). The major β-glucosidase component in the hindgut may have been represented by the right shoulder of the second minor β-glucosidase peak eluted at 48 ml (Fig. 1b).

A peak of the endo-β-1,4-glucanase was eluted at 71 ml from Superdex 75

from the whole body extract, and a protein peak corresponded to this activity peak (Fig. 1a). An endo-β-1,4-glucanase peak was also eluted from the hindgut extract from Superdex 75 at the same elution volume (71 ml), but the amount of activity was so small that no protein peak coincident with the activity peak was seen (Fig. 1b), indicating that the specific activity of endo-β-1,4-glucanase was lower in the hindgut extract than in the whole body extract.

The endo-β-1,4-glucanase eluted from the whole body extract from Superdex 75 was subsequently separated into two endo-β-1,4-glucanase components, YEG1 (Yamato-shiroari endo-β-1,4-glucanase 1) and YEG2, on hydroxylapatite (Fig. 2a). Both components were considered pure after hydroxylapatite chromatography, as they stained as single bands on SDS-PAGE (Fig. 3). Two endo-β-1,4-glucanase peaks, present in the salivary gland extract, eluted at similar concentrations of potassium phosphate on hydroxylapatite (Fig. 2b). These endo-β-1,4-glucanase components constituted more than a third (35%) of the soluble protein in salivary gland extracts, as calculated from the peak area (Fig. 2b).

The molecular weights, M_r, of YEG1 and YEG2 were estimated, by comparison with standards on SDS-PAGE, to be 42kDa and 41kDa, respectively (Fig. 3). The purification of YEG1 and YEG2 is summarized in Table 1. YEG1 and YEG2 were purified 22 and 25 fold, respectively, from the crude extract (Table 1).

Characterisation of endo-β-1,4-glucanase components

Both YEG1 and YEG2 had optimal activity at pH 6.0. YEG1 retained over 80% of the maximum activity from pH 5.3 to 7.0 while for YEG2, this range was from pH 4.9 to 7.4. YEG1 and YEG2 retained 32% and 46% of their respective maximal activities at pH 9.0, but the activity dropped more sharply in the acidic range and was lost at pH 4.0 and 3.5, respectively. Both components showed maximum activity at 50°C (Fig. 4a and b). They were stable up to 40°C during a 30 min preincubation, but the activity decreased dramatically when the preincubation

temperature was increased to 45°C and was lost above 55°C (Fig. 4c and d).

YEG1 and YEG2 hydrolyzed not only CMC but also cellodextrins and crystalline cellulose (Sigmacell Type 20). The specific activities of YEG1 and YEG2 on crystalline cellulose were 1:3100 and 1:7600 of that on CMC. The ratios of glucose to reducing sugars in the products of crystalline cellulose were 1:17 and 1:10 for YEG1 and YEG2, respectively (Table 2). TLC analysis resolved that YEG1 and YEG2 hydrolyzed crystalline cellulose to glucose and cellobiose, and the ratios of glucose to cellobiose in the products were 1:25 for YEG1 and 1:8 for YEG2. YEG1 and YEG2 also showed differences in substrate specificity (Table 3). YEG2 weakly hydrolyzed cellotriose to cellobiose and glucose, cellotetraose to cellobiose and small amounts of cellotriose and glucose. YEG1 was inactive on cellotriose and cellotetraose. YEG1 hydrolyzed cellopentaose to equimolar amounts of cellobiose and cellotriose. YEG2 hydrolyzed cellopentaose to cellotriose, cellobiose and a trace of glucose, which was probably produced as a hydrolytic secondary product from cellotriose produced in the reaction. No cellotetraose was produced from cellopentaose by either YEG1 or YEG2.

The K_m and V_{max} values for YEG1 and YEG2 are shown in Table 3. The K_m and V_{max} values on CMC for both components were similar, but YEG1 showed a smaller K_m value than that of YEG2 on cellopentaose, despite the fact that YEG2 hydrolyzed shorter cellodextrins (cellotriose and cellotetraose), which were not hydrolyzed by YEG1 (Table 3).

Immunological detection of endo-β-1,4-glucanase components

Western blotting demonstrated that YEG1 and YEG2 were present in the salivary glands but not in other tissues of the gut. Anti-YEG2 serum reacted with purified YEG1 and YEG2, crude salivary gland extract, and whole midgut extract (Fig. 5b). The salivary gland extract showed a single broad band spreading over the apparent molecular weight range of YEG1 and YEG2 (Fig. 5a). This broad protein

band reacted with anti-YEG2 mouse serum on the membrane (Fig. 5b). Coomassie Brilliant Blue staining of whole midgut extract showed a large amount of protein on SDS-PAGE, but there was only a weak reaction to anti-YEG2 serum in Western blotting (Fig. 5a and b). Midgut tissue showed only a small amount of protein on SDS-PAGE and no antigenicity with anti-YEG2 serum (Fig. 5b).

Anti-YEG2 serum reacted immunohistochemically with some of the acinar cells of the salivary glands (Fig. 6a). In the intestinal lumen, the anti-YEG2 serum reacted only with the food debris, which appeared to adsorb YEG1 and YEG2, in the middle part of the midgut (Fig. 6b). In the foregut and the anterior third of the midgut, from which food debris was absent, there was no binding of anti-YEG2 serum.

Discussion

The results showed that the cellulase of *R. speratus* consisted of two endo-β-1,4-glucanase components, and one major and three minor β-glucosidase components. Chromotography on Superdex 75 showed that the major β-glucosidase component was prominent in the whole body extract but not in the hindgut extract, while for the minor components the opposite was true. As secretion from the hindgut is unknown in insects (Snodgrass, 1993), these minor components are likely to be of protozoan origin, while the prominence of the major component in the whole body extract suggests that it is endogenous. The cellulase system of *R. speratus* is thus similar to another lower termite *Coptotermes lacteus*, which has both endogenous and protozoan β-glucosidase components (Hogan et al., 1988a).

Single peaks of endo-β-1,4-glucanase were eluted at the same elution volume from Superdex 75, from both whole body and hindgut extracts. These peaks were considered to be identical chromatographically. As the results of gel filtration (on Superdex 75) also indicated that the specific activity of the endo-β-1,4-glucanase was considerably lower in the hindgut extract than in the whole body extract, the origin of this activity was not thought to be in the hindgut. This activity is thus likely to have

originated from upper regions of the intestinal lumen. No other endo-β-1,4-glucanases were eluted from the hindgut extract, implying that protozoan endo-β-1,4-glucanase components were absent from this extract. This is possibly because the protozoan components were bound to protozoan cell-membranes and were not solubilized during the extraction procedure. The endo-β-1,4-glucanase in the gut of *R. speratus* was subsequently separated into two components, YEG1 and YEG2. Many endo-β-1,4-glucanases consist of multiple components, such as EG1 and EG2 from the cockroach *Panesthia cribrata* (Scrivener and Slaytor, 1994b), and three components from the fungus *Aspergillus japonicus* (Kundu et al., 1988) and three (Okada, 1975, 1976) or four components (Kim et al., 1994) from the fungus *Trichoderma viride*.

