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Ethylene glycol chitin을 이용한 진드기 *H. longicornis* 재조합 CHT1 단백의 키틴분해능 검정 연구

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A simple and sensitive assay for chitinolytic activity of the recombinant CHT1 proteins from the hard tick *H. longicornis* using ethylene glycol chitin

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Abstract: To determine effectively the chitinolytic activity of rCHT1 from the hard tick *H. longicornis* expressed in baculovirus-mediated *Spodoptera frugiperda* (Sf) 9 cells, a simple and sensitive assay system was established in solid phase using agarose gel containing ethylene glycol chitin as substrate. The various factors affecting the efficacy of the assay were also investigated. The effects of various temperature, dosages of proteins, pH of media and time courses of reaction were examined to verify the sensitivity of assay for chitinolytic activity of rCHT1 protein. It was found that the optimal reactive conditions were 37℃ of temperature, 12 to 15 hours of reactive times, 0.1μg of protein concentration and pH 5 to 7 of media. Using the assay system designed, the functional activities of *H. longicornis* rCHT1 protein could be evaluated simply and sensitively.

Key words: *H. longicornis*, recombinant CHT, chitinolytic activity, simple assay, ethylene glycol chitin

Introduction

Polysaccharide metabolism has been known as a very ancient and widespread biological activity. Because of its central role in energy flux, a wide range of enzymes have been evolved over a time to catalyze these reactions. Among the enzymes involved in breakdown of sugar-based polymers are cellulases, chitinases, chitosanases and lysozymes[1]. These have been cataloged, based on amino acid sequences, into nearly 50 families of glycohydrolases[1].

Chitinases act by hydrolytically cleaving the â-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. Chitinases have been classified in two major categories[2]. Endochitinases cleave chitin randomly at internal sites, generating soluble low molecular mass multimers of GlcNac, such as chititetraose, chitotriose and the dimmer di-acetylchitobiose[2]. Exo-chitinases can be divided into two subcategories: chitobiosidases[3], which catalyze the progressive

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release of di-acetylchitobiose starting at the nonreducing end of the chitin microfibril; and 1-4-â-N-acetylglucosaminidases, which cleave the oligomeric products of endochitinases and chitibiosidases generating monomers of GlcNAc[2,3]. Chitin is second only to cellulose in biomass and it is an important component of many cell wall structures[4]. Chitin is a complex polymer consisting of N-acetylglucosamine chains arranged in an antiparallel or a parallel configuration with different degrees of deacetylation[4].

In arthropods, chitin microfibrils are complex with proteins forming chitinous structures of the cuticle and the peritrophic matrix (PM) that lines the gut[4,5]. Insect chitinase is induced by ecdysteroids at the time of molting and metamorphosis of the larvae to degrade most of the older chitin[6,7]. 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[8]. Ticks also require chitinolytic enzymes to remove old cuticle and synthesize a new one to allow for continuation of growth and development in their life cycles[9].

The increasing awareness of the potential uses of chitin and its derivatives in the field of biotechnology gives the need for establishment of a sensitive and simple assay system capable of differentiating functional activities of the recombinant chitinases[9]. Chitinase assays based on the release of 4-methylumbelliferone (4MU) from chitin substrates have been investigated by a number of groups[9,10]. The carboxymethyl remazol brilliant violet chitin has been also used as substrate to measure the chitinolytic activity of *Manduca sexta* chitinase[11,12]. Recently, ethylene glycol chitin has been applied as a substrate to measure the chitinase activity of sweet potato[13] and rodent malaria parasite chitinase[14], and it was reported that the assay was highly sensitive and effective to differentiate the activities of those chitinases.

In the previous works[15,16], we have cloned *Haemaphysalis longicornis* (*H. longicornis*) CHT1 gene, and demonstrated that the CHT1 genes encode chitinases[15]. The enzymatic activities of recombinant CHT1 (rCHT1) from *H. longicornis* were proved in liquid phase using a spectrophotometry with 4MU-(GlcNAc)3 as substrate[15,16]. However it was necessary to develope more simple and sensitive assay for determination of chitinolytic activity of the rCHT1 protein originated from *H. longicornis*. In this study, to measure effectively the chitinolytic activity of rCHT1 from the hard tick *H. longicornis* expressed in baculovirus-mediated Sf 9 cells, we have designed a simple and sensitive assay system in solid phase using agarose gel containing ethylene glycol chitin as substrate. The various factors affecting the efficacy of the assay were also evaluated.

