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Recombinant Expression and Enzyme Activity of Chymotrypsin-like Protease from Black Soldier Fly, *Hermetia illucens* (Diptera: Stratiomyidae)

Kwan Ho Park*, Young Cheol Choi, Sung Hee Nam, Won Tae Kim, A Young Kim, and Sin Young Kim
National Academy of Agricultural Science, RDA, Suwon 441-100, Republic of Korea

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Chymotrypsin serine protease is one of the main digestive proteases in the midgut of and is involved in various essential processes. In a previous study, a gene encoding a chymotrypsin-like protease, Hi-SP1, was cloned from the larvae of *Hermetia illucens* and characterized. In this study, we produced the recombinant chymotrypsin-like protease Hi-SP1 in *Escherichia coli* cells. The molecular weight of the recombinant Hi-SP1 was estimated to be approximately 26 kDa by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western-blotting. Chymotrypsin activity was detected when AAPF was used as the substrate. Examination of the effects of temperature and pH revealed that the proteolytic activity of recombinant Hi-SP1 decreased markedly at temperatures above 30°C, and the optimum pH was found to be 10.0.

Key words: *Hermitia illucens*, Chymotrypsin-like protease, Recombinant protease

Introduction

Chymotrypsin is a serine protease that has been extensively studied both structurally and functionally (Blow, 1971). It specifically cleaves peptide bonds on the carboxyl side of phenylalanine, tyrosine, and tryptophan residues. It is also known to play an important role in protein digestion (Balti *et al*., 2012). Much research has focused on midgut enzymes of insects, and special attention has been directed toward trypsin and chymotrypsin, which belong to the trypsin subfamily (Rawlings and Barrett, 1994). Trypsin and chymotrypsin are the major proteases found in most insect species, which take part in a number of physiological processes such as coagulation, immunity, fibrinolysis, embryonic development, and digestion (Noriega and Wells, 1999). As in mammals, insect chymotrypsins are SPs that cleave substrates at the carboxyl termini of hydrophobic amino acids such as tyrosine, tryptophan, leucine, or phenylalanine (Mazumdar-Leighton and Broadway, 2001). In insects, the most abundant and best studied group of SPs contains those expressed in the larval midgut, which are thought to be involved in the digestion of dietary protein (Herrero *et al*., 2005). The black soldier fly (BSF), *Hermetia illucens*, belongs to the polyphagous insect group (Kim *et al*., 2011). These flies can devour large amounts of garbage and food scraps in just a few hours, converting the waste into organic material that can be used as fertilizer (Diener *et al*., 2009). BSF is economically important as animal feed. The prepupae and larvae have an estimated value comparable to fishmeal can be used as specialty feed for a variety of pets, including reptiles; however, pretreatment with BSF digestive enzymes is required for their use as animal feed. In a previous study, we cloned and characterized a trypsin- and a chymotrypsin-like protease gene from BSF. The full-length cDNA of *H. illucens* chymotrypsin-like protease (Hi-SP1) is 895 bp with an open reading frame of 804 bp. The Hi-SP1 cDNA encodes a polypeptide of 267 amino acids (aa), consisting of a 16-aa signal peptide and a trypsin domain (34–263 aa) (Kim *et al*., 2011).

To the best of our knowledge, no recombinant protease of BSF has previously been purified and characterized. Here, we report the production and purification of Hi-SP1 from BSF. The recombinant protease was characterized with respect to its biochemical properties.

*To whom the correspondence addressed
Department of Agricultural Biology, National Academy of Agricultural Science, RDA, Suwon 441-100, Republic of Korea.
Tel: +82-31-290-8575; Fax: +82-31-290-8543;
E-mail: nicegano@korea.kr
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Materials and Methods

Expression and purification of recombinant chymotrypsin-like Hi-SP1 in *E. coli*

To express Hi-SP1 in *E. coli* cells (Rosetta (DE3) pLysS), Hi-SP1 cDNA was cloned into the pET-22b vector. The encoding DNA fragment was confirmed by sequencing. The Rosetta (DE3) pLysS were maintained on Luria-Bertani (LB) medium supplemented with 50 mg/ml ampicillin at 37°C with agitation. A 25 μl aliquot of the overnight culture was added to 25 ml LB medium containing 50 mg/ml of ampicillin. Protein expression was induced by adding 1 mM isopropylthio-b-galactoside (IPTG) for overnight at 37°C, when the cells reached the exponential growth [optical density (OD)600 0.6]. The *E. coli* cells were harvested by centrifugation (3000 rpm, 10 min, 4°C) and lysed by sonication in 50 ml lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole). The sonicated cells were shaking with lysozyme (0.1 mg/ml) and 0.1% triton X-100 in ice for I hr. After centrifugation (20000 rpm, 15 min), the supernatant were applied to 5 ml of beads into a Poly-Prep chromatography column (Bio-Rad Laboratories, Hercules, CA, USA) at 4°C. Proteins were eluted with 3 ml of 500 mM imidazole elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole).

