Journal of Marine Science and Technology

Volume 20 | Issue 4 Article 4

FRAGMENTATION AND CHARACTERISTICS OF RECOMBINANT ENDOCHITINASE EXPRESSED IN ESCHERICHIA COLI

Wei-Ming Chen
Department of Food Science, National Taiwan Ocean University, Keelung, Taiwan, R.O.C. Taiwan Agricultural Chemicals and Toxic Substances Research Institute, Taichung, Taiwan, R.O.C.

Gen-Hung Chen
Department of Cosmetic Science, Providence University, Taichung, Taiwan, R.O.C.

Shann-Tzong Jiang
Department of Food Science, National Taiwan Ocean University, Keelung, Taiwan, R.O.C. Department of Food and Nutrition, Providence University, Taichung, Taiwan, R.O.C., stjiang@pu.edu.tw

Journal of Marine Science and Technology, Vol. 20, No. 4, pp. 369-375 (2012)
DOI: 10.6119/JMST-011-0225-1

FRAGMENTATION AND CHARACTERISTICS OF RECOMBINANT ENDOCHITINASE EXPRESSED IN *ESCHERICHIA COLI*

Wei-Ming Chen[1,2], Gen-Hung Chen[3], and Shann-Tzong Jiang[1,4]

Key words: expression, endochitinase, *Escherichia coli*, proteolysis.

ABSTRACT

A recombinant endochitinase fused with hexahistidine (rEC-H) was expressed and characterized. After Ni affinity chromatography, the recovery, purification-fold and specific activity of rEC-H were 88.8%, 13.4 and 142.1 U/mg, respectively. The purified rEC-H had optimal pH and temperature at pH 7.5 and 60°C, respectively and was stable at pH 4.0-9.0 and <50°C. It was activated by Ca^{2+}, Sr^{2+}, Ba^{2+}, Co^{2+} and β-mercaptoethanol, but highly inhibited by Cu^{2+}, Hg^{2+} and SDS. The thiol group may play an important role on rEC-H and rEC-H may easily be destructed when hydrophobic interaction is disrupted by sodium dodecyl sulfate. The molecular mass was lower than that of expected due to cleavage at 2 specific sites, ^{33}Ala-^{34}Asp and ^{472}Glu-^{473}Leu within rEC-H, suggesting the fragmentation of rEC-H occurred during expression.

I. INTRODUCTION

Chitin, the polymer of β-1,4-linked *N*-acetyl-*D*-glucosamine, is one of the major components in the cell walls of fungi and exoskeleton of arthropod. It is the second abundant and renewable polysaccharide in the world. Marine waste, such as shells of prawn and crab, is the most important sources of chitin. However, the waste is traditionally utilized in low-economic products, for instance, fish silage and pet foods [11]. Chitin is chemically and biochemically stable as well as biocompatible and non-allergenic [25]. Base on the properties, chitin can be applied on biomaterials for cell attachment [14, 26] and bone formation accelerator [17], though it may need to be degraded or modified. In addition, chitin and its derivatives, which under specific degree of polymerization (DP), play an critical role on pharmaceutical delivery [16]. Furthermore, they have been applied on antitumor, immunoadjuvant, hypolipidemic, hemostatic, antimicrobial, acceleration of wound-healing, and growth of probiotics [1, 18].

Chitinases (EC 3.2.1.14) are mild and efficient in degrading chitin into chito-oligosaccharide or *N*-acetyl-*D*-glucosamine. It has been detected in most of organisms, even those without chitin structure [29]. Chitinases in vertebrate are considered to be associated with immune system. Acidic mammalian chitinases were observed in asthmatic patients that were referred to inflammation and interleukin pathway [38]. Chitinases from plants were utilized for protecting from pathogenic fungi or insects' attacks [15]. In agriculture, chitinases has been well studied and developed for biocontrol against fungi [23, 37] and insects [7, 8]. Accordingly, investigation of the properties and functions of chitinases from various species is very important and also necessary on the applications in many fields.

Many studies on proteins or enzymes depend on expressing a cloned gene product in bacterial cells. Because expression vectors with strong promoters had been well studied and *Escherichia coli* is considered to be an ideal model system, it is wildly employed for fast expressing recombinant proteins though the misfolding or inclusion body and following digestion may occur during expression [3]. In this study, we expressed the hexahistidine fused rEC (rEC-H) in *E. coli*. The difference of molecular mass (Mr) was observed. We identified the specific proteolytic sites for clarifying the changes in Mr of target protein and determined the characteristics of rEC-H.

II. MATERIALS AND METHODS

1. Materials

Bacillus cereus BCRC 10603 was obtained from the Bioresources Collection and Research Center (BCRC), Food Industry Research and Development Inst., Hsinchu, Taiwan. *Escherichia coli* Top10F' was purchased from Invitrogen Co. (Carlsbad, Ca, USA) and *E. coli* AD494(DE3)pLysS was from Novagen Inc. (Darmstadt, Germany). All media for bacteria cultivation were obtained from Difco Laboratories

Paper submitted 09/02/10; revised 01/03/11; accepted 02/25/11. Author for correspondence: Shann-Tzong Jiang (e-mail: stjiang@pu.edu.tw).
[1] *Department of Food Science, National Taiwan Ocean University, Keelung, Taiwan, R.O.C.*
[2] *Taiwan Agricultural Chemicals and Toxic Substances Research Institute, Taichung, Taiwan, R.O.C.*
[3] *Department of Cosmetic Science, Providence University, Taichung, Taiwan, R.O.C.*
[4] *Department of Food and Nutrition, Providence University, Taichung, Taiwan, R.O.C.*