Materials and Methods

rCHT1 from *H. longicornis*

rCHT1 protein was prepared as described by You *et al*[15,16]. In brief, the 2790 bp EcoRI PCR fragment from *H. longicornis* CHT1 containing the open reading frame was inserted at the EcoRI site of pBlueBac4.5/V5-His to produce the transfer plasmid pBlueBac4.5/V5-His-CHT1. A 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). 2 ml 50% Ni-NTA slurry (Invitrogen, SanDiego, Cal) was centrifuged (5 min at 500g) and washed twice with binding buffer. After incubation for 1 hour 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 determination

The protein concentration was determined using the BCA protein assay reagent (Pierce, Rockford, Il) with bovine serum albumin as a standard.

Preparation of agarose gel containing ethylene glycol chitin

Ethylene glycol chitin supplied by Seikagaku corporation (Tokyo) was swelled in 0.1 M phosphate buffer (pH 5.6) in a refrigerator for one night and 0.2% solution in same buffer was prepared and filtered through a glass filter. The

agarose gel (1.0%, Amresco, Ohio) in PBS was melted and added ethylene glycol chitin by 0.01%. The mixtures were applied on a small gel marker plate(Cosmo Bio, Tokyo, 52Wx60L) with comb. When the gel solidified, the combs were removed, and the rCHT1(10$\mu\ell$) at various protein concentrations were applied into the comb wells.

Assay in various temperatures

After overnight incubation of the plates at 4℃, 25℃, 37℃ or 48℃, the gels were stained for 5 min in PBS containing 0.01% Fluorescent Brightener 28 (Sigma, Mo) and destained in distilled water. Chitin hydrolysis was visualized under UV light. The chitinase activity was expressed by the longest diameter of the vertical length (mm) of the dark circle. The data represent the mean of triplicate.

Reaction by time course

Kinetics of enzyme action were observed by monitoring the diameter of hydrolyzed zone by time course. All reactions were carried out in 1% agarose gels in PBS containing 0.01% ethylene glycol chitin (Seikagaku, Tokyo). Assays were carried out under the same conditions as described above. The linear portion of the time course was applied to calculate the specific activity of the chitinase enzyme system against ethylene glycol chitin.

pH dependence

The optimal pH of rCHT1 activity in the assay system was investigated in a wide range of buffers (pH 4.0-10)[17] at 37℃. The diameters of hydrolyzed zone were measured at 12 hours after addition of the rCHT1.

Results

In order to measure the chitinolytic activity of rCHT1 from the hard tick *H. longicornis* expressed in baculovirus-mediated Sf 9 cells, a simple and sensitive assay system was designed in solid phase using agarose gel containing ethylene glycol chitin as substrate. The effects of various temperatures and pH, reactive times and dosages of the recombinant protein which are affecting the efficacy of the assay were evaluated.

To verify the chitinolytic activity, *Serratia marcescens* chitinase A (0.05μg, Sigma) was applied as a positive control. As shown in Fig 1. the specific reaction circles were demonstrated in well No 1 for *Serratia marcescens* chitinase A and well No 3 for the rCHT1 (0.05μg) expressed in AcMNPV/CHT1-infected Sf 9 cells. No color reaction was observed in well No 2 loaded with 20 mM sodium phosphate buffer as control.

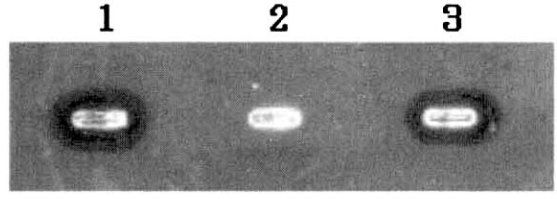

Fig. 1. Features of chitinolytic activity of *Serratia marcescens* chitinase A (0.04μg, Sigma) in well No 1 and the rCHT1(0.04μg) expressed in AcMNPV/CHT1-infected Sf 9 cells in well No 3 using ethylene glycol chitin as substrate. Well 2 was loaded with 20 mM sodium phosphate buffer as control. Reaction temperature, at 25℃. Reaction time, for 10 hour.