The properties of YEG1 and YEG2 were similar to endo-β-1,4-glucanase components from fungi and bacteria. Thus, the weakly acidic optimal pH is comparable to that of endo-β-1,4-glucanase components from *Aspergillus niger*, 4.0 (Okada, 1985), or those from *A. japonicus*, 4.5 (Kundu et al., 1988), *Clostridium thermocellum* (Mori, 1992), *Paecilomyces* sp. S28 (Liu et al., 1992) and *Robillarda* sp. Y-20 (Yoshigi et al., 1988), which all had pH optima of 5.0. The optimal and stable temperatures were also comparable to fungal endo-β-1,4-glucanases, like those from *Trichoderma viride*, which had optimal temperatures greater than 50°C and were stable at temperatures higher than 40°C (Kundu et al., 1988; Okada, 1975; Yoshigi et al., 1988).

Estimated K_m values showed that YEG1 and YEG2 had increasing affinity for longer cellodextrins over smaller ones. The specific activity of YEG1 and YEG2 against CMC was higher than on cellodextrins, which was demonstrated by V_{max} value for the two components. YEG1 hydrolyzed neither cellotriose nor cellotetraose, but did hydrolyze cellopentaose. YEG2 showed the smallest K_m values on cellopentaose among the cellodextrins examined. The same tendency was also demonstrated for endo-β-1,4-glucanase components from *P. cribrata* (Scrivener and Slaytor, 1994b). YEG1 and YEG2 are thus typical endo-β-1,4-glucanase components,

in that they exhibit increasing affinity (decreasing K_m values) with increasing cellodextrin chain length.

The hydrolytic products of YEG1 and YEG2 from cellodextrins and crystalline cellulose were mainly cellobiose, as is typical of most endo-β-1,4-glucanase components (Klesov, 1991), and a small amount of glucose. The endo-β-1,4-glucanase component from *N. walkeri* produced cellobiose, cellotriose and a small amount of glucose from Sigmacell Type 20 (Schulz et al., 1986). EG1 and EG2 from *P. cribrata* produced roughly equimolar amounts of cellobiose and cellotriose from Sigmacell Type 20, but no glucose (Scrivener and Slaytor, 1994b). The endo-β-1,4-glucanase components from these species resemble each other in that they exhibit low activity on crystalline cellulose and have little (or no) ability to produce glucose (Schulz et al., 1986; Scrivener and Slaytor, 1994b). Klesov (1991) has suggested that the ability of endo-β-1,4-glucanase components to digest crystalline cellulose is directly related to their ability to produce glucose by transglycosylation. The activity of YEG1 and YEG2 against crystalline cellulose was very small, and they produced relatively very small amount of glucose comparing to the amount of cellobiose. Thus, the current results go along with this Klesov's hypothesis. YEG1 and YEG2 are typical endo-β-1,4-glucanase components which are less active against crystalline cellulose, like those from *P. cribrata* and *N. walkeri*.

YEG1 and YEG2 from *R. speratus* cellulose were several thousand times less active against crystalline than against CMC, as were the endo-β-1,4-glucanase components (EG1 and EG2) from *P. cribrata* (Scrivener et al., 1989). Slaytor (1992) concluded that endogenous insect cellulases have little or no cellobiohydrase activity which is a prerequisite for the hydrolysis of crystalline cellulose, and therefore can attack crystalline cellulose only with low efficiency. *P. cribrata* compensates for the inefficiency of crystalline cellulose digestion by secreting enormous amounts of the endo-β-1,4-glucanase components, which comprised 13% of the soluble protein in the foregut and midgut contents (Scrivener and Slaytor, 1994b). Also in *R. speratus*, the endo-β-1,4-glucanase components (YEG1 and YEG2) were less active against

crystalline cellulose. They were as much as to contribute one third of the soluble protein in the salivary glands extract, but still a possible presence of another factors (likely different kinds of cellulolytic components) promoting crystalline cellulose digestion of the hindgut has to be considered. The ratios of filter paper and cotton hydrolyzing activity/carboxymethylcellulose hydrolyzing activity are 7/1000 and 5/1000, respectively in the salivary glands, while, those values in the precipitation of the protozoa and hindgut tissue are 55/1000 and 40/1000, respectively [calculated from Yamaoka and Nagatani (1975)]. Thus, these values suggest the presence of some factors promoting crystalline cellulose hydrolyzing activity in the hindgut. Still at present, the factors compensating the inefficient activity of YEG1 and YEG2 in *R. speratus* has not been solved. The contribution of the hindgut activity to the total physiological requirement of cellulose digestion and the components consisting of the hindgut activity are still unclear. In addition, whether some particular exocellobiohydrase components are included in the cellulase system is also not clear in this species. Lower termites are not considered to be able to live on crystalline cellulose without the hindgut protozoan fauna (Breznak and Brune, 1994). A precise observation is needed about the function of the hindgut and its protozoan fauna to know how the cellulase system of this species accomplish crystalline cellulose digestion.

In Western blotting analysis, anti-YEG2 serum cross-reacted with YEG1, as strongly as with YEG2, indicating that they were related immunologically and suggesting that they have been derived from a common genetic ancestry. This also suggests that YEG1 and YEG2 have both been detected in immunohistochemistry and Western blotting. These immunological techniques showed that the endo-β-1,4-glucanase components were present in the salivary glands, but absent from the midgut epithelium of *R. speratus*. Yamaoka and Nagatani (1975) reported that the carboxymethylcellulose hydrolyzing activity (endo-β-1,4-glucanase) of the salivary glands contributed 59% of the total activity in *R. speratus*. The presence of endo-β-1,4-glucanase in the salivary glands has been shown in other termites, such as

Mastotermes darwiniensis (Veivers et al., 1982) and *Coptotermes formosanus* (Azuma and Nishimoto, 1984). Indeed, 38% of the total endo-β-1,4-glucanase was found in the salivary glands of *M. darwiniensis* (Veivers et al., 1982). The results of the present study show that YEG1 and YEG2 are the major proteins in the salivary glands of *R. speratus* and that these same proteins are also present in the midgut lumen. As the salivary glands are a known secretory site for digestive enzymes, it can be concluded that YEG1 and YEG2 are endogenous endo-β-1,4-glucanase components which are produced by the salivary glands and are then secreted into the alimentary canal.

Slaytor (1992) weighed the role of the midgut more heavily than that of the salivary glands in the secretion of endo-β-1,4-glucanase components in xylophagous termites and cockroaches. This is because endo-β-1,4-glucanase is mainly located in the foregut and midgut of several lower termites, as well as in the midgut contents and epithelia of the higher termite *Nasutitermes walkeri* and the wood-eating cockroach *P. cribrata*. The data from the present study support the conclusion that the midgut epithelium of *R. speratus* plays little or no role in endo-β-1,4-glucanase secretion. Instead, the current study proposed the production and secretion of the endo-β-1,4-glucanase components is undertaken only by the salivary glands in *R. speratus*.

TABLE 1. Purification of the two endo-β-1,4-glucanase components, YEG1 and YEG2 from *R. speratus*

Fraction	Component	Activity* (Units†)	Protein (mg)	Specific activity (Units†/mg protein)	Purification (fold)	Recovery (%)
Crude extract	YEG1 and YEG2	1750	526.0	3.3	1	100.0
Acetone precipitate	YEG1 and YEG2	1470	40.0	36.8	11	84.0
Superdex 75	YEG1 and YEG2	305	5.4	56.5	17	17.4
Hydroxylapatite	YEG1	34	0.5	73.6	22	1.9
Hydroxylapatite	YEG2	64	0.8	83.4	25	3.7

*Activity on carboxymethylcellulose was measured.

†Units are μmol reducing sugar/min.