(BD Co., MD, USA). The synthesis of ligonucleotide primers and amino acid N-terminal sequencing were serviced by Mission Biotech Inc. (Taipei, Taiwan). ExSel, high fidelity DNA polymerase, was the product of JMR Holdings (JMR Holdings, Inc., London, UK). T4 DNA ligase, pGEM-T Easy Vector, protein markers and all restriction enzymes were obtained from Promega Co. (Madison, WI, USA), while the pET23a(+) vector was purchased from Novagen (Novagen Inc., Darmstadt, Germany). Chitin, X-Gal, Isopropyl β-D-thiogalactopyranoside (IPTG), ampicillin, kanamycin, chloramphenicol and other chemicals were the products of Sigma-Aldrich Inc.. The resins Ni Sepharose Fast Flow was purchased from Amersham Biosciences (GE Healthcare Bio-Sciences Corp., MA, USA).

2. Isolation of Genomic DNA

The genomic DNA was isolated according to Sambrook and Russel [31]. Aliquots of 1.5~3.0 ml of *Bacillus cereus* BCRC 10603 cells, incubated in Tryptic Soy Broth (TSB) overnight, was re-suspended in 50 μl STES buffer (containing 10 mM EDTA, 0.1% SDS, 0.5 M NaCl and 0.2 M Tris-HCl, pH 7.6). To the re-suspended sample, 0.3 g acid-washed glass beads (dia. 0.4 mm) with 20 μl TE buffer (Tris-HCl buffer with EDTA, containing 20 mM Tris-HCl and 5 mM EDTA, pH 7.6) and 60 μl phenol-chloroform (1:1, v/v) were added and vortexed violently to grind the bacterial cells. After 15 min centrifugation at 15,000 ×g, 0°C, the supernatant was then transferred to another clean microfuge tube containing 1.5 × volume of ethanol. The DNA pellet was collected after 15 min centrifugation at 15,000 ×g and 0°C. The precipitated DNA pellet was rinsed with 70% ethanol and stored in 70% ethanol at -20°C. It was dried prior to use and re-dissolve in 40 μl TE buffer (pH 7.6) for the further cloning.

3. Molecular Cloning with pGEM-T Easy Vector

The genomic DNA of *Bacillus cereus* BCRC 10603 was used as template. Primers were designed according to Ivanova *et al*. [13]. The sense primer was with oligonucleotide sequences of 5'-(ATG GCT AGC TCT CAA AAA TTC ACA CTG CTA TTA)-3', while the antisense primer was with oligonucleotide sequences of 5'-(GTG CTC GAG GTT TTC GCT AAT GAC GGT ATT TAA AAG)-3'. The underlined sequences in sense and antisense primers were recognition sites for restriction enzymes (*Nhe*I and *Xho*I, respectively). Amplification using ExSel high fidelity DNA polymerase was performed by using polymerase chain reaction (PCR) for 25 cycles (denaturation: 95°C for 30 s, annealing: 50°C for 45 s, and extension: 72°C for 90 s) in a DNA thermal cycler (2720 Thermal Cycler, Applied Biosystems). The PCR product was ligated onto pGEM-T easy vector and transformed to *E. coli* Top 10F'. Transformants were selected according to antibiotics resistance.

4. Construction of pET-23a(+)-Endochitinase Expression Vector

After blue/white selection and sequencing, the endochitinase gene fragment on pGEM-T easy vector was digested by *Nhe*I and *Xho*I. After agarose gel electrophoresis, the endochitinase gene fragment was isolated and eluted prior to the ligation with pET-23a(+) by T4 DNA ligase. After being transformed to *E. coli* Top 10F' and mass production, the ligated pET-23a(+)-*endochitinase* was transformed to *E. coli* AD494(DE3)pLysS expression host. The transformants were selected with LB agar (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl, and 15 g/l agar) containing 100 μg/ml ampicillin, 15 μg/ml kanamycin, and 34 μg/ml chloramphenicol. The bacterial colonies resistant to antibiotics were selected for protein expression.

5. Cultivation of *E. coli* Transformant and Extraction of Crude Enzyme

The *E. coli* AD494(DE3)pLysS transformant was cultivated in 50 ml LB broth containing 100 μg/ml ampicillin, 15 μg/ml kanamycin and 34 μg/ml chloramphenicol at 37°C for 16 hr with 200 rpm shaking. Fifty ml of transformant were inoculated into 1.0 l LB broth with 3 antibiotics under the same condition for 3 hr to refresh. When the absorbance at 600 nm (OD_{600}) reached 0.6, IPTG was added to a final concentration of 0.1 mM for inducing protein expression. After 3 hr IPTG induction, the bacterial cells were collected by centrifugation at 4000 ×g for 30 min. The harvested cells were then re-suspended in 30 ml Buffer A (TA buffer, 20 mM Tris-acetate buffer, pH 7.5) and sonicated (200 cycles with pulse on: 5s and pulse off: 15s).