The effects of temperatures on chitinolytic activity of the rCHT1 expressed in AcMNPV/CHT1-infected Sf 9 cells were examined. As shown in Fig 2, the temperature at 37℃ showed significantly larger reaction circle than at 4℃, 25℃ and 48℃, while the temperature at 4℃ revealed the smallest diameter of reaction. Afterwards the chitinolytic activity of rCHT1 using ethylene glycol chitin was tested at 37℃.

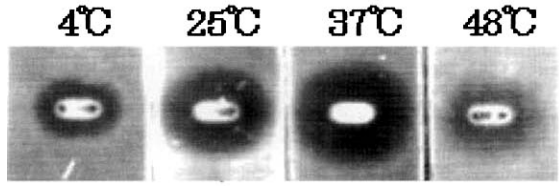

Fig. 2. The effects of temperatures on chitinolytic activity of the rCHT1 expressed in AcMNPV/CHT1-infected Sf 9 cells. rCHT1 at 0.1μg in 10$\mu\ell$ was loaded into well.

The chitinolytic activity of the rCHT1 (1.0μg) expressed in AcMNPV/CHT1-infected Sf 9 cells by time course was examined. At 3 to 6 hours the circle diameters were rapidly increased reaching 17 mm, and at 12 hour the maximum level of diameter were observed. Afterwards the length of circle diameter keeps parallel (Fig 3).

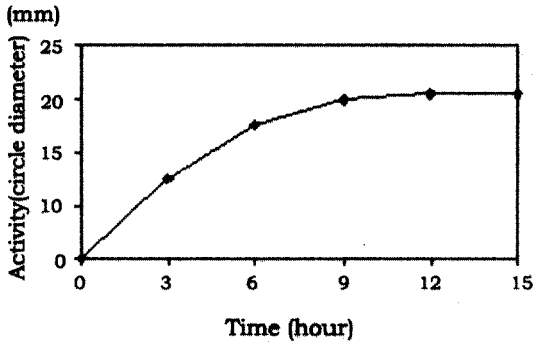

Fig 3. The chitinolytic activity of the rCHT1 (0.1μg) expressed in AcMNPV/CHT1-infected Sf 9 cells by time course. The reaction temperature was 37℃.

The chitinolytic activity of the rCHT1 expressed in AcMNPV/CHT1-infected Sf 9 cells was examined by dose-dependent manner at 37℃. As shown in Fig 4. the circle diameters were increased proportionally by increasing dosages of rCHT1, resulting in 18 to 20 mm at 0.08 to 0.16μg of rCHT1. And the optimal concentration of protein for the assay was determined as 0.1μg in 10$\mu\ell$.

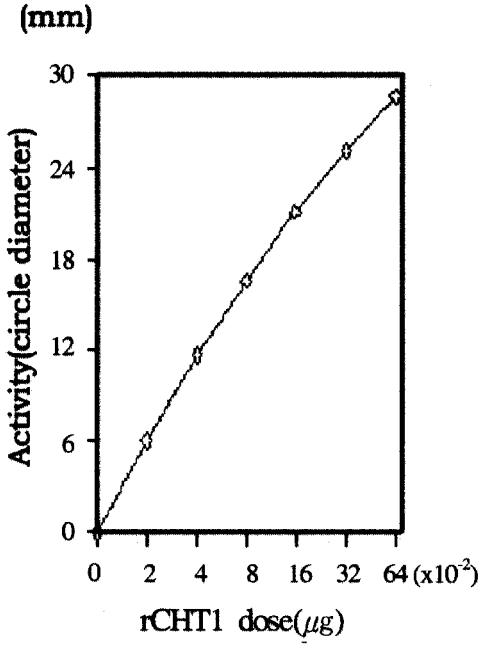

Fig. 4. The chitinolytic activity of rCHT1 at various dosages expressed in AcMNPV/CHT1-infected Sf 9 cells. The reaction temperature was 37℃.

The effects of various pH on chitinolytic activity of rCHT1 were studied. When 1.0μg of rCHT1 was loaded into well and reacted at 37℃. the buffers at pH 5 to 7 showed the highest activity showing the diameter of 18 to 21mm, while the acidic (pH<5) or alkaline (pH>10) conditions resulted in the lower activities (Fig 5).