TABLE 2. Specific activities of YEG1 and YEG2 from *R. speratus* on crystalline cellulose and ratios relative to the activity on carboxymethyl cellulose

Componetnt	Reducing sugars (units*/mg protein)	Glucose (units†/mg protein)	Relative activity‡
YEG1	0.024	0.0014	1:3100
YEG2	0.011	0.0011	1:7600

*Units are μ mol reducing sugar/min.

†Units are μ mol glucose/min,.

‡Specific activity on crystalline cellulose relative to that on carboxymethylcellulose.

TABLE 3. Kinetic constants and products of the endo-β-1,4-glucanase components from *R. speratus*

	K_m(mM)			K_m (mg/ml)	V_{max} (units † /mg protein)				Products		
Component	G3	G4	G5	CMC	G3	G4	G5	CMC	G3	G4	G5
YEG1	n.d.	n.d.	0.7	1.83	n.d.	n.d.	114	527	None	None	G2=G3
YEG2	22.7	6.1	4.1	1.48	172	77	164	540	G1=G2	G2>>G3	G2>G3>G1

G1 indicates glucose. Cellodextrins are abbreviated according to the number of glucose they contain (e. g. G5=cellopentaose).

*Units are μ mol reducing sugar/min. n.d.: not detected. >,= indicate relative amount of cach product compared to others.

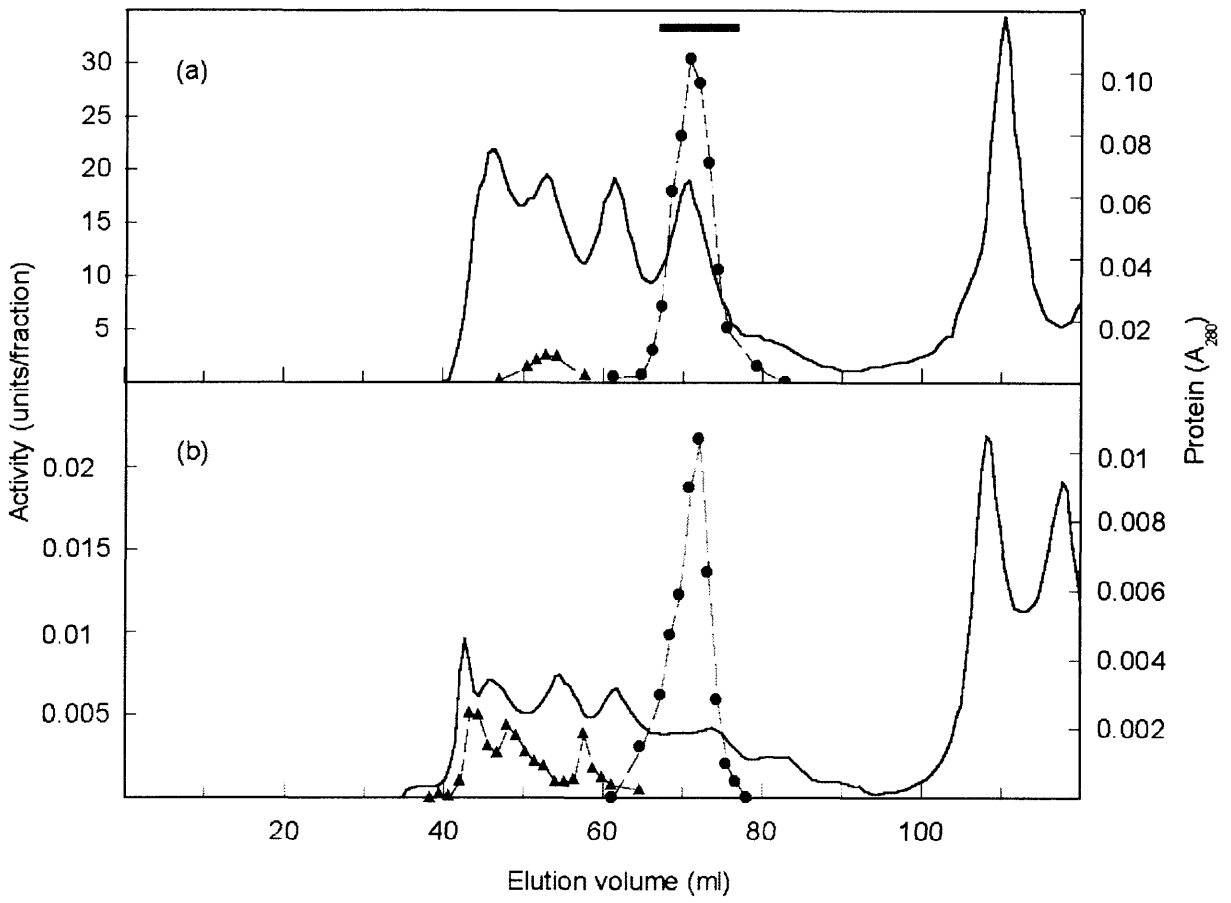

Figure 1. Elution profile of (a) whole body and (b) hindgut extract from *R. speratus* on Superdex 75. (J) Endo-β-1,4-glucanase activity; (H) β-glucosidase activity; (–) protein was measured by absorbance at 280 nm. Units of activity are defined in Materials and Methods. Bar indicates pooled fractions.

