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Cellulose Digestion in the Wood-Eating Higher Termite, *Nasutitermes takasagoensis* (Shiraki): Distribution of Cellulases and Properties of Endo-β-1,4-glucanase

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ABSTRACT—β-Glucosidase [EC 3.2.1.21] and endo-β-1,4-glucanase [EC 3.2.1.4] activities were measured in the wood-eating higher termite *Nasutitermes takasagoensis*. β-Glucosidase activity was present mainly in the salivary glands (66.7%) and midgut (22.2%), whereas endo-β-1,4-glucanase activity was detected mainly in the midgut (90.1%). Specific activity of endo-β-1,4-glucanase was also the highest in the midgut, indicating that cellulose is digested in the midgut. The major endo-β-1,4-glucanase component of *N. takasagoensis* was purified from whole termites by gel filtration on Sephacryl S-200 HR, Superdex-75 and hydroxyapatite column chromatography. Subsequently, the endo-β-1,4-glucanase activity from a crude midgut extract was eluted in an identical volume (Kd=0.68) to that from whole termites, suggesting the purified endo-β-1,4-glucanase is identical to that in the midgut. The molecular weight of the purified endo-β-1,4-glucanase was 47 kDa, and its specific activity was 1,200 units/mg. The optimal pH and temperature were 5.8 and 65°C, respectively. The K_m and V_{max} values on carboxymethyl cellulose were 8.7 mg/ml and 2,222 units/mg, respectively. The purified endo-β-1,4-glucanase hydrolyzed cellopentaose to cellotriose and cellobiose, and cellotetraose to cellobiose and a trace of cellotriose and glucose, but cellotriose and cellobiose were not hydrolyzed. The activity and stability on pH and temperature of the purified endo-β-glucanase are prominent among those from various organisms.

INTRODUCTION

Cellulose is one of the most abundant renewable biomasses in the biosphere. It is a polysaccharide composed of β-D-glucopyranosyl units joined by 1,4-glycosidic bonds. There are three major types of enzyme involved in hydrolysis of cellulose (Wood, 1985). Endo-β-1,4-glucanases [EC 3.2.1.4] hydrolyze mainly amorphous cellulose, resulting in a rapid decrease in chain length together with a slow increase in reducing sugars. β-Glucosidases [EC 3.2.1.21] hydrolyze cellobiose and soluble cellooligosaccharides to glucose. Cellobiohydrolases [EC 3.2.1.91] degrade cellulose by splitting off cellobiose units from the non reducing end of cellulose chain, but not attack substituted cellulose like carboxymethyl cellulose. Native cellulose has been supposed to be hydrolyzed by cooperative action of the enzymes. However, insects never appear to be capable of forming cellobiohydrolases except the possible case of the silverfish (Prins and Kreulen, 1991).

Cellulolytic systems of insects have been mainly studied in lower termites and wood-eating cockroaches which were closely related to each other phylogenetically (Kambhampati, 1995). It has been widely known that lower termites and wood-eating cockroaches have numerous protozoa within an enlarged part of their hindgut, so-called "paunch". In early studies on cellulose digestion in termites, Cleveland (1923, 1924, 1925, 1934) proposed that intestinal protozoa were essential for the lower termite *Reticulitermes flavipes* and the wood-eating cockroach *Cryptocercus punctulatus*. Yokoe (1964) first demonstrated in *Leucotermes* (=*Reticulitermes*) *speratus* that termites have their own cellulase activity. Subsequently, Yamaoka and Nagatani (1975) demonstrated that cellulolytic activity is present in both the salivary glands and hindgut of *R. speratus*. Similar reports suggesting the presence of the salivary and hindgut cellulases were obtained in *Mastotermes darwiniensis* (Veivers *et al.*, 1982), *Neotermes bosei* (Mishra, 1980), *Coptotermes lacteus* (O'Brien *et al.*, 1979) from the late 1970s to the beginning of 1980s. Therefore,

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Yamaoka (1989) assumed in *R. speratus* that cellulose was degraded in food vacuoles of the symbiotic protozoa by synenergistic action of both protozoan C_1-cellulase (cellobiohydrolase) and endogenous Cx-cellulase (endo-β-1,4-glucanase) from the salivary glands. In 1994, Scrivener and Slaytor separated and characterized endogenous endo-β-1,4-glucanase and β-glucosidase components from the wood-eating cockroach *Panesthia cribrata*. Subsequently, Watanabe *et al.* (1997) reported purification and properties of endogenous endo-β-1,4-glucanase components from the lower termite *R. speratus*. Thus, enzymatic properties of cellulases in lower termites and xylophagous cockroaches have started to be clarified.

In higher termites, it has been considered that their cellulolytic systems differ from that of lower termites because higher termites have no symbiotic protozoa in spite of numerous bacteria within their gut (Bignell *et al.*, 1979, 1980a, b, 1983; Czolij *et al.*, 1985; Anklin-Mühlemann *et al.*, 1995). Until the beginning of 1980s, it had been assumed that hindgut bacteria of higher termites substituted for protozoa of lower termites in the role of cellulose digestion (Breznak, 1982). However, several investigations indicated that a large amount of cellulase activity was present in the midgut (Potts and Hewitt, 1973; Martin and Martin, 1979; O'Brien *et al.*, 1979; Malaka, 1986; Hogan *et al.*, 1988; Chararas and Noirot, 1988; Veivers *et al*, 1991). In addition, there are no affirmative reports on bacterial cellulose degradation except one study which suggested the possible role of bacteria in cellulose degradation of wood-eating termites (French, 1975). The presence of a large amount of cellulase activity independent of any symbionts has led to the proposal that cellulose is digested in the midgut by endogenous cellulases in xylophagous higher termites although it is controversial on the role of fungus in cellulose digestion of fungus-growing termites which ingests fungal nodules growing on special faeces of termites (Martin, 1991; Slaytor, 1992). Cellulases were not fully purified from a xylophagous higher termite although a few of them were partially purified from *Trinervitermes trinervoides* (Potts and Hewitt, 1974a, b) and *Nasutitermes walkeri* (Schulz *et al.*, 1986). Characterization of the enzymatic properties is a key to understand the cellulolytic systems of termites and cockroaches. Since higher termites are more evolved species than *R. speratus* or *P. cribrata*, and are the most successful species in the tropical area (Wood, 1988), the clarification of the cellulolytic system in the higher termite is of importance in understanding not only cellulolytic system of termites but also the evolution of the interaction between termites and their intestinal symbionts.