6. Purification Procedure

The crude enzyme was purified by Ni sepharose Fast Flow affinity chromatography. The pH of crude enzyme (30 ml) extracted from cytoplasm of *E. coli* AD494(DE3)pLysS was adjusted to 7.5 by using 0.2 M Tris-acetate buffer. The resulted sample was chromatographed on a Ni sepharose Fast Flow (2.6 cm × 5 cm), which was pre-equilibrated with Buffer A. Elution was performed using a linear gradient of 0 to 0.5 M imidazol in Buffer A at the flow rate of 1 ml/min. Fraction of 3 ml/tube was collected.

7. Preparation of Colloidal Chitin

Colloidal chitin was prepared by HCl swelling [30]. About 10 g chitin from crab shell was suspended in concentrate HCl (200 ml) and stirred violently at 4°C for 24 h. The resulted chitin suspension was mixed with 2 l ethanol at 4°C with rapid stirring overnight and then centrifuged at 1000 ×g for 20 min. Chitin pellet was washed with cold water until the pH was neutral. The washed sample was freeze-dried and stored at room temperature until use.

8. Assay of Chitinase Activity

One % colloidal chitin suspended in 0.1 M phosphate buffer (pH 7.0) was employed as substrate for chitinase activity assay. To 0.5 ml of colloidal chitin suspension, 0.5 ml enzyme solu-

tion was added and incubated at 50°C for 30 min with violent shaking. One ml of 2,4-dinitrosalicylic acid (DNS) reagent (containing 0.5 g/l DNS, 8 g/l NaOH and 150 g/l sodium, potassium tartarate) was added to stop the reaction and boiled for 5 min to develop color [24]. After cooling in ice water for 5 min, samples were diluted with 8 ml distilled water and measured the absorbance at 540 nm. One unit (U) of chitinase activity was defined as the amount of enzyme that can hydrolyze colloidal chitin and release 1 μg N-acetyl glucosamine equivalent within 1 min at 50°C

9. Determination of Protein Concentration

Protein concentration was determined by Lowry method. Bovine serum albumin was used as standard protein.

10. SDS-PAGE Analysis

The profiles of protein contents during purification were observed by SDS-PAGE. Enzyme samples were mixed with dissociation buffer (62.5 mM Tris-HCl buffer, pH 6.8, containing 2% SDS, 5% β-mercaptoethanol and 0.01% bromophenol blue) and heated at 95°C for 3 min. The resulting samples were analyzed by SDS-PAGE using 12.5% polyacrylamide gel [10] and then stained by Coomassie Brilliant Blue G-250 [27]. Broad molecular weight protein kit containing 10, 15, 25, 35, 50, 75, 100, 150 and 225 kDa standard proteins was employed as markers.

11. Optimal pH and pH Stability

The optimal pH for enzymatic activity of recombinant endochitinase was determined in various buffers with pH 3.5-6.05 (50 mM sodium acetate buffer), pH 6.0-8.0 (50 mM sodium phosphate buffer) and pH 8.0-9.5 (50 mM Tris-HCl buffer) [4].

The pH stability of recombinant endochitinase was measured after 30 min incubation in various buffers as mentioned above at 37°C. After incubation, equal volume of 0.2 M sodium phosphate buffer (pH 7.0) was added, and the residual activity was measured according to Miller [24].

12. Optimal Temperature and Thermal Stability

The optimal temperature of recombinant endochitinase was determined at temperatures between 30 and 70°C in 50 mM sodium phosphate buffer (pH 7.0). For the determination of thermal stability, the recombinant endochitinase in 50 mM sodium phosphate buffer (pH 7.0) was incubated at various temperatures (30-60°C) for 30 min [4]. After cooling in ice water for 30 min, the residual activity was measured according to Miller [24].

13. Effect of Metal ions and Chemicals

Purified recombinant endochitinase in 50 mM sodium acetate buffer (pH 5.5) with 5.0 mM of various metal ions (Li^+, Na^+, Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}, Cd^{2+}, Cr^{3+}, Mn^{2+}, Co^{2+}, Cu^{2+}, Hg^{2+}, Ni^{2+}, Zn^{2+}) and chemicals (β-mercaptoethanol, SDS, urea, EDTA) were incubated individually at 37°C for 30 min [4].

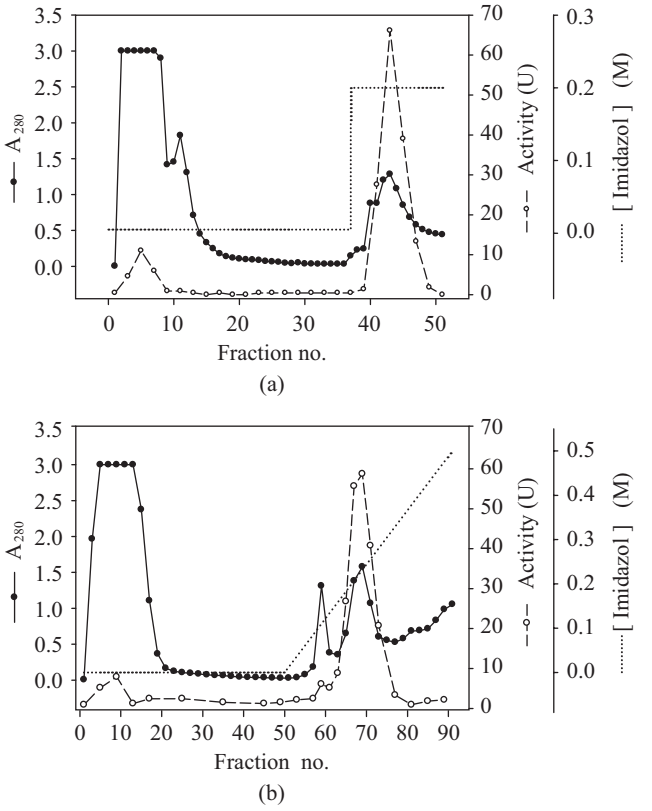


Fig. 1. Chromatogram of rEC-H on Ni Sepharose FF affinity chromatography. (a) one-step elution by using 0.2 M imidazol and (b) gradient elution by using 0-0.5 M imidazol.