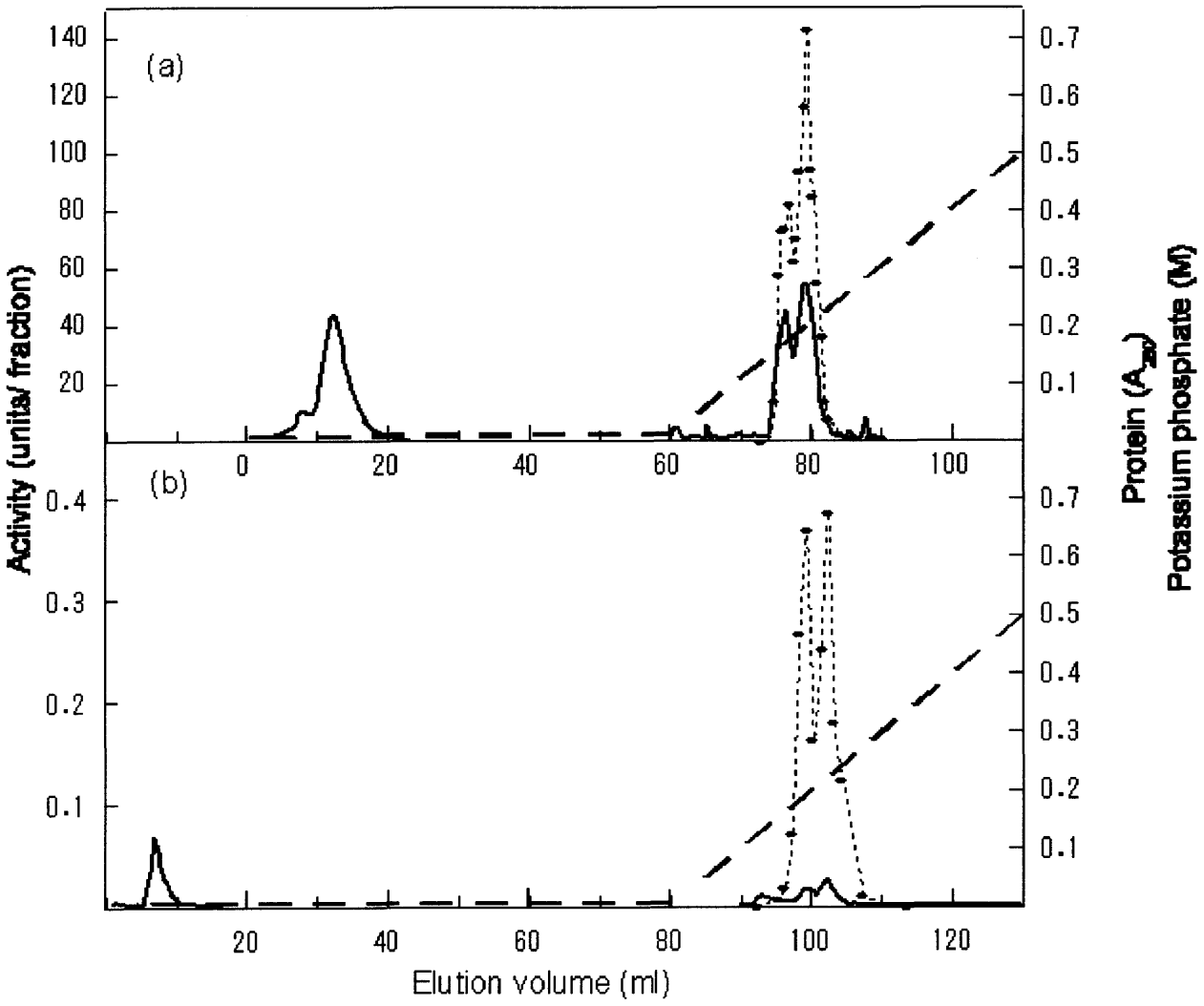

Figure 2. Chromatography of endo-β-1,4-glucanase activity on hydroxylapatite. (a) Endo-β-1,4-glucanase activity from Superdex 75; (b) crude salivary gland extract from *R. speratus* (J) Endo-β-1,4-glucanase activity; (K) protein measured by A_{280}; (---) potassium phosphate gradient.

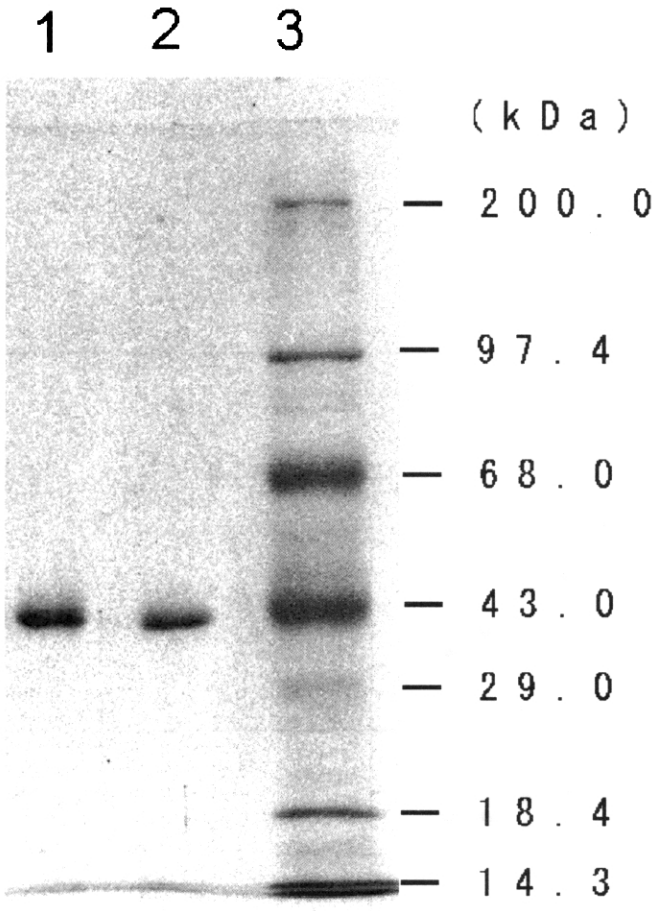

Figure 3. Estimation of molecular weights of endo-β-1,4-glucanase components from *R. speratus* on SDS-PAGE stained with Coomassie Brilliant Blue R250. Lanes are (1) YEG1, (2) YEG2, (3) molecular weight markers.

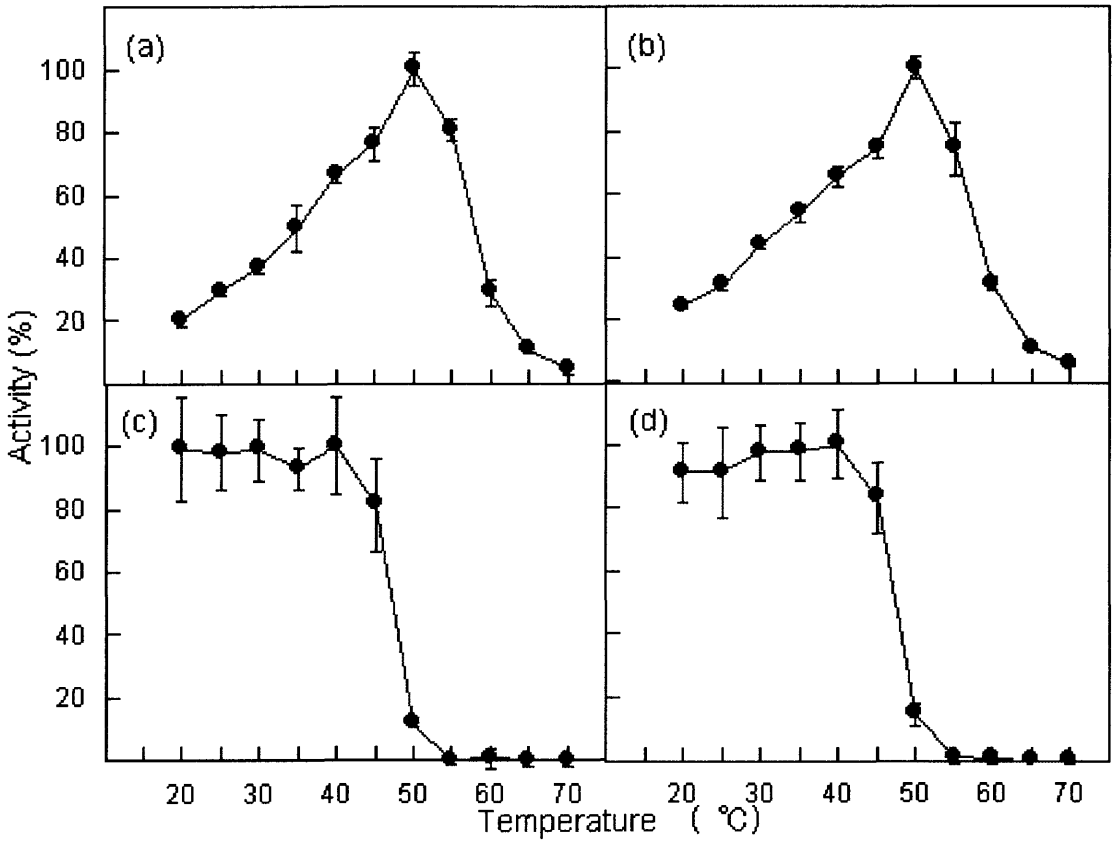

Figure 4. Effect of temperature on the activity of YEG1 (a) and YEG2 (b) and effect of temperature on the stability of YEG1 (c) and YEG2 (d). Each point is the average of five determinations ± S.D.