In the present study, we report cellulose digestion, purification and properties of the major endo-β-1,4-glucanase component from the wood-eating higher termite, *Nasutitermes takasagoensis* (Shiraki) (Isoptera: Termitidae: Nasutitermitinae).

MATERIALS AND METHODS

Termites

Arboreal nests and logs inhabited by *Nasutitermes takasagoensis* (Shiraki) were collected at Iriomote-island in Okinawa prefecture located in the subtropical region of Japan. Termites were kept at room temperature with nest materials.

Preparation of extracts for enzyme assays

0.1 M Sodium acetate buffer, pH 5.5, was used in the preparation of all extracts and in all assays, unless otherwise indicated. All procedures were carried out at 4°C.

The salivary glands and whole gut were removed from 20 termites and divided into the foregut, midgut, mixed segment, first proctodeal segment, paunch and colon with the rectum. The salivary glands and each of gut sections were homogenized in 10 μl of buffer in microcentrifuge tubes (1.5 ml) and centrifuged at 20,100 × g for 20 min. Supernatants were collected and diluted to 600 μl with buffer, then which were referred to as enzyme extracts.

Enzyme assays

Endo-β-1,4-glucanase. Enzyme extract (25 μl) was incubated with 200 μl of 2% (w/v) sodium carboxymethylcellulose (CMC; standard molecular weight: 250,000, degree of carboxymethyl substitution: 0.7 (w/v); Aldrich) in buffer at 37°C for 30 min. Reducing sugars were detected with tetrazolium blue (Sigma) (Jue and Lipke, 1985) and expressed as glucose equivalents.

β-Glucosidase. Enzyme extract (25 μl) was incubated with 200 μl of 2% (w/v) cellobiose (Nakalai Tesque) in buffer at 37°C for 30 min. Glucose production was detected with a GOD-mutarotase reagent kit (Glucose CII Test Wako; Wako Pure Chemical).

Protein measurement

The protein contents of samples were determined by the direct UV method of absorbance at 280 and 260 nm (Layne, 1957) or at 215 and 225 nm (Murphy and Kies, 1960) using bovine serum albumin as a standard.

Definition of enzyme unit

One unit of enzyme activity is defined as the amount of enzyme which produced 1 μmol of reducing sugar (glucose equivalents) or glucose/minute. Specific activity is defined as units/mg protein.

Column chromatography

Worker and soldier termites (15 g) were homogenized in 150 ml of distilled water, and centrifuged at 24,000 × g for 30 min. The supernatant was recovered as a crude extract. Ammonium sulfate was added to the crude extract and centrifuged at 24,000 × g for 30 min to collect precipitation. Precipitation from 35 to 70% (w/v) ammonium sulfate solution was collected and dissolved in 30 ml of 0.3 M ammonium acetate buffer, pH 5.0. This is referred to as 70% precipitation and was applied to column chromatography.

All chromatography was carried out at 4°C. Fractions containing endo-β-1,4-glucanase activity were concentrated using an ultrafiltration cell (Model 8003, 8010 or 8050; Amicon, Grace Japan K.K.) with a UK-10 membrane (PVDF, molecular cut off 10,000, Advantec TOYO). Protein was monitored at 280 nm using a UV-1 monitor (Pharmacia).

The 70% precipitation (5 ml at once) was applied to Sephacryl S-200 HR (Pharmacia) gel filtration column (26 × 900 mm), equilibrated and eluted with 0.3 M ammonium acetate buffer, pH 5.0, at a flow rate of 1.0 ml/min, with the collection of 5 ml fractions. Fractions containing endo-β-1,4-glucanase activity from 6 replications of the gel filtration were concentrated to 1 ml using the ultrafiltration cell, and then chromatographed on a HiLoad 16/60 Superdex-75 prep grade column (Pharmacia). The column was equilibrated and eluted with 0.3 M ammonium acetate buffer, pH 5.0, at a flow rate of 0.6 ml/min, with the collection of 1.2 ml fractions. Active fractions were recovered,

desalted and concentrated to 1 ml using the ultrafiltration cell with distilled water. The concentrated sample was adsorbed on a hydroxyapatite (DNA grade, Bio-Rad) column (10 × 50 mm). The column was washed with 40 ml of starting buffer (20 mM sodium phosphate buffer, pH 5.5), and eluted with a 50 ml linear gradient to 1 M sodium phosphate, pH 5.5, at a flow rate of 0.2 ml/min, with the collection of 1.0 ml fractions. Active fractions were desalted and concentrated to 1 ml using the ultrafiltration cell, and again adsorbed on hydroxyapatite, then eluted by the same procedure.

Midguts from 100 workers were homogenized in 1 ml of buffer in a microcentrifuge tube, and centrifuged at 20,100 × g for 30 min. The supernatant, which is referred to as a crude midgut extract, was similarly chromatographed on the Superdex-75 column.

Activity during the purification steps of endo-β-1,4-glucanase was measured using 25 μl of appropriately diluted sample and 200 μl of 2% (w/v) CMC at 37°C for 10 min.

Electrophoresis

The purified endo-β-1,4-glucanase was examined by SDS-PAGE according to the method of Laemmli (1970). The sample and protein standards (GIBCO BRL, Life Technologies, Inc.) were run on a 10% (w/v) acrylamide gel at 15 mA. Proteins were detected with silver stain kit (Bio-Rad).