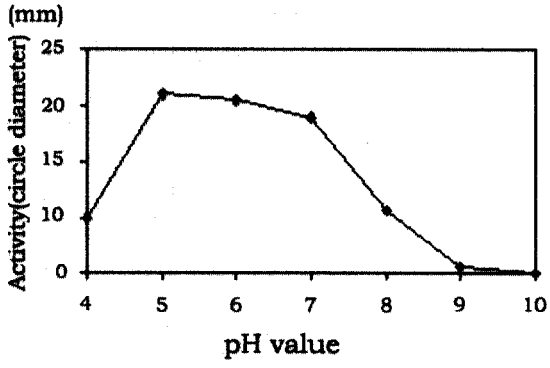

Fig. 5. The effects of pH on chitinolytic activity of the rCHT1 expressed in AcMNPV/CHT1-infected Sf 9 cells. rCHT1 at 0.1μg in 10$\mu\ell$ was loaded into well, The reaction temperature was 37℃.

Discussion

Chitin, the (1-4-â-linked homopolymer of N-acetylglucosamine), is produced in enormous quantities in the biosphere[18], and its degradation and recycling is of paramount importance in the maintenance of carbon and nitrogen cycles. Mineralization of chitin has been demonstrated by microbial populations[19,20], where it can serve as sole source of carbon and nitrogen[21]. In insects chitinase is produced in molting fluid and gut tissues subsequent to feeding at the end of a larval instar in preparation for a molt[22,23]. Chitinases act by hydrolytically cleaving the â-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[24]. The peritrophic matrix (PM) formed in the midgut lumen of blood-sucking arthropods after a blood meal[25] may act as a barrier for invasion of ingested microorganisms[26]. Chitin is a key structural component of the PM[27].

The ethylene glycol chitin which is a mono-hydroxyethyl ether of chitin in which only the primary hydroxyl groups

of chitin were selectively glycolated, are prepared by the glycolation of chitin with ethylene oxide[13]. In this paper we have demonstrated that the chitinolytic activity of a 6 x His-tagged recombinant *H. longicornis* CHT1 protein expressed in Sf 9 insect cells infected with AcMNPV/CHT1 was assayed effectively by using ethylene glycol chitin as substrate. It was indicated that rCHT1 protein had apparent endochitinase activity being similar activity to *Serratia marcescs* chitinase (Fig 1). The similar results have been also reported in some chitinases[14,28]. These results imply that the rCHT1 expressed in Sf 9 insect cells infected with AcMNPV/CHT1 has a function of chitinase. Establishment of more simple and sensitive assay system for measurement of specific enzyme activity of rCHT1 originated from tick has been recognized very important for identifying potential antigens for biological defense and vaccine development.

By the assay system designed in this work, the chitinolytic activities of purified rCHT1 were increased by time course (Fig 3) and dose-dependent manner (Fig 4), indicating that the enzymatic activity of *H. longocornis* rCHT1 depends on the protein concentration used and reactive time. The optimal concentration of protein for the assay was determined as 0.1μg in 10$\mu \ell$. According to the experiments on the effects of temperatures on the enzymatic activity of rCHT1, it was evident that the rCHT1 was most active at the temperature at 37℃, and less active in 4℃, 25℃ and 48℃. Previously, we have reported the similar results using 4MU-(GlcNAc)3 as substrate[15]. The purified rCHT1 showed a maximum activity at 45℃ and unstable around 50℃ in the liquid phases[15]. These results indicate that the activity of *H. longocornis* rCHT1 is unstable around 48℃. In the experiments on the effects of pH of the buffers, the chitinolytic activity of rCHT1 marked maximum activity in pH 5 to 7 (Fig 5), and weak activity in acidic (pH<5) and alkalic (pH>7) media. The similar results have been reported in the chitinases originated from *Spodoptera litura*[28] and *Maduca sexta*[12], indicating that *H. longocornis* rCHT1 is unstable under acidic and alkaline conditions .

In this study, we have established a simple and sensitive assay system for determination of chitinolytic enzymes from the *H. longicornis* CHT1 using ethylene glycol chitin as substrate under solid phase. It was assumed that the designed assay technique could be simpler and more straightforward methods than the other assays for chitinases. And by using this assay we could also conclude that the rCHT1 expressed in AcMNPV/CHT1-infected Sf 9 cells hydrolyzed ethylene glycol chitin, being functionally active.

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