SDS-PAGE and Western-blot analysis

To characterize the recombinant proteases produced by the transformants of pET-22b, SDS-PAGE was used for analysis. For SDS-PAGE analysis, the concentration of stacking gel was 7% and the concentration of separating gel was 12%. The protein bands were made visualised by staining them with Coomassie Brilliant Blue. Western-blot analysis was used to confirm the expressed recombinant Hi-SP1. Following electrophoresis, the proteins transferred to nitrocellulose transfer membranes (Schleicher & Schuell, Germany). After blotting, the membrane was blocked by incubation in 5% skimmilk blocking solution, incubated with His-probe antibody (1:10000 v/v) at room temperature for 3 hr and washed in TBS-T (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.05% (v/v) Tween-20). The membrane was conjugated with Goat antimouse IgG-HRP (1:10000 v/v) for 1 hr as secondary antibody. After repeated washing, the band visualization was achieved via chemiluminescence.

Determination of protease activity and enzyme assay

Recombinant Hi-SP1 activity was measured by zymography. Casein (Novex 12% Zymogram, Casein, Invitrogen) zymography was performed as manufacturer's instructions. Briefly, recombinant Hi-SP1 was mixed with Tris glycine SDS sample buffer and ran for 90 min at 125 V at 4°C. After electrophoresis, the gels were incubated in renaturing buffer for 30 min at room temperature, followed with incubation in developing buffer at 37°C overnight. Finally, the gel was stained with 0.1% Coomassie brilliant blue staining solution and destained in destaining solution containing 10% methanol and 5% acetic acid. Lytic band visualized by placing the gels on a view box were then photographed. Chymotrypsin activity was measured using the synthetic substrate N-succinyl-ala-ala-pro-phe-nitroanilide (AAPF). Sample contained 100 μl of 50 mM Tris-HCl buffer, pH 7.9 and 10 μl of substrate (200 mM AAPF) dissolved in DMSO. Reactions were initiated by the addition of 10 μl of recombinant Hi-SP1, and carried out at 39°C for 20 to 120 min. The rate of formation of the hydrolysis product was measured at 410 nm in a spectrophotometer. Separate control with no recombinant Hi-SP1 was carried for substrate and experimental values were corrected for background hydrolysis of the substrate. Each enzyme reaction was repeated in triplicate.

Effects of temperature and pH on the activity and stability of the recombinant Hi-SP1

The effect of temperature on the activity of recombinant Hi-SP1 was determined by incubating the recombinant enzyme between 10 and 90°C using the standard assay conditions used for the determination of the protease activity. The reaction mixture, composed of 1 mL of azocasein (2 mg/mL in 0.2 M Tris–HCl buffer, pH 7.8) and 20 μL of recombinant Hi-SP1 (640 μg/mL), was incubated at 37°C. After 1 hr, 0.25 ml of the mixture was transferred to a 1.5 mL tube containing 1 mL of 5% (w/v) trichloroacetic acid and was mixed well. The tubes were then centrifuged at 11,000 g for 5 min and the absorbance of the supernatant was measured at 450 nm. In a similar way, the effect of pH values on the recombinant Hi-SP1 was investigated by incubating the recombinant enzyme at different pH values (2.0–12.0). In various buffers with pH ranges from 2 to 12 Hi-SP1 of 20 μl (0.1 μg/μl) was added to 80 μl of 50 mM Tris–HCl buffer for pH 2–8 and 50 mM glycine-NaOH buffer for pH 10–12, respectively. After incubating for 1 hr at 37°C, the remaining protease activity (%) was measured using azocasein as a substrate. The relative activity at different pH values and temperatures was calculated when the recombinant Hi-SP1 activity obtained at the pH of 10.0 and the temperature of 10°C was used as a reference (relative activity was 100%).