Characterization of the purified endo-β-1,4-glucanase

Optimal temperature and pH. Activity of the pure endo-β-1,4-glucanase was measured at pH 5.5 over the range from 20 to 70°C with 5°C intervals for 5 min to determine the optimal temperature. To evaluate thermal stability, enzyme samples were incubated at constant temperature from 20 to 70°C with 5°C intervals for 30 min and then assayed at 37°C for 5 min. In order to measure optimal pH, 2% (w/v) CMC in three kinds of buffers was used for enzymatic assays: 0.04 M phosphoric acid, 0.04 M acetic acid and 0.04 M boric acid mixture - 0.2 M NaOH buffer (pH 1.8 to 12.0); citrate - phosphate buffer (pH 2.2 to 8.0; McIlvaine, 1921); 0.2 M boric acid and 0.2 M potassium chloride - 0.2 M sodium carbonate buffer (pH 7.4 to 11.0).

Hydrolytic products from cellodextrins by TLC. Samples (10 μl) were incubated with 10 μl of 60 mM solutions of cellopentaose, cellotetraose, cellotriose, cellobiose (Sigma) at 37°C. Aliquots (2 μl) were collected at 0 time and 30 min for estimation of kinetic constants, and 0 time and 18 hr for analysis of hydrolytic products. The products were analyzed by TLC as described by Hansen (1975). The TLC plates for hydrolytic product analysis were scanned by a GT-5000 scanner (EPSON). The density of each spot was estimated using NIH Image software on Macintosh computer (Apple Computer Inc.).

Estimation of kinetic constants. For the evaluation of K_m and V_{max} values, solutions of cellopentaose (0.25 to 6.0 mM), cellotetraose (0.2 to 2.0 mM) and CMC (1 to 40 mg/ml) in buffer were used as substrates. Reducing sugars were detected as described in enzyme assays.

RESULTS

General view of the alimentary canal

Figure 1 shows a schematic drawing of the alimentary canal and the salivary glands in *N. takasagoensis*. The alimentary canal was composed of the foregut, midgut, and hindgut. The midgut elongated toward the hindgut, and formed the mixed segment. The hindgut was further subdivided into five segments; the first proctodeal segment (P1), enteric valve (P2), paunch (P3), colon (P4), and rectum (P5).

Cellulase activity in the alimentary canal

The distribution of cellulase activity through the gut of *N. takasagoensis* is shown in Table 1. β-Glucosidase activity was dominant in the salivary glands (66.7%), while 22.2% of β-glucosidase activity was detected in the midgut. The level of β-glucosidase activity was lower in the mixed segment (1.2%) than that in the midgut, and it was hardly detected in the first proctodeal segment. Only a small amount of β-glucosidase activity was detected in the paunch (4.9%). β-Glucosidase activity was not detected in the colon and rectum. Specific activity of β-glucosidase was the highest in the midgut, and was slightly higher than that in the salivary glands.

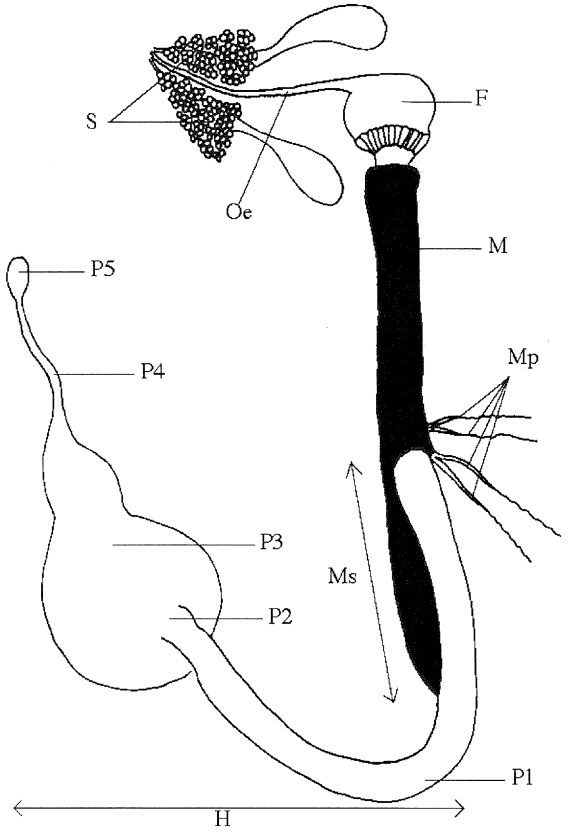

Fig. 1. General view of the alimentary canal and salivary glands in *N. takasagoensis*. Oe, oesophagus; S, salivary glands; F, foregut; M, midgut; Ms, mixed segment; Mp, Malpighian tubules; H, hindgut; P1, first proctodeal segment; P2, enteric valve; P3, paunch; P4, colon; P5, rectum.

More than 90% of endo-β-1,4-glucanase activity was localized in the midgut. The level of endo-β-1,4-glucanase activity was much lower in the foregut (3.6%) and mixed segment (2.3%) than in the midgut, and it was hardly detected in the salivary glands and first proctodeal segment (0.4%). Endo-β-1,4-glucanase activity was absent in the paunch, where was considered as the main site of cellulose digestion in lower termites. A small amount of endo-β-1,4-glucanase activity was detected in the colon and rectum (3.2%). Specific activity of endo-β-1,4-glucanase was the highest in the midgut. It was 2.9 and 240 times higher than those of the foregut and salivary glands, respectively.

Table 1. Distribution of cellulase activities in the gut of *N. takasagoensis*

section	endo-β-1,4-glucanase			β-glucosidase		
	total activity (units)	(%)	specific activity (units/mg)	total activity (units)	(%)	specific activity (units/mg)
salivary glands	0.01 ± 0.00	0.4	0.01 ± 0.00	0.32 ± 0.04	66.7	0.34 ± 0.04
foregut	0.08 ± 0.00	3.6	0.83 ± 0.03	0.03 ± 0.01	4.9	0.07 ± 0.03
midgut	1.99 ± 0.09	90.1	2.40 ± 0.10	0.11 ± 0.06	22.2	0.36 ± 0.21
mixed segment	0.05 ± 0.00	2.3	0.03 ± 0.00	0.01 ± 0.00	1.2	0.01 ± 0.00
P1	0.01 ± 0.00	0.4	0.01 ± 0.00	0	0	0
P2-P3	0	0	0	0.02 ± 0.00	4.9	0.11 ± 0.01
P4-P5	0.07 ± 0.00	3.2	0.07 ± 0.00	0	0	0

Values are means of 5 determinations ± S.D.
One unit is the amount of enzyme which produced either 1 μmol of glucose or reducing sugar (glucose equivalents)/min.

