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*E. coli*와 baculovirus-mediated Sf 9 세포에서 발현된 진드기 *H. longicornis*의 CHT1 단백의 효소활성 비교

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Comparison of enzymatic activities between the recombinant CHT1 proteins from the hard tick *Haemaphysalis longicornis* expressed in *E. coli* and baculovirus-mediated Sf 9 cells

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Abstract: A chitinase cDNA named CHT1 was cloned from the hard tick, *Haemaphysalis longicornis*, and the enzymatic properties of its recombinant proteins were characterized. The CHT1 cDNA encodes 930 amino-acid (aa) residues including a 22 aa putative signal peptide, with the calculated molecular mass of the putative mature protein 104 kDa. The *E. coli*-expressed rCHT1 exhibited weak chitinolytic activity against 4MU-$(GlcNAc)_3$. The rCHT1 protein with higher activity was obtained using recombinant *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV), which expresses rCHT1 under polyhedrin promoter. These findings suggest that the rCHT1 expressed in baculovirus-mediated Sf 9 cells has a high activity than *E. coli*-expressed rCHT1.

Key words: tick, *Haemaphysalis longicornis*, chitinase, recombinant protein

Introduction

Chitinase is an essential component in the hydrolysis of chitin, the β-1,4-linked homopolymer of N-acetylglucosamine. Chitin, an insoluble structural polysaccharide that occurs in the exoskeletal and gut linings of insects, is a metabolic target of selective pest control agents. In arthropods chitin microfibrils are complexed with proteins forming chitinous structures of the cuticle and the peritrophic matrix that lines the gut[1,2]. Insect chitinase is induced by ecdysteroids at the time of molting and metamorphosis of the larvae to degrade most of the older chitin[3,4]. The degradation of chitin is performed mainly by two types of enzymes, endo-splitting chitinase and exo-splitting β-N-acetylglucosaminidase, with the former being the rate-limiting one[5]. Several insect chitinase inhibitors, such as allosamidin isolated from *Streptomyces* spp.[6] were shown to prevent larval ecdysis, but no practical insect growth regulator inhibiting chitinase has been developed so far. Chitinase activity is involved in the developmental process of cuticle degradation at different larval stages. Events during the molting cycle have been followed biochemically and morphologically[7]. Ticks

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also require chitinolytic enzymes to remove old cuticle and synthesize a new one to allow for continued growth and development.

The tick also transmits a number of diseases, *Babesia, Theileria sergenti /buffeli/orientalis*, which causes economically important diseases in cattle[8-10]. The problems and challenges of successful ectoparasite control are epitomized by the situation with *Haemaphysalis longicornis* (*H. longicornis*) in North-East Asia. The increase in legislation to combat the detrimental effect of residues of acaricides on the environment[11] has emphasized the need to assess a variety of alternatives to tick vector control. Applications of biological tick control agents are an attractive alternative to chemical acaricides since they do not pose the environmental and health risks associated with chemical residues. Previously, we have cloned *H. longicornis* CHT1 gene, and demonstrated that the recombinant CHT1 protein had the activities of chitinolytic enzyme[12]. Chitinases have been indicated as an important candidate for a selective insect-control protein[5,7,18], because it is quite safe and readily degradable in environment[13].

To facilitate the use of insect chitinases as a component in an integrated pest management system, it is important to gain more knowledge on these enzymes. This report describes here the comparison of enzymatic activities between the recombinant CHT1 (rCHT1) proteins from the hard tick *H. longicornis* expressed in *Escherichia coli* (*E. coli*) and baculovirus-mediated *Spodoptera frugiperda* (Sf) 9 cell line.

Materials and Methods

Ticks

The parthenogenetic Okayama strain of the tick *H. longicornis*[8] has been maintained by feeding on rabbits and mice for several generations in our laboratory since 1997.