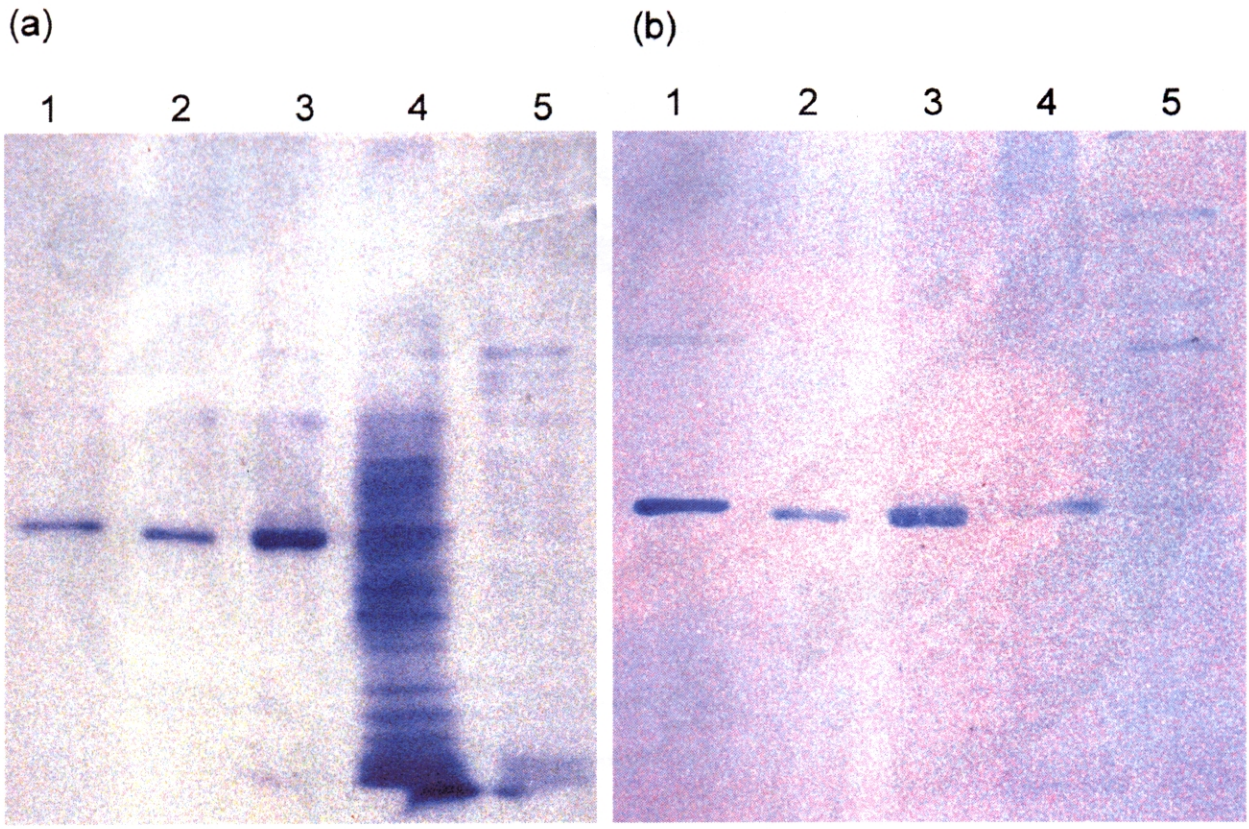

Figure 5. SDS-PAGE and Western blotting using anti YEG2 polyclonal mouse serum of the purified endo-β-1,4-glucanase components and crude extracts from *R. speratus*. (a) SDS-PAGE stained with Coomassie Brilliant Blue R250. (b) Western blotting. Lanes are (1) YEG1, (2) YEG2, (3) crude extract from the salivary glands, (4) crude midgut extract including the luminal contents and (5) crude midgut tissue extract. Salivary glands and midguts were prepared from the same number of termites.

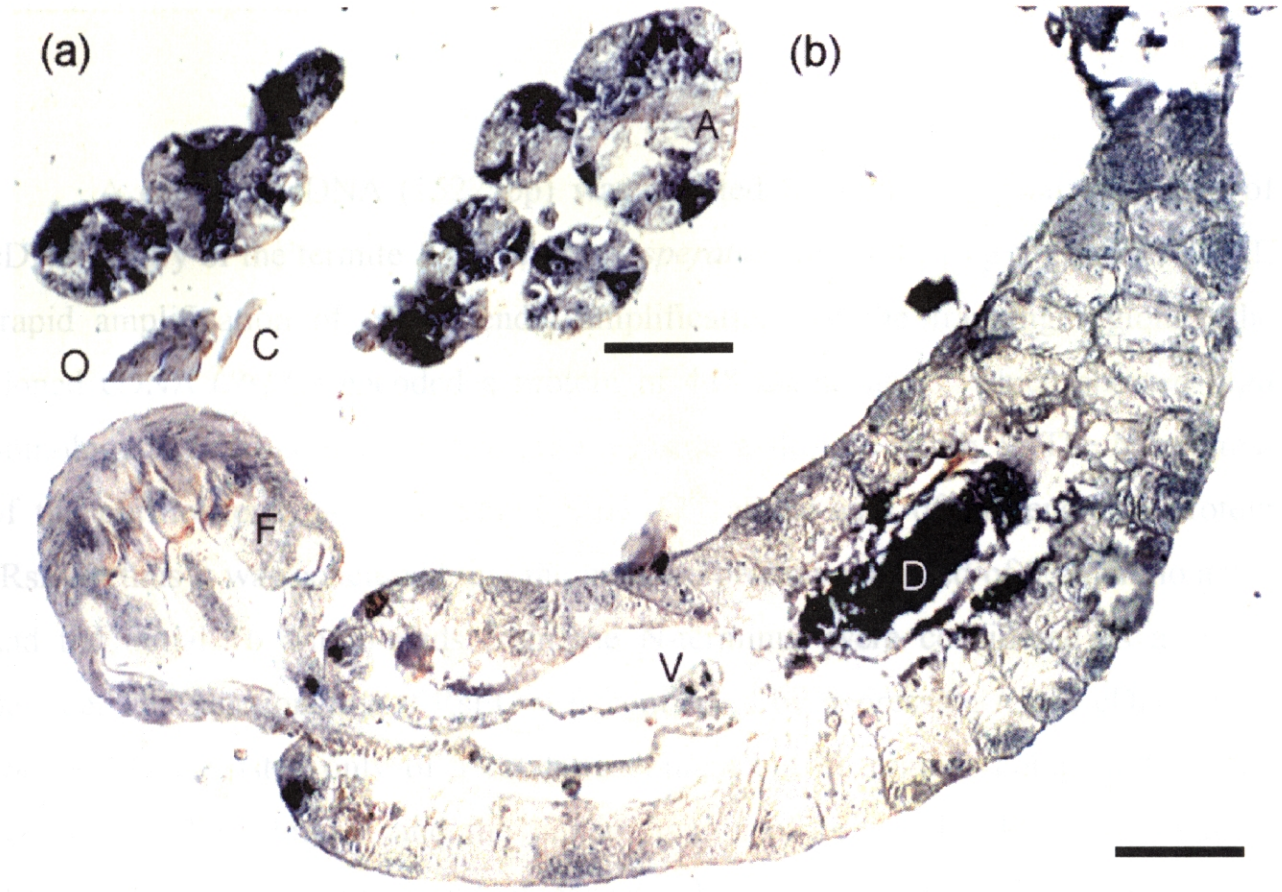

Figure 6. Immunohistochemistry of endo-β-1,4-glucanase activity in the digestive system of *R. speratus*. (a) salivary glands; (b) foregut and midgut. (O) oesophagus; (A) an acinus; (C) salivary canal; (D) food debris; (F) foregut; (M) midgut tissue; (T) trachea; (V) stomodaeal valve. Dark areas indicate binding of anti-YEG2 mouse serum.
Bars indicate 100 μm.