After incubation, the residual activity was measured according to Miller [24].

III. RESULTS AND DISCUSSION

1. Expression and Purification of the rEC-H in *E. coli*

The constructed expression vector was transformed into *E. coli* AD494(DE3)pLysS expression host, and the transformant was then induced by IPTG at 37°C for 3 h. The cell of *E. coli* transformant was collected and then lysed using sonication. The main of hexahistidine fused endochitinase (rEC-H) was observed in the soluble cell lysate fraction, not in insoluble inclusion body (data not shown). The soluble *E. coli* cell lysate was collected as crude enzyme for the further purification.

The crude enzyme with a specific activity of 10.6 U/mg was applied on Ni Sepharose Fast Flow. Under one-step elution by 0.2 M imidazol, an A_{280} peak with chitinase activity was collected and analyzed. Although protein peak and enzyme activity fit each other with high correlation on the chromatogram (Fig. 1(a)), two major bands in SDS-PAGE, at the positions approximately of 69 kDa and 22 kDa, were observed (Fig. 2, lane 2). The N-terminal sequence of higher molecular weight protein is Asp-Ser-Pro-Lys-Gln-Ser-Gln-Lys, while the lower molecular weight one is Leu-Leu-Gly-Gly-Pro-Ile-Ser-Gln

Table 1. Summary of purification of rEC-H expressed in *E. coli* AD494(DE3)pLysS.

Purification procedure	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Recovery (%)	Purification fold
Crude enzyme	94.6	999.2	10.6	100	1
Ni Sepharose FF	6.24	886.9	142.1	88.8	13.4

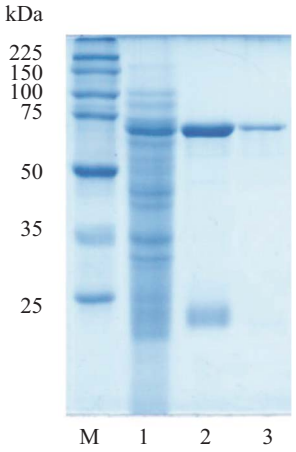
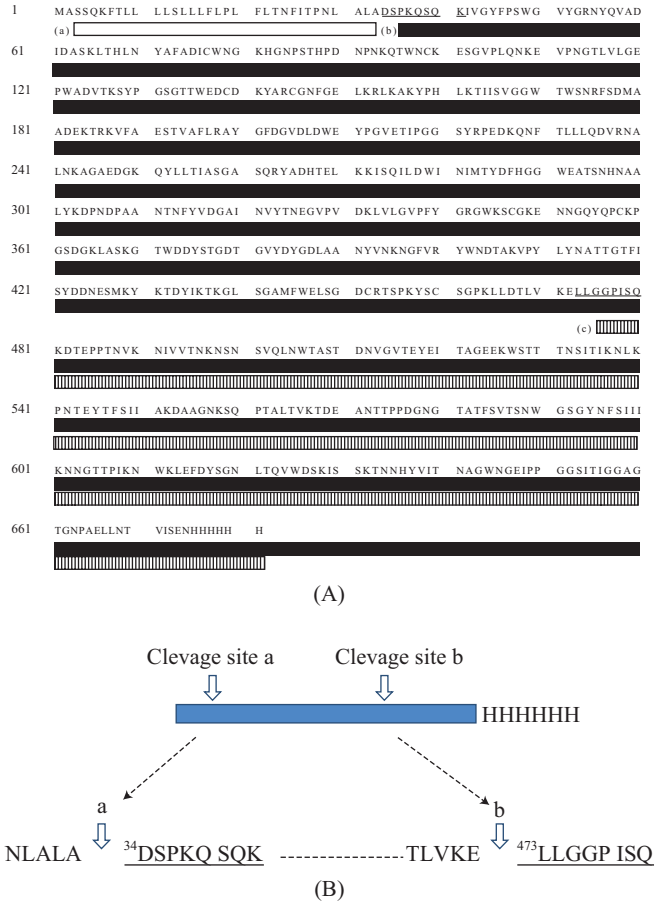


Fig. 2. SDS-PAGE profile of rEC-H during purification (M, marker; lane 1, crude enzyme; lane 2, the partial purified enzyme which collected from one-step elution on Ni affinity chromatography; lane 3. Purified rEC-H collected from gradient elution on Ni affinity chromatography).



(A)

(B)

Fig. 3. Protein sequence encoded by the cloned rEC-H gene. (A) Full protein sequence encoded. The underline sequences, DSPKQSQK and LLGGPISQ, are the results of N-terminal sequencing from Fig. 2, lane 2. And sequence (b) and (c) were polypeptides inferred from full cloned sequence and the results of N-terminal sequencing. (B) Two putative cleavage sites of rEC-H expressed in *E. coli*.