Isolation and expression of the CHT1 gene in *E. coli*

The 2790bp *Eco*RI PCR fragment from *H. longicornis* chitinase containing the open reading frame was inserted at the EcoRI site of pET28a expression vector (Novagen), yielding pET28a/CHT1[12]. Restriction enzyme analysis was performed to identify the construct containing the insert in the correct orientation[12]. BL21 (DE3) *E. coli* cells were transformed with pET28a/CHT1, and cultured in LB medium containing 50 μg/ml kanamycin. The expression of the 6 x His-tagged protein was induced by the addition of IPTG (final conc. 1 mM). The product, which was present mainly as inclusion bodies, was dissolved in the buffer (20 mM Tris, 0.5 M NaCl, 5 mM imidazole, 6 M urea, pH 7.9) and isolated with a His-bind Quick column (Novagen, Madison, Wis.) under denaturing conditions following the supplier's protocol. The final eluent containing the isolated protein was dialyzed against 10 mM Tris-HCl buffer (pH 8.0) containing 1% Triton X-100 at 4°C and stored at -20°C until further analysis.

SDS-PAGE and immunoblot analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis under reducing conditions were performed as described previously[14]. Proteins were loaded with 15 μg/lane for SDS-PAGE and immunoblot. The membranes were incubated with the mouse anti-CHT1 serum at dilution of 1:100. After washing with TBS-Tween 20, the membranes were incubated for 1 h with alkaline-phosphatase-conjugated goat anti-mouse IgG (Stratagene, La Jolla, Cal.) at a dilution of 1:15,000.

Production of anti-rCHT1 sera

One hundred micrograms of the recombinant fusion protein were injected into mice (BALB/c, 8 weeks old) intraperitoneally in Freund's complete adjuvant. The same antigen in Freund's incomplete adjuvant was injected intraperitoneally into the mice on days 14 and 28. The sera from immunized mice were collected 10 days after the last immunization.

Cells and viruses

Sf 9 cells were propagated at 27℃ in TC-100 and SF-900 II serum-free insect cell culture medium (GIBCO BRL, Grand Island, NY). The transfer vector pBlueBac 4.5/V5-His (Invitrogen, San Diego, Cal.) was used. Cell culture techniques were employed as described by You *et al*[12, 14]. Viral infection of insect cells were carried out using the Bac-N-Blue Transfection Kit (Invitrogen, San Diego, Cal.).

Construction of recombinant baculovirus containing the *H. longicornis* CHT1 gene

The pBlueBac4.5/V5-His (5027 bp) contains the promoter for the polyhedrin gene followed by a polylinker with a unique restriction site for *Eco*RI. The 2790 bp *Eco*RI PCR fragment from *H. longicornis* CHT1 containing the open reading frame was inserted at the EcoRI site of pBlueBac 4.5/V5-His to produce the transfer plasmid pBlueBac4.5/ V5-His-CHT1. Restriction enzyme analysis was performed to identify the construct containing the insert in the correct orientation. Transfection technique was performed as described previously[13].

Purification of *H. longicornis* CHT1

A 30 ml culture supernatant of Sf 9 cells infected with the recombinant virus (AcMNPV/CHT1) or as a control infected with the wild type (AcMNPV) were dialyzed against binding buffer (20 mM sodium phosphate, 500 mM NaCl, 5 mM imidazole, pH = 7.4) and concentrated by ultrafiltration (concentrator 10/20, exclusion size 30 kDa). Two ml of 50% Ni-NTA slurry (Invitrogen, San Diego, Cal.) was centrifuged (5 min at 500g) and washed twice with binding buffer. After incubation for 1 h at 4℃ under continuous shaking, the slurry was centrifuged and the supernatant decanted. The pellet was washed at 4℃ with 20 ml washing buffers (20 mM sodium phosphate, 500 mM NaCl, pH = 6.0) containing 50 mM imidazole. Elution was performed with 5 ml of the following elution buffer (pH 6.0) containing 350 mM imidazole. The final eluent containing the isolated protein was dialyzed against 20 mM sodium phosphate (pH 6.0) at 4℃ and stored at -80℃ until further analysis.

Protein and enzyme assay

The protein concentration was determined using the BCA protein assay reagent (Pierce, Rockford, IL) with bovine serum albumin as a standard. The enzymatic activity of the recombinant chitinase was quantified using 4MU-$(GlcNAc)_3$ (Sigma, St Louis, MO) as substrates[12,17]. The enzyme solutions (100 μl) diluted in 50 mM sodium-phosphate buffer (pH 7.0) were incubated with substrate (final conc. 40 μM) at 37°C for 20 min. After the addition of 120 μl of 1 M glycine-NaOH buffer (pH 10.6), the fluorescence of 4 MU liberated in the reaction was measured with a FP-770 Spectrophotometer (Japan) with excitation at 350 nm and emission at 450 nm by continuously recording changes in fluorescence.