CHAPTER 2

Cloning and sequencing of endogenous endo-β-1,4-glucanase gene from the termite *Reticulitermes speratus*

Abstract

A cellulase cDNA (1522 bp) was isolated by immunological screening of cDNA library of the termite *Reticulitermes speratus* with following 5'- and 3'-RACE (rapid amplification of cDNA ends) amplifications of the franking regions. The cloned cDNA (*RsEG*) encoded a protein of 448 amino acids, which showed high homology to other cellulases from the glycoside-hydrolase family 9. The N-terminal of the purified enzyme was started from 17th amino-acid of the encoded protein (RsEG), thus it was concluded that the matured protein consisted of 332 amino acids and the initial 16 amino-acids from the N-terminus were considered as a signal peptide. The functionality of RsEG was confirmed by expressing *RsEG* cDNA in *E. coli*. RsEG consisted only of a catalytic domain but no regions comparable to the cellulose-binding domain and the linker domain of bacterial cellulases, which is characteristic of many plant endoglucanases. The endogenous origin of *RsEG* gene was confirmed by Southern blotting and the isolation of an intron (450 bp) in the open-reading frame.

Introduction

The traditional view of cellulose digestion in animals is that they are incapable of producing their own cellulase, and thus rely upon symbiotic micro-organisms in the gut to hydrolyze cellulose. In recent decades, evidence has accumulated for the production by some wood-feeding insects of endogenous cellulase components (Beguin and Aubert, 1994; Breznak and Brune, 1994). Whilst cellulase genes have been extensively studied in bacteria, fungi and plants (Henrissat

The content of this chapter has been published in; H. Watanabe *et al.* (1998) A cellulase gene of termite origin. Nature **394**: 330-331.

and Bairoch, 1993), investigations on putative animal cellulases using molecular techniques have been scarce. In this chapter, a molecular-level evidence for the presence a cellulase (endo-β-1,4-glucanase) gene in the genome of the termite *Reticulitermes speratus* and its expression in the salivary glands are given.

Materials and methods

Termites

R. speratus were collected and maintained as described in Chapter 1.

DNA sequencing

PCR products were purified (Microspin S-300 HR Columns, Pharmacia), cloned into the *EcoRV* site of pBluescript II plasmid vector (Stratagene) and transformed into E. coli strain JM109. PCR was performed on positive colonies with Reverse and Ml 3 primers and following purification fragments were sequenced with T3 and T7 dye-labeled primers using the ABI 373A DNA sequencing System (Perkin Elmer). A minimum of 3 clones were sequenced for each PCR product initially cloned.

Preparation of mRNA and cDNA synthesis

Worker termites (20 g) were powdered in a ceramic mortar containing liquid nitrogen and total RNA was extracted (Total RNA Separator Kit, Clontech). mRNA was subsequently obtained (mRNA Separator Kit, Clontech) and used for synthesis of double stranded (ds) cDNA (cDNA Synthesis System, Amersham). dscDNA was methylated with EcoRI methylase and ligated to EcoRI linkers (Takara, Japan). Methylated dscDNA was subsequently digested and cloned into the EcoRI site of λgtll phage vector (Stratagene).

Screening of positive Clones

Culture and expression of λgtll was performed as per the manufacturer's instructions (Stratagene). Plaques containing expressed phage material were transferred to a PVDF membrane (Bio-Rad) and screened with anti-YEG2 serum, prepared as described in Chapter 1. Positive plaques were detected using a Vectastain ABC-Alkaline Phosphatase Kit (Vector Laboratories). The inserts of 9 positive clones were subcloned into pBluescript II and 3 of these sequenced. Surprisingly, these identical inserts were only 387 bp long (Fig. 1) and lacked the expected EcoRI-linker flanking sequences. Thus, initial digestion at EcoRI sites within *RsEG (YEG2)* cDNA proceeded despite apparent methylation, and the resulting 387 bp fragment was cloned into λgtll at a site which enabled expression of a RsEG (YEG2) truncated peptide recognized by anti-YEG2.

Cloning of 5'- and 3'- cDNA ends

The complete *RsEG* cDNA sequence was obtained using an adaptor mediated 'Rapid Amplification of cDNA Ends' (RACE) method (Marathon cDNA Amplification Kit, Clontech). Primers for amplification of 5'- and 3'-cDNA ends [positions 441-420 and 729-748 of *RsEG* cDNA respectively (Fig. 1)] were designed from the initial EcoRI fragment. Following ligation of anchor adaptors to *R. speratus* dscDNA, PCR was performed using one of the above primers in combination with primer AP-1 (Clontech). The amplified fragments were purified and sequenced, providing the full *RsEG* cDNA sequence.

Genomic RsEG DNA Cloning

The heads of 100 workers termites were powdered in liquid nitrogen and

genomic DNA extracted using conventional techniques (Blin and Stafford, 1976). Termite head tissue was chosen to avoid contamination by resident gut microorganisms. With primers designed from *YEG2* cDNA sequence, fragments of *YEG2* gene containing partial ORF and full intron sequences were amplified by PCR, cloned and sequenced. To be sure no introns in the RsEG gene were overlooked, PCR was performed using genomic DNA with different combinations of primers and the expected size of fragments checked by agarose gel electrophoresis.

Genomic Southern blotting

Termite head DNA (15 μl) was digested with one of the restriction enzymes PstI, XbaI, and EcoRI (Takara, Japan) and blotted onto a Hybond-N membrane (Bio-Rad) using conventional upward capillary transfer techniques (Meinkoth and Wahl, 1984). For negative controls, genomic DNA extracted from a degutted body of the wood-eating cockroach *Panesthia angustipennis* digested by EcoRI and molecular size markers (double strand DNA fragments) were used. The membrane was hybridized with a DIG-labeled (DIG Nucleic Acid Detection Kit, Boehringer Mannheim) 203 bp probe corresponding to positions 1109-1312 of *RsEG* cDNA (Fig. 1). Hybridization was carried out at 65°C for 12 hours and detection of alkaline phosphatase complexed probe was carried out as per the manufacturer's instructions (Boehringer Mannheim).

RT-PCR

cDNA was prepared as described above from *R. speratus* salivary gland, foregut, midgut, hindgut and head (negative control) tissue. Primers corresponding to positions 398-419 and 836-816 on *RsEG* cDNA (GenBank Accession: AB008778) were used in PCR with the aforementioned samples to detect the site of *RsEG*

expression.

N-terminal amino acid sequencing

YEG2 protein was purified as described in Chapter 1, and blotted onto a Hybond-N membrane (Bio-Rad) following SDS-PAGE. N-terminal sequencing was performed using a LF-3000 protein sequencer (Beckman).