(Fig. 3(A)). Both sequences were encoded from the DNA sequence we cloned.

The rEC-H was purified to electrophoretic homogeneity with a specific activity of 142.1 U/mg after Ni affinity chromatography eluted by a linear gradient of 5 mM-500 mM imidazol (Fig. 1(b) and Fig. 2, lane 3). The recovery and purification fold were 88.8% and 13.4, respectively (Table 1).

Base on well studied of strong promoter and regulation on protein expression, *E. coli* was extensively utilized as expression host to produce recombinant proteins, and the expressed proteins were regularly matched with predicted Mr in most of reports. By using Ni affinity chromatography, the rEC-H could be ensured if no changes occurred in C-terminal of rEC-H. According to the data obtained in this study, rEC-H could attach to Ni affinity resin and the Mr of purified rEC-H was lower than that of expection (Fig. 2). However, an additional protein band was observed at the position of 22 kDa on SDS-PAGE. Since affinity chromatography has high specificity to His-tag, the 22-kDa protein should be derived from rEC-H.

The N-terminal sequences of 2 protein bands in Fig. 2 were identified and confirmed that they are derived from our target protein. Two cleavage sites, ^{33}Ala−^{34}Asp and ^{472}Glu−^{473}Leu, were recognized (Fig. 3(B)). According to the model of chitinase [29], the 33 amino acids leading in N-terminal are signal peptides, which can be recognized by *E. coli*, and the shortened protein is similar to chiCW [12, 20]. Therefore, these 2 cleavage sites were completely assured, since no uncut recombinant protein was observed. Degradation of insoluble or misfolding protein, inclusion body, in *E. coli* was frequently reported [3]. The cleavage site b, ^{472}Glu−^{473}Leu, indicated the cutting site was specific for Cathepsin G and Chymotrypsin C [6, 9]. However, the activity of degraded rEC-H was still left and also soluble form. This phenomenon might be due to the consumption of nutrients during expression of heterogeneous proteins and, consequently decreased

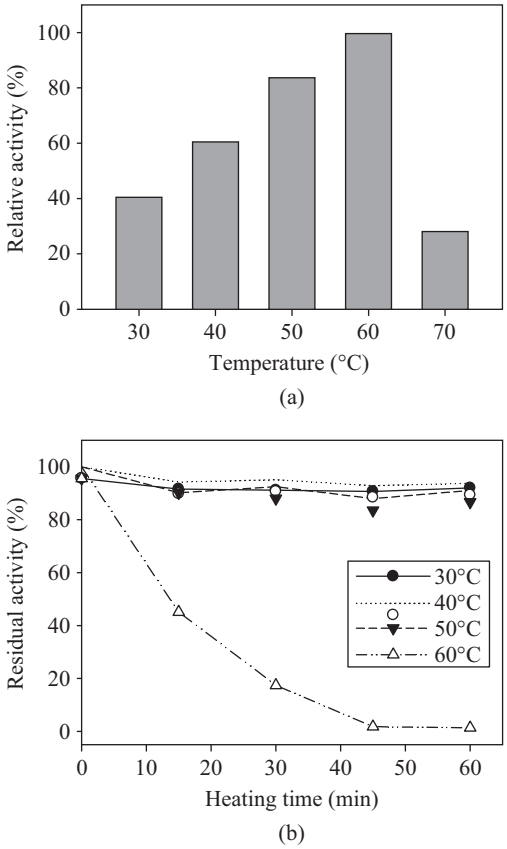


Fig. 4. Effect of temperature on rEC-H. (a) optimal reaction temperature. The activity of rEC-H was determined at various temperatures, and the highest one was defined as 100%; (b) thermostability of rEC-H. The residual activity of rEC-H was determined after 0-60 min incubation at 30, 40, 50 and 60°C.

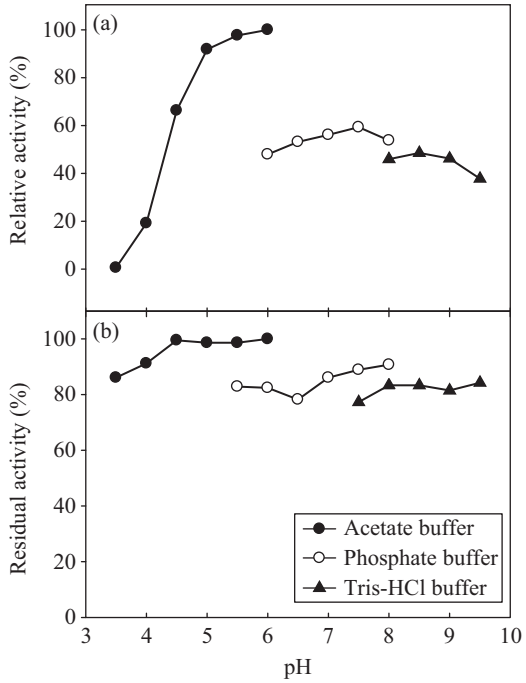


Fig. 5. Effect of pH on rEC-H. (a) optimal pH. The activity was determined at pH 3.5-9.5; (b) pH stability of rEC-H. The residual activity of rEC-H was determined after 30 min incubation at 37°C and pH 3.5-9.5. The highest activity was defined as 100%.

the growth of *E. coli*. Proteolytic system in cytoplasm was, therefore, developed to recycle the heterogeneous proteins and ensure the normal growth of cell [2].