Purification of the major endo-β-1,4-glucanase component

A major endo-β-1,4-glucanase component was purified from the whole termites. The major endo-β-1,4-glucanase activity was eluted on Sephacryl S-200 HR at 365 ml (Kd=0.75). However, the protein profile did not show any peaks at a corresponding volume to the activity peak because the sample contained only a small amount of endo-β-1,4-glucanase protein (Fig. 2). Active fractions in each replication were combined and chromatographed on a Superdex-75 column (Fig. 3). The endo-β-1,4-glucanase activity was eluted at 87.6 ml (Kd=0.68). The protein profile also showed an identical peak to the endo-β-1,4-glucanase activity. In hydroxyapatite chromatography, the endo-β-1,4-glucanase activity was eluted at 49 ml (175 mM sodium phosphate) (Fig. 4). Active fractions were again applied to the same hydroxyapatite column. Then, the endo-β-1,4-glucanase activity was also eluted at 49 ml (175 mM sodium phosphate). The purified endo-β-1,4-glucanase was pure after the duplicated hydroxyapatite chromatography because it was detected as a single band on SDS-PAGE by silver staining (Fig. 5). The molecular weight of the major endo-β-1,4-glucanase component was estimated to be 47 kDa by comparing its mobility with standard proteins on SDS-PAGE. The purification of the major endo-β-1,4-glucanase component was summarized in Table 2. The endo-β-1,4-glucanase was purified 6,000-fold from the crude extract.

The crude midgut extract was also chromatographed on Superdex-75 (Fig. 6). Endo-β-1,4-glucanase activity was eluted in an identical volume (Kd=0.68) to that from whole termites.

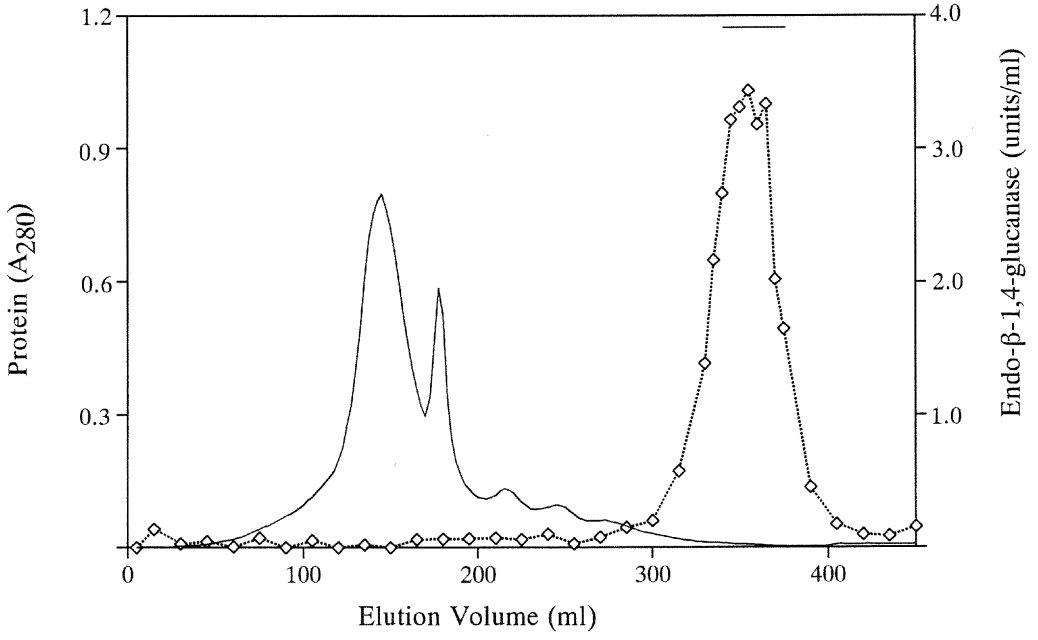

Fig. 2. Elution profile of crude extract on Sephacryl S-200 HR. (◇) Endo-β-1,4-glucanase activity; (–) protein measured by absorbance at 280 nm. One unit is the amount of enzyme which produced 1 μmol of reducing sugar/min. Bar indicates pooled fractions.

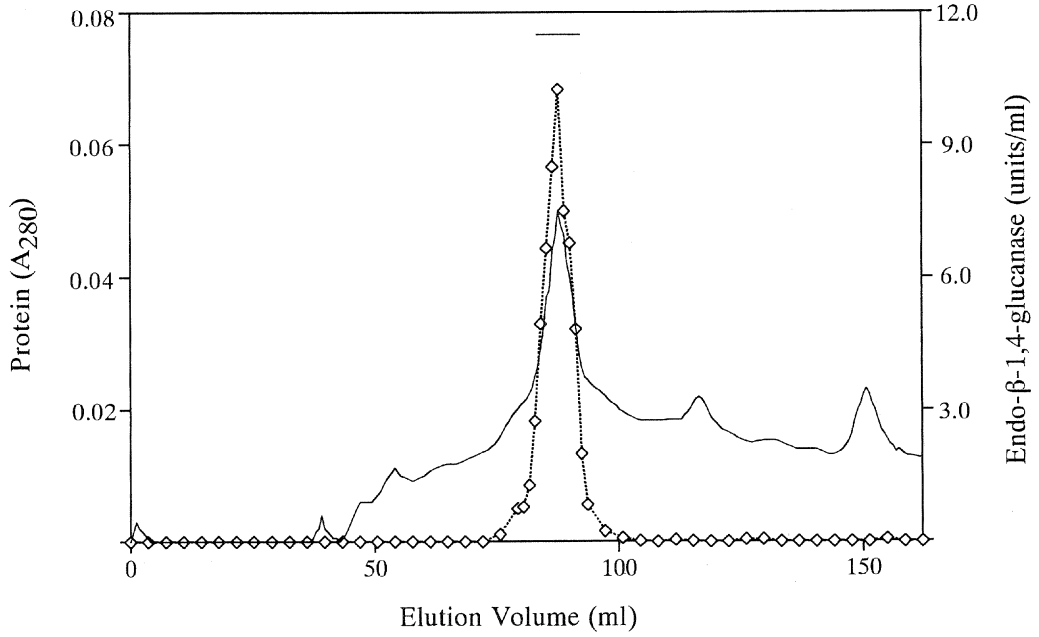

Fig. 3. Elution profile of the pooled fractions of Sephacryl S-200 HR on Superdex-75. (◇) Endo-β-1,4-glucanase activity; (–) protein measured by absorbance at 280 nm. Bar indicates pooled fractions.