Results and discussion

The traditional view of cellulose digestion in animals is that they cannot produce their own cellulase, and so rely on gut micro-organisms to hydrolyze cellulose. A classic example of this symbiosis is that between phylogenetically lower termites and the unicellular organisms (protists) that colonize their hindguts: cellulose fermented to acetate by the protists can be used as an energy source by the termite (Breznak and Brune, 1994). There is evidence for the production of endogenous cellulase components by termites and other wood-feeding insects (Slaytor, 1992); however, an unambiguous origin for such enzymes (Breznak and Brune, 1994) has not been established. Here the first insect cellulase-encoding gene was identified as *RsEG*, which encodes an endo-β-1,4-glucanase (EC 3.2.1.4) in the termite *Reticulitermes speratus*. Using antiserum raised against an endo-β-1,4-glucanase purified from *R. speratus* (Watanabe et al., 1997), a recombinant phage complementary DNA library from this species was screened and identified a partial sequence encoding a peptide with similarity to cellulases from glycosyl hydrolase family 9 (GHF9) (Henrissat and Bairoch, 1993). The complete coding region of *RsEG* was obtained by rapid amplification of complementary DNA ends.

Although the source of messenger RNA for this study was the salivary glands,

a part of termites that lacks microorganisms (Slaytor, 1992), the endogenous origin of the gene was confirmed by Southern blot analysis of DNA extracted from degutted termites (results not shown) and with amplification by the polymerase chain reaction of a partial *RsEG* genomic fragment (Fig. 1). This fragment contains an exon sequence identical to that of *RsEG* cDNA and is interrupted by a 450-base-pair intron with typical eukaryotic splicing sites (Fig. 2). In addition, the expression of RsEG in the salivary glands was confirmed by RT-PCR (Fig. 3).

The predicted mature RsEG protein shares 52%, 48% and 42% identity with the catalytic domains of selected bacterial, protist and plant members of GHF9, respectively (Fig. 2). The first 16 of the 448 amino acids of RsEG represent a secretory peptide, deduced from the amino-terminal sequences of two chromatographically distinct endo-p-l,4-glucanases purified from *R. speratus* (Watanabe et al., 1997) these amino-terminal ends are identical. It is not clear at present which of these proteins is RsEG.

The amino-acid residues thought to be involved in catalysis and substrate-binding in all GHF9 members (Brummell et al., 1994) are conserved in RsEG, further confirmation that RsEG encodes an active cellulase.

A conspicuous feature of the termite endo-β-l,4-glucanase is the absence of any motif similar to the ancillary modules found in most bacterial and fungal cellulase components (Beguin and Aubert, 1994). The presence of a cellulose- binding domain in such cellulases is thought to enhance activity towards crystalline cellulose (Tomme et al., 1995). RsEG appears to consist only of a single catalytic domain, a characteristic of plant endo-β-l,4-glucanases. However, unlike the latter enzymes, which have no apparent activity towards crystalline cellulose (Brummell et al., 1994), the termite endo-β-1,4-glucanase exhibits low exo-β-l,4-glucanase activity towards this substrate (Watanabe et al., 1997), producing cellobiose and glucose. The specific

activity of this reaction is similar in magnitude to that of bacterial endoglucanases from GHF9 (Tomme et al., 1996).

Thus the endogenous cellulase system of *R. speratus*, including an apparent β-glucosidase (Inoue et al., 1997), is secreted in the salivary glands and is capable of producing glucose from crystalline or amorphous cellulose present in ingested wood. Although this endogenous cellulase is not 'complete' (able to exhaustively digest native cellulose), the production of glucose from crystalline cellulose by this cellulase (estimated in vitro) can account for a substantial amount of the carbon dioxide expired by whole insects (Inoue et al., 1997).

Cellulose not hydrolyzed in the anterior portion of the gut then travels to the hindgut, where it can be endocytosed by symbiotic protists and fermented to produce acetate, an important energy source and precursor for biosynthesis in the termite (Breznak and Brune, 1994). Use of this dual cellulase system explains why wood-glucan assimilation in lower termites can be greater than 90% (Breznak and Brune, 1994).

The evolutionary origin of cellulases in termites is intriguing. Although RsEG exhibits marked similarity to bacterial and protist members of GHF9, the fact that *RsEG* contains introns makes the prospect of recent horizontal gene transfer from these species unlikely. Indeed, the amino-terminal amino-acid sequences of two putatively endogenous cockroach endoglucanases (Scrivener et al., 1997) both exhibit over 75% identity with the mature RsEG amino terminus. If these enzymes prove to be of insect origin, it is likely that an ancestral GHF9-like cellulase gene was present before the divergence of cockroaches and termites, an estimated 250 million years ago (Bandi et al., 1995).