Considering the proposed model that functions of chitinase sequence usually containing leading sequence in the front, catalytic domain in the middle, and chitin-binding domain in the tail [29]. We supposed that the catalytic domain of rEC-H was between cleavage site a and b, and the chitin-binding domain was following cleavage site b. Binding domain affects the activity and property of chitinase [33]. However, no other obvious activity was observed in chromatogram in this study (Fig. 1), suggesting that catalytic and binding domains are still left on rEC-H

2. Characteristics of rEC-H

The purified rEC-H had optimal reaction pH and temperature at pH 7.5 and 60°C respectively, and was stable at pH 4.0-9.0 and <50°C (Figs. 4 and 5). It was highly inhibited by Cu^{2+}, Hg^{2+} and SDS, moderate inhibited by Mg^{2+}, Mn^{2+}, Cd^{2+}, but activated by Ca^{2+}, Sr^{2+}, Ba^{2+}, Co^{2+} and β-mercaptoethanol (Table 2).

Optimal reaction temperature of rEC-H was observed at 60°C and the activity of the rEC-H decreased sharply when the temperature was over 60°C (Fig. 4(a)). The rEC-H was stable at the temperature below 50°C, and lost 50% of activity within 15 min at 60°C (Fig. 4(b)). The optimal temperature of rEC-H is higher than those from papaya (37°C), thermophilic fungi (50°C) [21], *Bombyx mori* (50°C) [22], *Sanguibacter antarcticus* (37°C) [19], *Serratia ureilytica* (50°C) [35] and *Chaetomium cupreum* (45°C) [36]; however, the thermal stability of rEC-H is lower than that from thermophilic fungi [21].

The optimal pH of purified rEC-H was 7.5 (Fig. 5), which was higher than those from *Serratia ureilytica* (pH 6) [35], *Bombyx mori* (pH 7.0) [22], and *Avena sativa* (pH 7.0) [32], but similar to that from *Sanguibacter antarcticus* (pH 7.6) [19]. The highest activity was observed at pH 6.0 in acetate buffer. However, enzyme activity is somewhat affected by buffers. Similar phenomenon was also observed on the pH stability data (Fig. 5(b)). Therefore, the breaking point at pH 7.5 in phosphate buffer could be considered to be optimal pH (Fig. 5(a)).

The rEC-H was highly inhibited by Cu^{2+} and Hg^{2+}, indicating that it was very sensitive to oxidation and thiol group on rEC-H played a key role on its activity. Addition of 5 mM β-Me activated the activity of rEC-H up to 157% (Table 2), suggesting that antioxidants could not only protect rEC-H from oxidation, but recover the thiol group. The properties of rEC-H, in this study, were similar to those of chitinases from *Chaetomium cupreum* [36] and *Serratia* sp. [34]. No

Table 2. Effect of metal ions and chemicals on rEC-H.

		Relative activity (%)
Metals*	None	100.0**
	Li^{+}	103.7
	Na^{+}	114.0
	Mg^{2+}	84.1
	Ca^{2+}	131.8
	Sr^{2+}	124.3
	Ba^{2+}	123.4
	Mn^{2+}	62.6
	Co^{2+}	132.7
	Ni^{2+}	103.7
	Cu^{2+}	19.6
	Zn^{2+}	96.3
	Cd^{2+}	86.0
	Hg^{2+}	1.9
	Cr^{3+}	92.5
Chemicals*	EDTA	104.7
	Urea	108.4
	β-Me	157.0
	SDS	3.7

* The final concentration of reagents was 5 mM; EDTA: Ethylenedinitro tetraacetic acid disodium; SDS: Sodium dodecyl sulfate; β-Me: 2-Mercaptoethanol.

** The activity of rEC-H without any metal ion or chemical was defined as 100%.

significant effect of ENTA on rEC-H suggested it was independent on metal ions. However, Ca^{2+}, Sr^{2+}, Ba^{2+}, and Co^{2+} enhanced its activity. This is similar to Chi32 [28], but quite different from that from *Chaetomium cupreum* [36].

The rEC-H was stable in 5 mM urea, but lost its activity dramatically in 5 mM SDS. This phenomenon indicated that hydrophobic structure involved in rEC-H may be destructed, which consequently made it unstable in SDS [5]. Therefore, it would not suggest applying the rEC-H using in detergent formula.

In summary, rEC-H can be functionally expressed in *E. coli*. Two specific cleavage sites reported here would be novel. The cleavage on expressed protein not only observed in insoluble inclusion body [3], but also in soluble protein. According to the data provided, some techniques such as changing the specific sequences by protein engineering can be preventing the rEC-H from auto-lysis during expression in host cell, which is helpful for the further application of rEC-H.