Animal care and manipulations

Animal care and manipulations in this study were in accordance with the "National Research Center for Protozoan Diseases" and "Obihiro University of Agriculture and Veterinary Medicine" instructions and ordinances on Animal Welfare and adhered to the "Guide for the Care and Use of Laboratory Animals".

Results

Expression of rCHT1 by *E. coli*

The CHT1 gene was ligated into the bacterial expression vector pET28a, and then the CHT1 gene was expressed as a 6 x His-tagged fusion protein in *E. coli*. In SDS-PAGE, a major 104 kDa band was detected in the crude lysate of *E. coli* cells transformed with a plasmid carrying CHT1 cDNA (pET28a/CHT1), but not in the bacteria transformed with the original pET28a (Fig 1).

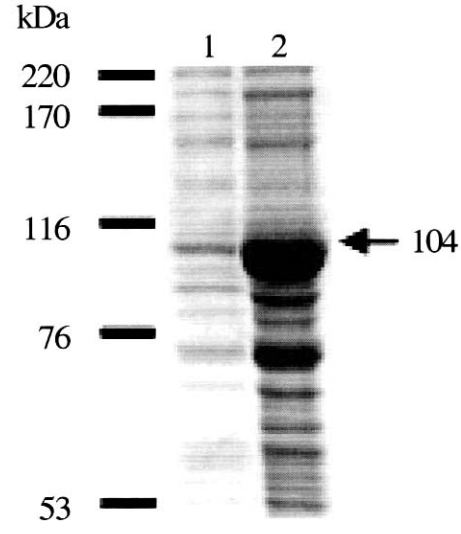

Fig. 1. SDS-PAGE analysis of rCHT1 expressed in *E. coli*. Lane 1: pET28a vector without insert. Lane 2: pET28a/CHT1. The gels were stained with coomassie brilliant blue.

Expression of rCHT1 by recombinant baculovirus-mediated Sf 9 cell line

We constructed a recombinant AcMNPV expressing the CHT1 gene under the control of the polyhedrin promoter (AcMNPV/CHT1) to obtain a recombinant chitinase with higher specific activity. To distinguish the chitinolytic activities of the *H. longicornis* chitinase and the viral chitinase, we purified the 6 x His-tagged rCHT1 from media of Sf 9 cells. The media of Sf 9 cells four days after infection with AcMNPV/CHT1 were analyzed using SDS-PAGE. The media of AcMNPV/CHT1-infected cells showed a 116 kDa band (Fig 2).

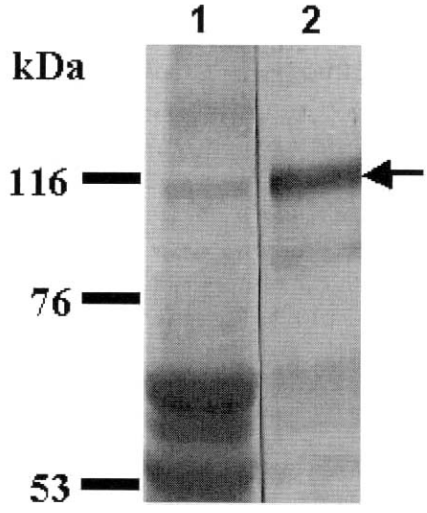

Fig. 2. SDS-PAGE and immunoblot analysis of rCHT1 expressed in AcMNPV/CHT1-mediated Sf 9 cell line. The media of AcMNPV/CHT1-infected Sf 9 cell line were used as antigen. Lane 1 was treated with amido black staining, and lane 2, with anti-rCHT1 fusion protein serum.

Enzymatic activity of 6 x His-tagged H. *longicornis* rCHT1 expressed in *E. coli* and baculovirus-mediated Sf 9 cell line

We prepared a 6 x His-tagged recombinant protein from the cDNA using an *E. coli*-based expression system (pET system, Novagen) and examined its chitinolytic activity to ensure that the isolated cDNA encodes a functional protein. Enzymatic activities in the media of insect cells four days after infection of recombinant viruses were quantified by a liquid assay using 4MU-$(GlcNAc)_3$ as substrate. The rCHT1 expressed in *E. coli* showed relatively low chitinolytic activity against 4MU-$(GlcNAc)_3$ compared to the rCHT1 expressed in baculovirus-mediated Sf 9 cell line (Fig 3). Remarkably, the chitinolytic activity of AcMNPV/CHT1-infected cells was detected in the media and the cell extracts of AcMNPV-infected insect cells.