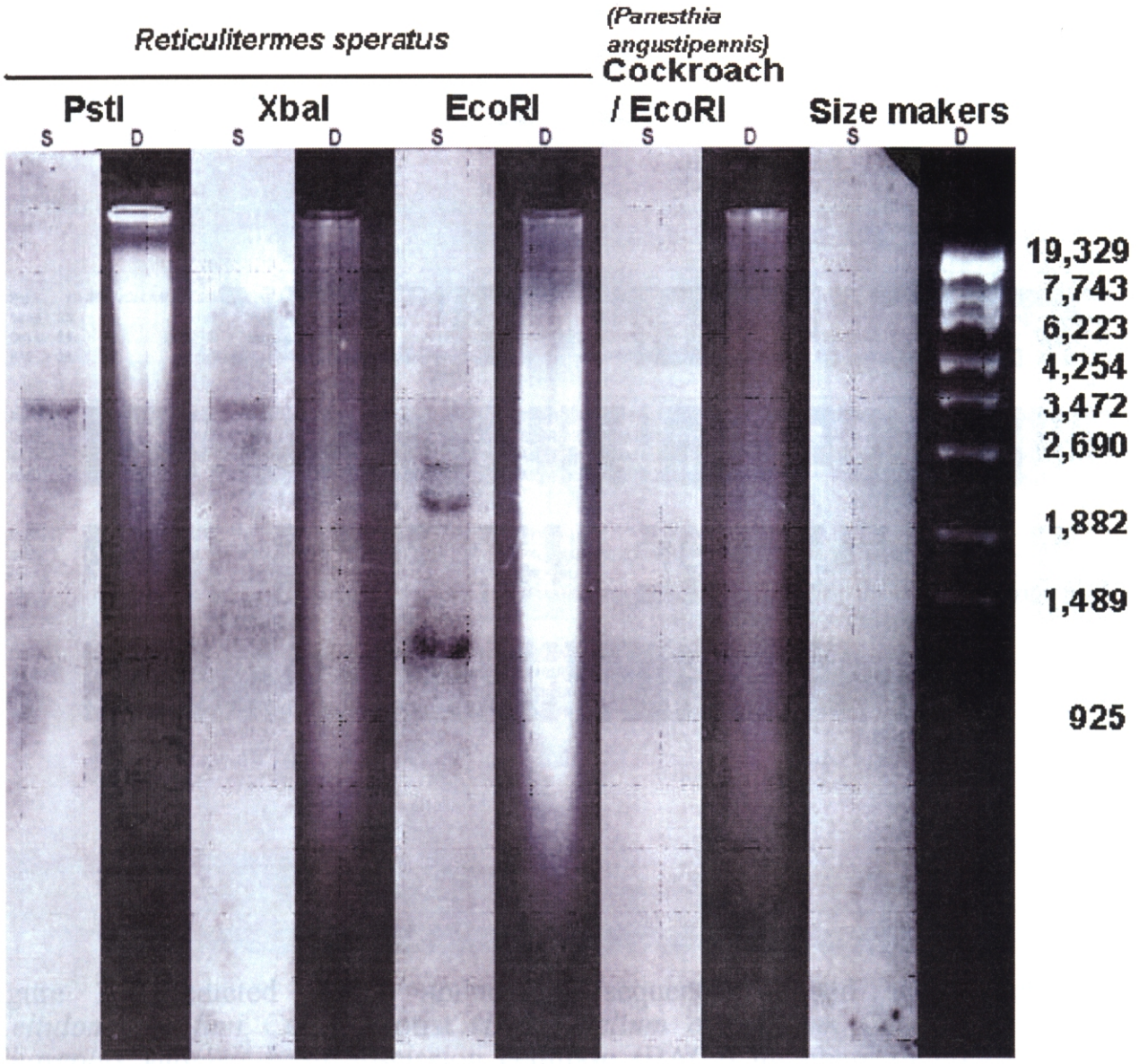

Fig. 1 Genomic Southern Blot analysis of RsEG using DIG labeled *RsEG* cDNA as a probe. The genomic DNA extracted from the head of the termite *Reticulitermes speratus* was digested by each one of the restriction enzymes PstI, XbaI, and EcoRI before agarose gel electrophoresis. For negative controls, genomic DNA of the wood-eating cockroach *Panesthia angustipennis* digested by EcoRI and molecular size markers (double strand DNA fragments) were used. Columns marked with "S" show results of Southern Blot analysis and columns marked with "D" show DNA fragments separated on agarose gel electrophoresis (1% agarose with Tris-borate-EDTA buffer, pH 8.0).

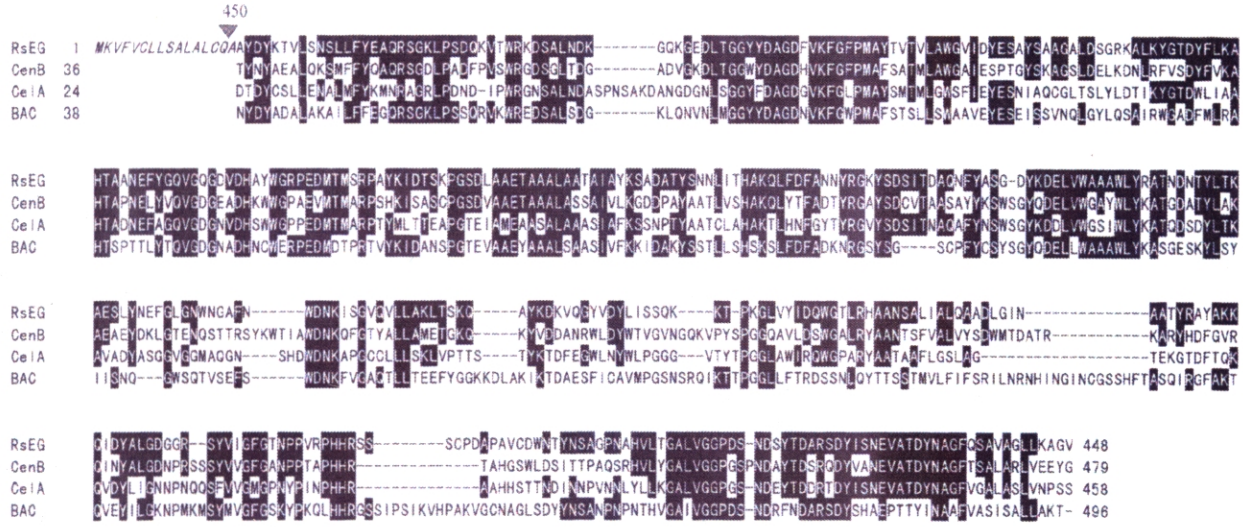

Figure 2. Predicted RsEG amino-acid sequence aligned with bacterial (*Cellulomonas fimi* CenB, protist (*Dictyostelium discoideum* CelA) and plant (*Phaseolus vulgaris* bean abscission cellulase (BAC)) members of GHF9. The alignment was done with the program Clustal W using mature RsEG. The putative 16-amino-acid RsEG signal peptide is shown in italics. The position of a 450-base-pair phase 1 intron present in an *RsEG* genomic fragment obtained by the polymerase chain reaction is indicated by an inverted triangle. Shaded amino acids show identity with RsEG. Coordinates have been deposited with the DNA Data Bank of Japan (RsEG, accession number AB008778), or GenBank (CenB, CelA and bean abscission cellulase, accession numbers M64644, M33861 and U34754, respectively).

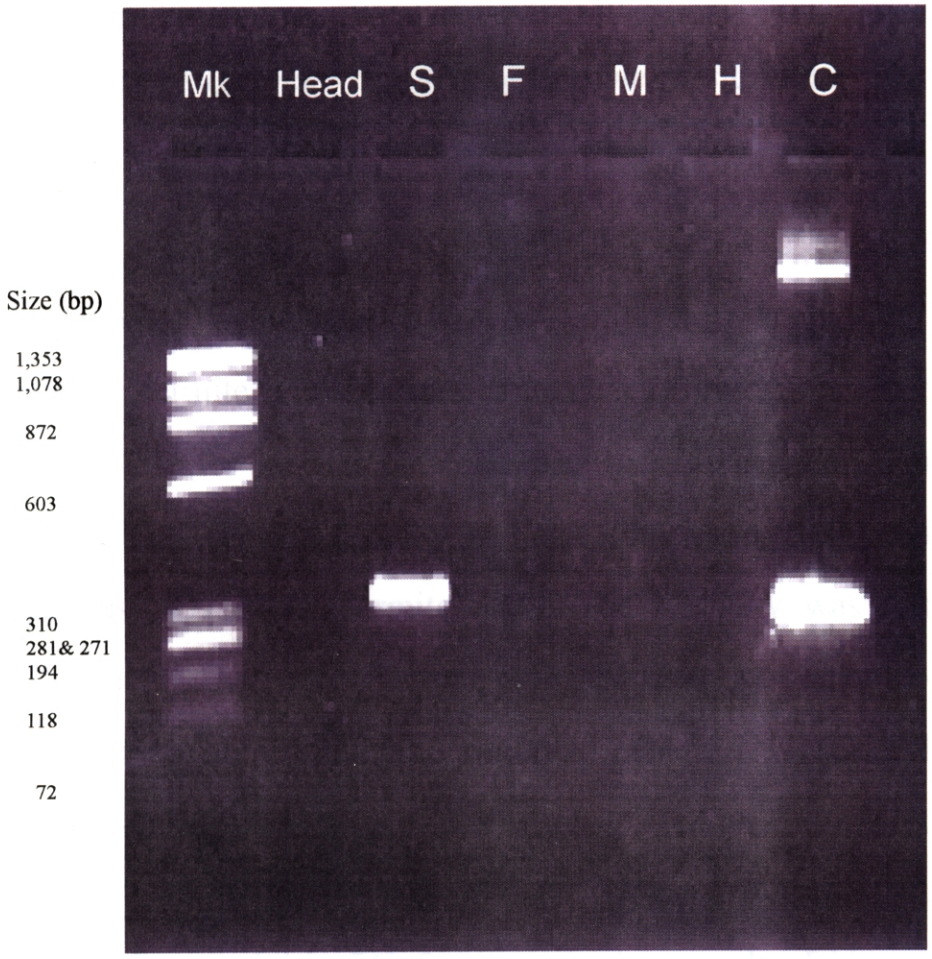

Fig. 3 RT-PCR analysis of the digestive system of the termite *Reticulitermes speratus*. mRNA was extracted from each part of the digestive system and the head (negative control) and the first strand cDNAs were produced with a oligo-dT primer. Lanes are Mk (DNA size markers, φX174 HindIII digets), Head: head, S: salivary glands, F: foregut, M: midgut, H: hindgut, C: positive control (*RsEG* cDNA).