REFERENCES

1. Aytekin, O. and Elibol, M., "Cocultivation of *Lactococcus lactis* and *Teredinobacter turnirae* for biological chitin extraction from prawn waste," *Bioprocess and Biosystems Engineering*, Vol. 33, No. 3, pp. 393-399 (2010).
2. Baneyx, F. and Georgiou, G., "Construction and characterization of *Escherichia coli* strains deficient in multiple secreted proteases: protease III degrades high-molecular-weight substrates *in vivo*," *Journal of Bacteriology*, Vol. 173, No. 8, pp. 2696-2703 (1991).
3. Baneyx, F. and Mujacic, M., "Recombinant protein folding and misfolding in *Escherichia coli*," *Nature Biotechnology*, Vol. 22, No. 11, pp. 1399-1408 (2004).
4. Bernfeld, P., "Amylase, α and β," In: Colowick, S. P. and Kaplan, N. O. (Eds.), *Methods in Enzymology*, Academic Press, New York, pp. 149-158 (1995).
5. Bhuyan, A. K., "On the mechanism of SDS-induced protein denaturation," *Biopolymers*, Vol. 92, No. 2, pp. 186-199 (2009).
6. Blow, A. M. and Barrett, A. J., "Actio of human cathepsin G on the oxidized B chain of insulin," *Biochemical Journal*, Vol. 161, No. 1, pp. 17-19 (1977).
7. Boldo, J. T., Junges, A., do Amaral, K. B., Staats, C. C., Vainstein, M. H., and Schrank, A., "Endochitinase CHI2 of the biocontrol fungus *Metarhizium anisopliae* affects its virulence toward the cotton stainer bug *Dysdercus peruvianus*," *Current Genetics*, Vol. 55, No. 5, pp. 551-560 (2009).
8. Fan, Y., Fang, W., Guo, S., Pei, X., Zhang, Y., Xiao, Y., Li, D., Jin, K., Bidochka, M. J., and Pei, Y., "Increased insect virulence in *Beauveria bassiana* strains overexpressing an engineered chitinase," *Applied and Environmental Microbiology*, Vol. 73, No. 1, pp. 295-302 (2007).
9. Folk, J. E. and Cole, P. W., "Chymotrypsin C: II. enzymatic specificity toward several polypeptides," *Journal of Biological Chemistry*, Vol. 240, No. 1, pp. 193-197 (1965).
10. Hames, B. D., "An introduction to polyacrylamide gel electrophoresis of proteins: A Practical Approach," In: Hames, B. D. and Rickwood, D. (Eds.), *Gel Electrophoresis of Proteins*, Oxiford University Press, New York, pp. 1-91 (1990).
11. Hayes, M., Carney, B., Slater, J., and Bruck, W., "Mining marine shellfish wastes for bioactive molecules: chitin and chitosan - Part A: extraction methods," *Biotechnology Journal*, Vol. 3, No. 7, pp. 871-877 (2008).
12. Huang, C. J., Wang, T. K., Chung, S. C., and Chen, C. Y., "Identification of an antifungal chitinase from a potential biocontrol agent, *Bacillus cereus* 28-9," *Journal of Biochemistry and Molecular Biology*, Vol. 38, No. 1, pp. 82-88 (2005).
13. Ivanova, N., Sorokin, A., Anderson, I., Galleron, N., Candelon, B., Kapatral, V., Bhattacharyya, A., Reznik, G., Mikhailova, N., Lapidus, A., Chu, L., Mazur, M., Goltsman, E., Larsen, N., D'Souza, M., Walunas, T., Grechkin, Y., Pusch, G., Haselkorn, R., Fonstein, M., Ehrlich, S. D., Overbeek, R., and Kyrpides, N., "Genome sequence of *Bacillus cereus* and comparative analysis with *Bacillus anthracis*," *Nature*, Vol. 423, pp. 87-91 (2003).
14. Jayakumar, R., Divya Rani, V. V., Shalumon, K. T., Kumar, P. T., Nair, S. V., Furuike, T., and Tamura, H., "Bioactive and osteoblast cell attachment studies of novel alpha- and beta-chitin membranes for tissue-engineering applications," *International Journal of Biological Macromolecules*, Vol. 45, No. 3, pp. 260-264 (2009).
15. Karasuda, S., Tanaka, S., Kajihara, H., Yamamoto, Y., and Koga, D., "Plant chitinase as a possible biocontrol agent for use instead of chemical fungicides," *Bioscience, Biotechnology, and Biochemistry*, Vol. 67, pp. 221-224 (2003).
16. Kato, Y., Onishi, H., and Machida, Y., "Application of chitin and chitosan derivatives in the pharmaceutical field," *Current Pharmaceutical Biotechnology*, Vol. 4, No. 5, pp. 303-309 (2003).
17. Kawai, T., Yamada, T., Yasukawa, A., Koyama, Y., Muneta, T., and Takakuda, K., "Biological fixation of fibrous materials to bone using chitin/chitosan as a bone formation accelerator," *Journal of Biomedical Materials Research Part B: Applied Biomaterials*, Vol. 88, No. 1, pp. 264-270 (2009).
18. Kurita, K., "Chemistry and application of chitin and chitosan," *Polymer Degradation and Stability*, Vol. 59, pp. 117-120 (1998).
19. Lee, S. G., Koh, H. Y., Han, S. J., Park, H., Na, D. C., Kim, I. C., Lee,