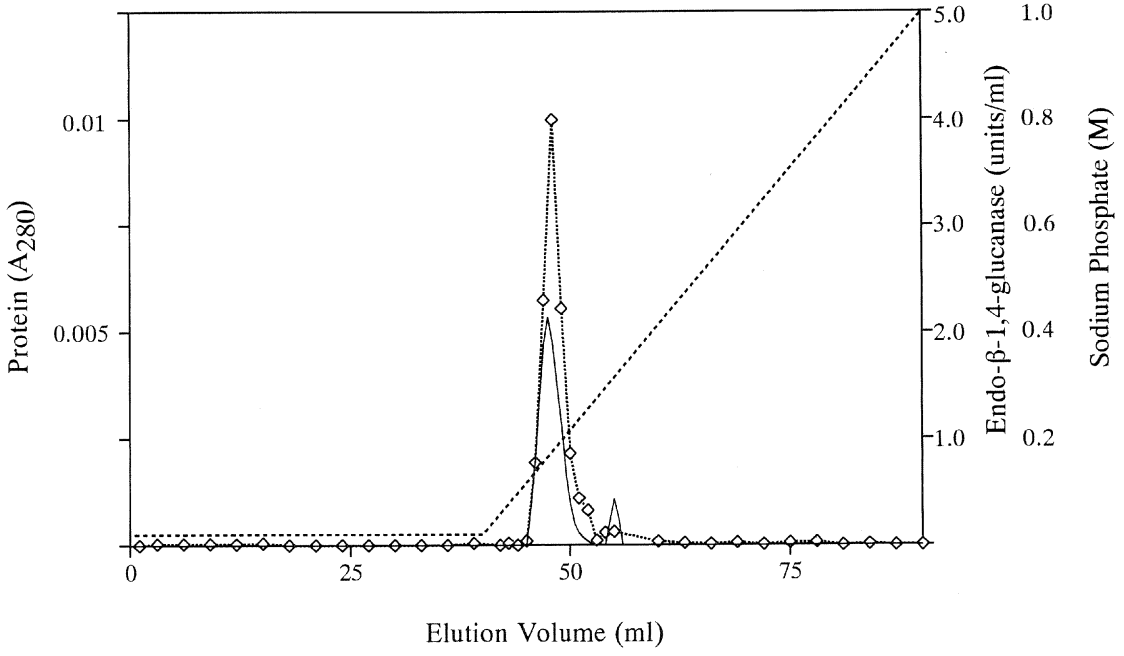

Fig. 4. Chromatography of the pooled fractions of Superdex-75 on hydroxyapatite. (◇) Endo-β-1,4-glucanase activity; (–) protein measured by absorbance at 280 nm; (---) sodium phosphate gradient.

Characterization of the purified endo-β-1,4-glucanase

Figure 7 shows the effect of pH on activity of the major endo-β-1,4-glucanase component. The major endo-β-1,4-glucanase component had optimal activity at pH 5.8, and retained more than 60% of the maximal activity from pH 5.0 to 9.2. However, the activity dropped sharply in more acidic or alkaline ranges. The activity was lost at pH 4.0 and 11.8.

The major endo-β-1,4-glucanase component had optimal temperature at 65°C (Fig. 8). The activity was stable up to 55°C during 30 min preincubation but was lost after preincubation at 65°C (Fig. 8).

Hydrolytic products by the major endo-β-1,4-glucanase component were examined using cellodextrins (Table 3). Cellopentaose was hydrolyzed to equimolar amounts of

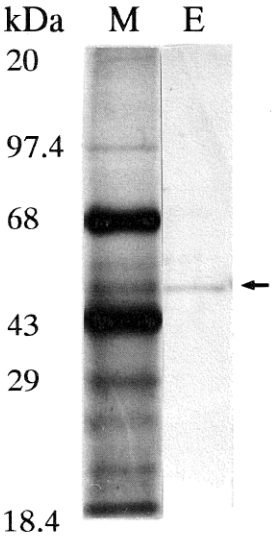

Fig. 5. SDS-PAGE for the purified endo-β-1,4-glucanase by silver staining. E, purified endo-β-1,4-glucanase (arrow); M, molecular weight standards consisting of myosin H-chain (20 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), and β-lactoglobulin (18.4 kDa). The molecular weight of the purified endo-β-1,4-glucanase was estimated to be 47 kDa.

cellotriose and cellobiose. Cellotetraose was hydrolyzed mostly to cellobiose, and to a trace of cellotriose and glucose. However, the major endo-β-1,4-glucanase component did not hydrolyze cellotriose or cellobiose.

Kinetic constants on CMC, cellopentaose and cellotetraose were shown in Table 3. K_m value on cellopentaose was smaller than that on cellotetraose. K_m value on CMC, 8.7 mg/ml, is almost equivalent to 34.8 μM according to the average molecular weight of CMC. Thus, the K_m value on CMC was extremely smaller than those on cellopentaose and cellotetraose. In contrast, V_{max} value on cellopentaose was larger than that on cellotetraose. Similarly, V_{max} value on CMC was much larger than those on cellopentaose or cellotetraose.