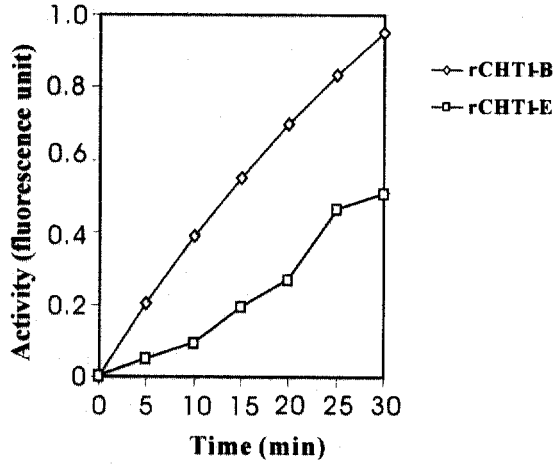

Fig. 3. Comparison of enzymatic activities between the purified rCHT1 expressed in *E. coli* (□; rCHT1-E) and baculovirus-mediated Sf 9 cell line (◇; rCHT1-B).

Discussion

Chitinases act by hydrolytically cleaving the β-glycosidic linkages between GlcNAc residues. In general, this hydrolysis can occur in one of two ways, either with retention of anomeric configuration in the product or with inversion[15]. Chitinase is produced in molting fluid and gut tissues subsequent to feeding at the end of a larval instar in preparation for molting[7,16].

We have shown that the *H. longicornis* CHT1 cDNA encodes a functional chitinase using a 6 x His-tagged recombinant protein expressed in *E. coli* and a highly sensitive chitinase assay with 4MU-$(GlcNAc)_3$ as substrates. The purified 6 x His-tagged rCHT1 show apparent endochitinase activity. Its specific activity, however, is extremely low when compared to the rCHT1 expressed in Sf 9 insect cells infected with recombinant baculoviruses (Fig 3), such as seen in the chitinases of some insects[13,17]. Further studies would be necessary to increase the specific activity of the rCHT1 expressed in bacteria, by improving

refolding from the inclusion bodies *in vitro*. We further confirmed the chitinolytic activity of rCHT1 by a baculovirus-based protein expression system. It is rather difficult to detect the chitinase activity of the recombinant protein in the baculovirus system, because of a high background of chitinolytic activity derived from the viral chitinase. We have solved this problem by purifying a 6 x His-tagged rCHT1.

From a practical point of view, the media of the AcMNPV/CHT1-infected cells provides a superior source of insect chitinase, which can be utilized for developing novel pest management agents. Chitinases have been known to enhance the insecticidal activity of entomopathogens such as *Bacillus thuringiensis*, probably by changing the permeability of the peritrophic membrane[18]. The rCHT1 thus can possibly be used as a supplement for biological control agents.

In this paper, we described the construction of a recombinant nonoccluded baculovirus, *Autographa californica* nuclear polyhedrosis virus (AcMNPV), containing the *H. longicornis* CHT1 cDNA under the control of the polyhedrin promoter. *S. frugiperda* cells infested with the virus produce and secrete an enzymatically active protein with molecular mass of 116 kDa into the culture media. We have shown in this experiment that the enzyme activities of rCHT1 expressed in *E. coli* were lower than baculovirus-mediated Sf 9 cell line (Fig 3). This indicates the importance of rCHT1 protein that was expressed in baculovirus-mediated Sf 9 cell line with respect to the vaccine of ticks both as a vector and as a pest and hence it is a candidate antigen for the vaccine against ticks. Mulenga *et al*[19,20] have demonstrated the possibility of the vaccine against *H. longicornis*. Further studies exploring the use of rCHT1 expressed baculovirus-mediated Sf 9 cell line as antigen for vaccine of tick need to be carried out.