H. K., and Yim, J. H., "Expression of recombinant endochitinase from the Antarctic bacterium, *Sanguibacter antarcticus* KOPRI 21702 in *Pichia pastoris* by codon optimization," *Protein Expression and Purification* Vol. 71, No. 1, pp. 108-114 (2010).
20. Lee, Y. S., Park, I. H., Yoo, J. S., Chung, S. Y., Lee, Y. C., Cho, Y. S., Ahn, S. C., Kim, C. M., and Choi, Y. L., "Cloning, purification, and characterization of chitinase from *Bacillus* sp. DAU101," *Bioresource Technology*, Vol. 98, No. 14, pp. 2734-2741 (2007).
21. Li, A. N., Yu, K., Liu, H. Q., Zhang, J., Li, H., and Li, D. C., "Two novel thermostable chitinase genes from thermophilic fungi: cloning, expression and characterization," *Bioresource Technology*, Vol. 101, No. 14, pp. 5546-5551 (2010).
22. Li, X. H., Wang, D., Zhou, F., Yang, H. J., Bhaskar, R., Hu, J. B., Sun, C. G., and Miao, Y. G., "Cloning and expression of a cellulase gene in the silkworm, *Bombyx mori* by improved Bac-to-Bac/BmNPV baculovirus expression system," *Molecular Biology Reports*, Vol. 37, No. 8, pp. 3721-3728 (2010).
23. Mamarabadi, M., Jensen, B., and Lubeck, M., "Three endochitinase-encoding genes identified in the biocontrol fungus *Clonostachys rosea* are differentially expressed," *Current Genetics*, Vol. 54, No. 2, pp. 57-70 (2008).
24. Miller, G. L., "Use dinitrosalicylic acid reagent for determination of reducing sugar," *Analytical Chemistry*, Vol. 31, pp. 426-428 (1959).
25. Muzzarelli, R. A., "Chitins and chitosans as immunoadjuvants and non-allergenic drug carriers," *Marine Drugs*, Vol. 8, No. 2, pp. 292-312 (2010).
26. Nagahama, H., Rani, V. V., Shalumon, K. T., Jayakumar, R., Nair, S. V., Koiwa, S., Furuike, T., and Tamura, H., "Preparation, characterization, bioactive and cell attachment studies of alpha-chitin/gelatin composite membranes," *International Journal of Biological Macromolecules*, Vol. 44, No. 4, pp. 333-337 (2009).
27. Neuhoff, V., Arold, N., Taube, D., and Ehrhardt, W., "Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250," *Electrophoresis*, Vol. 9, pp. 255-262 (1988).
28. Nguyen, V. N., Oh, I. J., Kim, Y. J., Kim, K. Y., Kim, Y. C., and Park, R. D., "Purification and characterization of chitinases from *Paecilomyces variotii* DG-3 parasitizing on *Meloidogyne incognita* eggs," *Journal of Industrial Microbiology and Biotechnology*, Vol. 36, No. 2, pp. 195-203 (2009).
29. Patil, R. S., Ghormade, V. V., and Deshpande, M. V., "Chitinolytic enzymes: an exploration," *Enzyme and Microbial Technology*, Vol. 26, No. 7, pp. 473-483 (2000).
30. Roberts, W. K. and Selitrennikoff, C. P., "Plant and bacterial chitinases differ in antifungal activity," *Journal of General Microbiology*, Vol. 134, pp. 169-176 (1988).
31. Sambrook, J. and Russel, D. W., *Molecular Cloning, a Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York (2001).
32. Sorensen, H. P., Madsen, L. S., Petersen, J., Andersen, J. T., Hansen, A. M., and Beck, H. C., "Oat (*Avena sativa*) seed extract as an antifungal food preservative through the catalytic activity of a highly abundant class I chitinase," *Applied Biochemistry and Biotechnology*, Vol. 160, No. 6, pp. 1573-1584 (2010).
33. Tanaka, T., Fukui, T., and Imanaka, T., "Different cleavage specificities of the dual catalytic domains in chitinase from the hyperthermophilic archaeon *Thermococcus kodakaraensis* KOD1," *Journal of Biological Chemistry*, Vol. 276, No. 38, pp. 35629-35635 (2001).
34. Wang, S. L., Li, J. Y., Liang, T. W., Hsieh, J. L., and Tseng, W. N., "Conversion of shrimp shell by using *Serratia* sp. TKU017 fermentation for the production of enzymes and antioxidants," *Journal of Microbiology and Biotechnology*, Vol. 20, No. 1, pp. 117-126 (2010).
35. Wang, S. L., Lin, C. L., Liang, T. W., Liu, K. C., and Kuo, Y. H., "Conversion of squid pen by *Serratia ureilytica* for the production of enzymes and antioxidants," *Bioresource Technology*, Vol. 100, No. 1, pp. 316-323 (2009).
36. Wang, Y. J. and Yang, Q., "Cloning and expression of a novel chitinase chi58 from *Chaetomium cupreum* in *Pichia pastoris*," *Biochemical Genetics*, Vol. 47, Nos. 7-8, pp. 547-558 (2009).
37. Xu, X., Qin, G., and Tian, S., "Effect of microbial biocontrol agents on alleviating oxidative damage of peach fruit subjected to fungal pathogen," *International Journal of Food Microbiology*, Vol. 126, Nos. 1-2, pp. 153-158 (2008).
38. Zhu, Z., Zheng, T., Homer, R. J., Kim, Y. K., Chen, N. Y., Cohn, L., Hamid, Q., and Elias, J. A., "Acidic mammalian chitinase in asthmatic Th2 inflammation and IL-13 pathway activation," *Science*, Vol. 304, No. 5677, pp. 1678-1682 (2004).