DISCUSSION

β-Glucosidase activity was found in the salivary glands and midgut. Specific activity was also high in the salivary glands and midgut. This is the first report suggesting that the salivary glands are the major secretion site of β-glucosidase as well as the midgut in higher termites.

The midgut had more than 90% of endo-β-1,4-glucanase activity, whereas a small amount of the activity was detected in the hindgut. This result is consistent with the case of other xylophagous higher termites (Potts and Hewitt, 1973; O'Brien *et al.*, 1979; Hogan *et al.*, 1988; Chararas and Noirot, 1988), supporting that the midgut is the main site of cellulose digestion in *N. takasagoensis*. Gel filtration profile from the crude midgut extract showed that the major endo-β-1,4-glucanase activity was eluted in a corresponding volume with that from whole termites, suggesting that the major endo-β-1,4-glucanase component was identical to that in the midgut. Although absence of symbionts was reported in the midgut of termites belonging to Nasutitermitinae (Potts and Hewitt, 1973; Czolij *et al.*, 1985), the specific activity in the midgut was higher than upstream and downstream parts of the alimentary canal (i.e. salivary glands, foregut and hindgut). These results indicate that endo-β-1,4-glucanase is secreted in the midgut tissue. Worthy to note, the main secreting site of endo-β-1,4-glucanase is the salivary glands in lower termites (Yokoe, 1964; Yamaoka and Nagatani, 1975; O'Brien *et al.*, 1979; Mishra, 1980; Veivers *et al.*, 1982; Mednicova and Tiunova, 1984; Watanabe *et al.*, 1997). It is probable that secretion sites of cellulase have been changed in the course of evolution of termites. In the present study, no endo-β-1,4-glucanase activity was detected in the paunch, where is considered as the main site of cellulose digestion in lower termites, suggesting that the paunch is not the site of cellulose digestion in *N. takasagoensis*. Although no bacterial cellulase was found in the hindgut of *N. exitiosus* (Hogan *et al.*, 1988), the presence of bacterial cellulase is not deniable in the hindgut of *N. takasagoensis* because the small amount of endo-β-1,4-glucanase was detected in the colon and rectum, where have no secretory cells but are associated with intestinal bacteria (Noirot and Noirot-Timothée, 1977; Yamaoka and Nagatani, 1978; Bignell *et al.*, 1980c; Czolij *et al.*, 1984).

In spite of numerous studies on purification and properties of cellulases from fungi, bacteria and plants, only a few insect cellulases have been purified and characterized. Endo-β-1,4-glucanase components have been purified from four species of insects; the fungus-growing higher termite *Macrotermes mülleri* (Rouland *et al.*, 1988), the lower termite *R. speratus* (Watanabe *et al.*, 1997), the wood-eating cockroach *P. cribrata*

Table 2. Purification of the major endo-β-1,4-glucanase component from *N. takasagoensis*

	total activity (units)	total protein (mg)	recovery (%)	specific activity (units/mg)	purification (fold)
crude extract	1100	6200	100	0.2	1
70% precipitation	755	383	68.6	2.0	10
Sephacryl S-200 HR	167	1.75	15.2	95.4	477
Superdex-75	57.2	0.27	5.2	210	1050
Hydroxyapatite 1	10.7	0.016	1.0	670	3350
Hydroxyapatite 2	6.0	0.005	0.5	1200	6000

One unit is the amount of enzyme which produced 1 μmol of reducing sugar(glucose equivalents)/min.

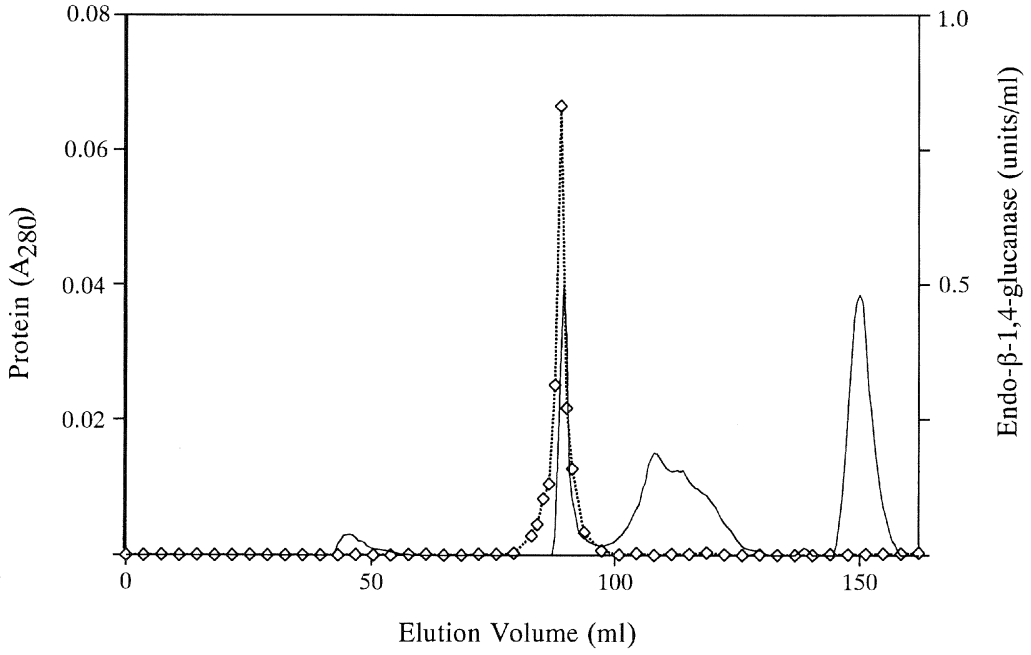

Fig. 6. Elution profile of crude midgut extract on Superdex-75. (◇) Endo-β-1,4-glucanase activity; (–) protein measured by absorbance at 280 nm. The endo-β-1,4-glucanase activity was eluted in an identical volume (Kd=0.68) to that from whole termites.