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References

1. Fristrom D, Fristorm JW. The metamorphic development of the adult epidermis. In Bate M, Arias AM, ed, Development of *Drosophila melanogaster*, Vol. 2, Cold Spring Harbor Laboratory Press, New York: 843-897, 1993.
2. Lehane MJ. Peritrophic matrix structure and function. *Annu Rev Entomol*, 42, 525-550, 1997.
3. Kimura S. The control of chitinase activity by ecdysterone in larvae of *Bombyx mori*. *J Insect Physiol*, 19, 115-123, 1973.
4. Koga D, Fujimoto H, Funakoshi T, *et al.* Immunoblot analysis of chitinolytic enzymes in integuments and molting fluids of the silkworm, *Bombyx mori*, and the tobacco hornworm, *Manduca sexta*. *Insect Biochem Mol Biol*, 22, 305-311. 1992.
5. Kamer KJ, Koga D. Insect chitin: physical state, synthesis, degradation and metabolic regulation. *Insect Biochem*, 16, 851-877. 1986.
6. Sakuda S, Isogai A, Matsumoto A *et al.* The structure of allosamidin, a novel insect chitinase inhibitor, produced by *Streptomyces* sp, *Tetrahedron Lett*, 27, 2475-2478, 1986.
7. Reynolds SE, Samuels RI. Physiology and biochemistry of insect moulting fluid. *Adv Insect Physiol*, 26, 157-232, 1996.
8. Fujisaki K. Development of acquired resistance and precipilating antibody in rabbits experimentally infested with females of *Haemaphysalis longicornis* (Ixodoidea: Ixodidae). *Natl Inst Anim Hlth Q (Japan)*, 18, 27-38, 1978.
9. Fujisaki K. A review of the taxonomy of *Theileria sergenti / buffeli/ orientalis* group parasites in cattle. *J Protozool Res*, 2, 87-96, 1992.
10. Fujisaki K, Kawazu S, Kamio T. The taxonomy of the bovine *Theileria* spp. *Parasitol Today*, 10, 31-33, 1994.
11. Willadsen P, Kemp DH, Vaccination with 'concealed' antigens for tick control. *Parasitol Today*, 4, 196-198, 1988.
12. You M, Xuan X, Tsuji N, *et al.* Identification and molecular characterization of a chitinase from the hard tick *Haemaphysalis longicornis*. *J Biol Chem*, 278, 8556-8563, 2003.
13. Gopalakrishnan B, Muthukrishnan S, Kramer KJ. Baculovirus-mediated expression of a *Manduca sexta* chitinase gene: properties of the recombinant protein. *Insect Biochem Mol Biol*, 25, 255-265, 1995.
14. You M, Xuan X, Tsuji N, *et al.*. Molecular charac-

terization of a troponin I-like protein from the hard tick *Haemaphysalis longicornis*. *Insect Biochem Mol Biol*, 32, 67-73, 2001.

15. Sinnott ML. Catalytic mechanisms of enzymatic glycosyl transfer. *Chem Rev*, 90, 1171-1202., 1990.
16. Kramer KJ, Corpuz LM, Choi H, *et al.* Sequence of a cDNA and expression of the gene encoding epidermal and gut chitinases of *Manduca sexta*. *Insect Biochem Mol Biol*, 23, 691-701, 1993.
17. Shinoda T, Kobayash J, Matsui M, *et al.* Cloning and functional expression of a chitinase cDNA from the common cutworm, *Spodoptera litura*, using a recombinant baculovirus lacking the virus- encoded chitinase gene. *Insect Biochem Mol Biol*, 31, 521-532, 2001.
18. Kramer KJ, Muthukrishnan S, Lowell J, *et al.* Chitinases for insect control. In Carozzi N, Koziel M, ed, Advances in insect control: the role of transgenic plants. Taylor and Francis, Bristol, pp.186-193, 1997.
19. Mulenga A, Sugimoto C, Ingram G, *et al.* Molecular cloning of two *Haemaphysalis longicornis* cathepsin L-like cysteine proteinase genes. *J Vet Med Sci*, 61, 497-503, 1999.
20. Mulenga A, Sugimoto C, Sako Y, *et al.* Molecular characterization of a *Haemaphysalis longicornis* tick salivary gland-associated 29-Kilodalton protein and its effect as a vaccine against tick infestation in rabbits. *Infect Immun*, 7, 1652-1658, 1999.