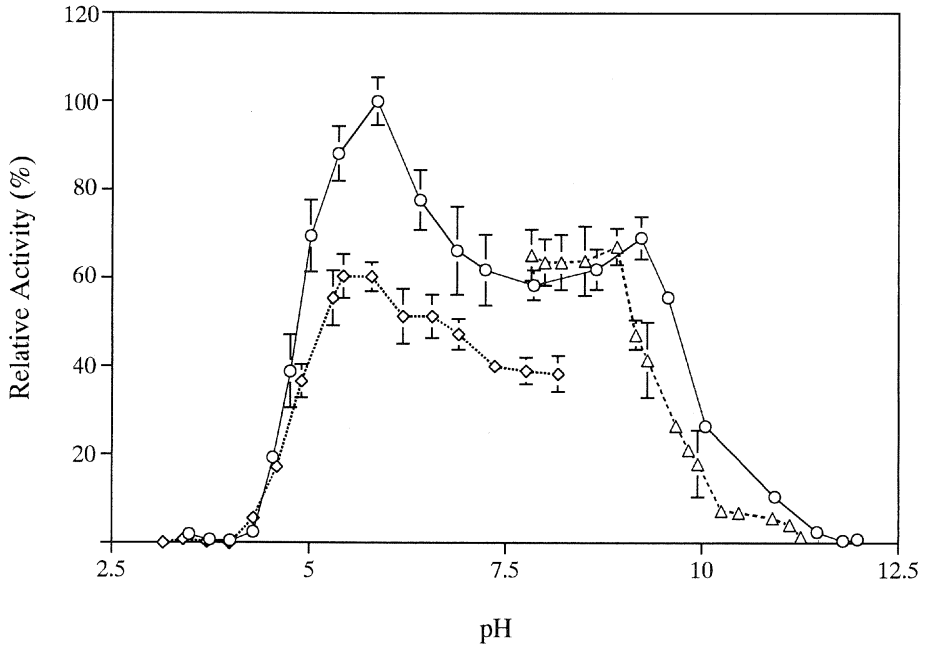

Fig. 7. Effect of pH on the purified endo-β-1,4-glucanase activity. Activity was measured using (○) acids mixture - NaOH, (◇) phosphate - citrate, or (△) boric acid, KCl - sodium carbonate buffer.

(Scrivener and Slaytor, 1994) and the wood-eating larvae of the longicorn beetle *Ergates faber* (Chararas *et al.*, 1983). Table 4 summarizes enzymatic properties among endo-β-1,4-glucanase components from different organisms. In the present study, molecular weight of the major endo-β-1,4-glucanase component from *N. takasagoensis* was estimated to be 47 kDa. This value is similar to the previously reported molecular weights from the insects, and is within the range of that of most fungal and plants endo-β-1,4-glucanases (Wood, 1991; Maclachlan and Carrington, 1991).

Specific activity of the major endo-β-1,4-glucanase component from *N. takasagoensis* is higher than those from other organisms. Since CMC which has universally used as substrate has varied in degree of polymerization and

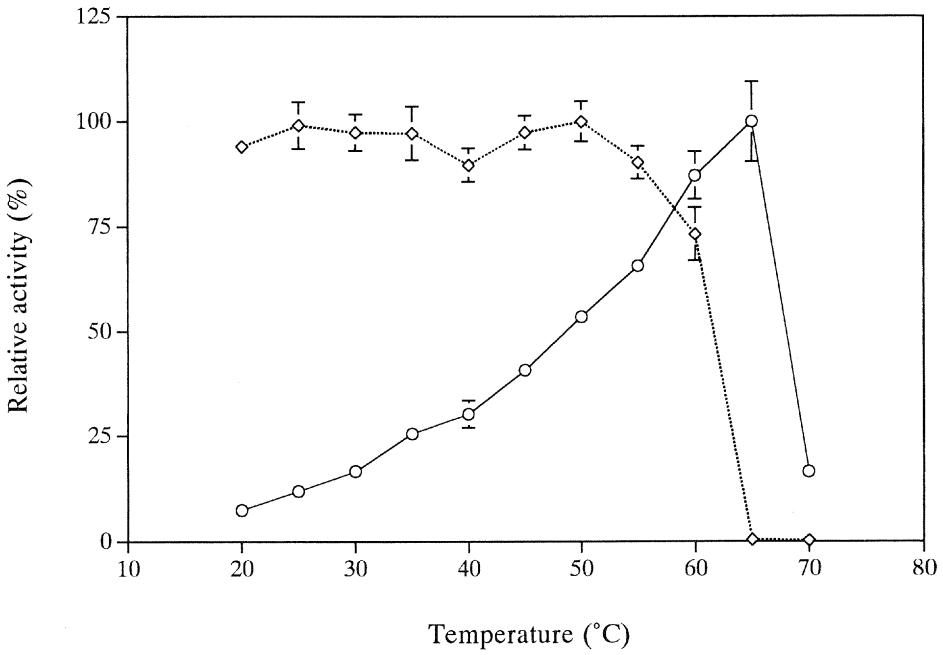

Fig. 8. Effect of temperature (○) and thermostability (◇) on the purified endo-β-1,4-glucanase activity. For measuring thermostability, enzyme samples were preincubated at each temperature for 30 min, and then assayed enzyme activity at 37°C.

Table 3. Kinetic constants and hydrolytic products for the major endo-β-1,4-glucanase component

substrate	K_m	V_{max}	products
CMC	8.7 mg/ml	2222 units/mg	
G5	1.1 mM	216 units/mg	G3=G2
G4	4.5 mM	101 units/mg	G2>>G3, G1
G3	–	–	n.d.
G2	–	–	n.d.

One unit is the amount of enzyme which produced 1 μmol of reducing sugar(glucose equivalents)/min.
CMC=carboxymethyl cellulose; G5=cellopentaose; G4=cellotetraose; G3=cellotriose; G2=cellobiose; G1=glucose
n.d.=not detected.

substitution among lots or suppliers, enzyme affinity and reaction velocity are affected by degree of them. Even if differences of enzyme affinity and reaction velocity are taken into account, specific activity of the major endo-β-1,4-glucanase component from *N. takasagoensis* is considered to be prominent among those from different organisms (Table 4).

Optimal pH of the major endo-β-1,4-glucanase component from *N. takasagoensis* is identical to that of *T. trinervoides* (Potts and Hewitt, 1974a), and is similar to those of *R. speratus* (Watanabe *et al.*, 1997), *N. exitiosus* (Schulz *et al.*, 1986), most anaerobic bacteria (Rapp and Beermann, 1991) and plants (Maclachlan and Carrington, 1991). Although activities of endo-β-1,4-glucanase components from the other insects decreased in alkaline pH, more than 60% of activity of the purified endo-β-1,4-glucanase from *N. takasagoensis* was retained even in pH 9.0. Optimal temperature of the major endo-β-1,4-glucanase component from *N. takasagoensis* was 65°C and stable at 55°C at least 30 min. The optimal temperature and high thermostability are similar to those of *N. exitiosus* (Schulz *et al.*, 1986) and *T. trinervoides* (Potts and Hewitt, 1974a), but the major endo-β-1,4-glucanase component from *N. takasagoensis* is stabler than those of other insects.

Hydrolytic properties of the major endo-β-1,4-glucanase component from *N. takasagoensis* are similar to those of partially purified endo-β-1,4-glucanase from *N. exitiosus*, which hydrolyses cellotetraose to cellobiose, but does not hydrolyze cellotriose or cellobiose (Schulz *et al.*, 1986). In *R. speratus*, YEG1 also shows similar properties to the major endo-β-1,4-glucanase component from *N. takasagoensis* though YEG2 hydrolyses cellotriose (Watanabe *et al.*, 1997). In *P. cribrata*, EG1 and EG2 also do not hydrolyze cellotriose or cellobiose (Scrivener and Slaytor, 1994). A trace of glucose production from cellotetraose by the major endo-β-1,4-glucanase component from *N. takasagoensis* was presumably due to transglycosidation as is reported in the case of EG1 of *P. cribrata* (Scrivener and Slaytor, 1994). As the K_m value decreased with higher polymerization degree of substrate, the purified endo-β-1,4-glucanase is considered to increase affinity to the substrate when polymerization degree become higher. The V_{max} values indicate that specific activity also become higher when polymerization degree become higher. Similar tendency was reported for endo-β-1,4-glucanase components from *R. speratus* (Watanabe *et al.*, 1997) and *P. cribrata* (Scrivener and Slaytor, 1994). Therefore, endo-β-1,4-glucanase activity from these species depends on the length of cellulose chains.

Table 4. Comparison of enzymatic properties among purified endo-β-1,4-glucanase components from various organisms

Species	MW (kDa)	Sp.act. (units/mg)	Opt.pH	Opt.temp. (°C)	Stable temp. (°C)	K_m (mg/ml)	V_{max} (units/mg)	[11]DS
HIGHER TERMITES								
Nasutitermes takasagoensis								
	47	1200	5.8	65	~60	8.7	2222	0.7
[1]*Macrotermes mülleri*								
Cellulase I_T	34	360	4.4	55	~55	7.5		0.71
Cellulase II	52	274	4.4	37	~42	1.0		0.71
LOWER TERMITE								
[2]*Reticulitermes speratus*								
YEG1	42	73.6	6.0	50	~40	1.83	527	0.55-0.65
YEG2	41	83.4	6.0	50	~40	1.48	540	
COCKROACH								
[3]*Panesthia cribrata*								
EG1	53.6	171.1				9.4	123.2	
EG2	48.8	318.2				6.8	490.1	
LONGICORN BEETLE								
[4]*Ergates faber*								
cellulase A	25		4.0-4.7		~60	20		0.71
FUNGI								
[5]*Aspergillus niger*								
	31	116.83	4.0	45-50		0.86		0.62-0.64
[6]*Tricoderma viride*								
Cellulase IIA	30	29.83	5.0	60	~60	0.81		0.62-0.64
Cellulase IIB	43	4.95	5.0	50	~50	0.96		0.62-0.64
Cellulase III	45	20.00	5.0	50		0.54		0.62-0.64
[7]*Robillarda* sp. Y-20								
CMCase I	56	17.0	5.0	60	~50	0.60		0.51
CMCase II	59	72.3	4.0-5.0	55	~50			
BACTERIA								
[8]*Thermomonospora fusca*								
E_1	94	768	6.0	74		0.36		
E_2	46	77	6.0	58		0.12		
[9]*Clostridium thermocellum*								
	83-94	65.1	5.2	62				
[10]51-kDa subunit of cellulosome	51	595	5.0	60				

One unit is the amount of enzyme which produced 1 μmol of reducing sugar(glucose equivalents)/min. Each endo-β-1,4-glucanase activity was measured at 37°C unless otherwise indicated. [1] Rouland *et al.* (1988). Cellulase I_T was purified from the whole termites but was supposed to be originated from fungus. [2] Watanabe *et al.* (1997). [3] Scrivener and Slaytor (1994). Activity was measured at 40°C. Original values were 1850 and 3440 units/mg (units were mg reducing sugar/hr) for specific activities of EG1 and EG2, respectively. V_{max} values were 22.2 and 88.3 units/mg (units were mg reducing sugar/min) for EG1 and EG2, respectively. [4] Chararas *et al.* (1983). [5] Okada (1985). Activity was measured at 30°C. [6] Okada (1975, 1976). Activity was measured at 30°C. [7] Yoshigi *et al.* (1988). [8] Calza *et al.* (1985). Activity was measured at 56°C. [9] Ng and Zeikus (1981), and [10]Mori (1992). Activity was measured at 60°C. [11]Degree of substitution for used CMC.

Our results demonstrated prominent efficiency of the major endo-β-1,4-glucanase component from *N. takasagoensis* in cellulose digestion among those from various organisms. This higher efficiency seems to compensate the lack of protozoan cellulases because higher termite lost cellulolytic protozoa in the course of evolution. The wood-eating higher termites must have increased adaptability to cellulose diet and have flourished by acquisition of highly efficient endo-β-1,4-glucanase. This higher efficiency also seems to partially explain why higher termites play more significant role than lower termites in keeping carbon balance in nature (Wood and Johnson, 1986).

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