Results and Discussion

Expression and purification of recombinant Hi-SP1

In order to obtain recombinant Hi-SP1 for further func-

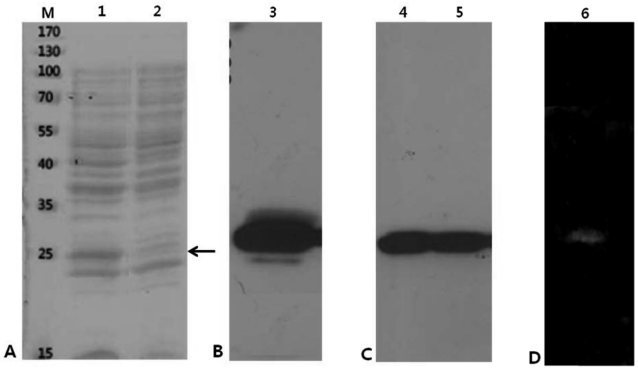

Fig. 1. SDS-PAGE analysis of expression of Hi-SP1 in *E. coli*: (A) Lane M, protein molecular weight markers; Lane 1, total protein extracts from IPTG-induced transformants harboring the plasmid pET22b; Lane 2, protein extracts from E. coli cell containing empty vector pET22b; (B) Lane 3, Western-blotting confirmation of recombinant protein with His-probe antibody and Goat antimouse IgG-HRP as second antibody; (C) Lane 4 and 5, protein extracts from IPTG -induced transformants harboring the plasmid pET22b; (D) Lane 6, zymogram of the purified recombinant Hi-SP1 chymotrypsin-like protease.

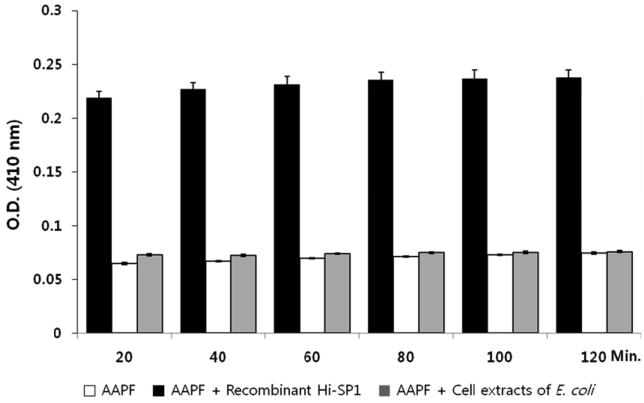

Fig. 2. Chymotrypsin activity measured in purified recombinant Hi-SP1. Activity measured using the AAPF substrate for chymotrypsin-like activity.

tional studies, we selected and *E. coli* expression system. Recombinant protein was expressed by inducing *E. coli* (Rosetta (DE3) pLysS) with 1 mM IPTG. The expected molecular mass of recombinant Hi-SP1 was approximately 25 kDa. Recombinant Hi-SP1 was purified using an Ni-NTA purification system (MCLAB, USA). Using a rather complex insect cell system, we analyzed the activity of purified Hi-SP1 toward the substrate azocasein and specific substrate AAPF. The system used here may well be applicable to other dipteran chymotrypsins, although the expression of some enzymes may be difficult to obtain a high yield without damage to the expression system. Trypsin and chymotrypsin are the major proteases in most insect species (Noriega and Wells, 1999). The estimated molecular mass of recombinant Hi-SP1, 25kDa, was similar to other mammalian and insect chymotrypsin proteinases (Gráf et al., 1998; Lee and Anstee, 1995; Terra and Ferreira, 1994). As seen in Fig. 1A, purified Hi-SP1 protein showed an estimated mobility of approximately 26 kDa, which is close to the molecular weight (25 kDa) of the mature Hi-SP1 protein, as predicted from the gene sequence (including the 6×His-tag). The estimated yield of purified recombinant Hi-SP1 was approximately 0.64 mg/l of medium. By Western-blot analysis of the proteins with a monoclonal antibody to the His-tag, we identified a specific protein band of the same size as that obtained by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE; Fig. 1B, 1C), indicating that the recombinant protein was fused with the His-tag. The recombinant proteases were purified by Ni-NTA affinity chromatography and were subjected to SDS-PAGE analysis. These results indicate a specific band with a molecular weight of approximately 26 kDa (Fig. 1B), suggesting that the purified protein was the recombinant Hi-SP1. Purified Hi-SP1 was used for analyzing the enzyme activity by the casein zymography assay using the specific substrate AAPF, and the enzyme activity was detected.

Determination of protease activity

Zymography is a sensitive and rapid assay method for analyzing protease activity, and the proteolytic activity of the recombinant Hi-SP1 protein was confirmed by this method. As shown in Fig. 1D, we observed a single clear hydrolyzed casein indicating homogeneity of the purified chymotrypsin. Chymotrypsin activity could be detected when AAPF was used as the substrate. The observed activity detected in a control experiment was less in the absence of the recombinant Hi-SP1 protein (Fig. 2).

Effects of temperature and pH on the stability of the recombinant Hi-SP1

Azocasein was used as the substrate in assays of the effects of temperature and pH on recombinant Hi-SP1 protease activity.

The results indicate that Hi-SP1 has azocaseinolytic activity within a broad range of pH values, from acidic to alkaline. The residual activity of recombinant Hi-SP1 was evaluated after heat treatment for 60 min at various temperatures, and the results are shown in Fig. 3A. Like other enzymes, the recombinant Hi-SP1 showed good stability when pre-incubated at lower temperatures (≤10°C), whereas the total protease activity did not change up to

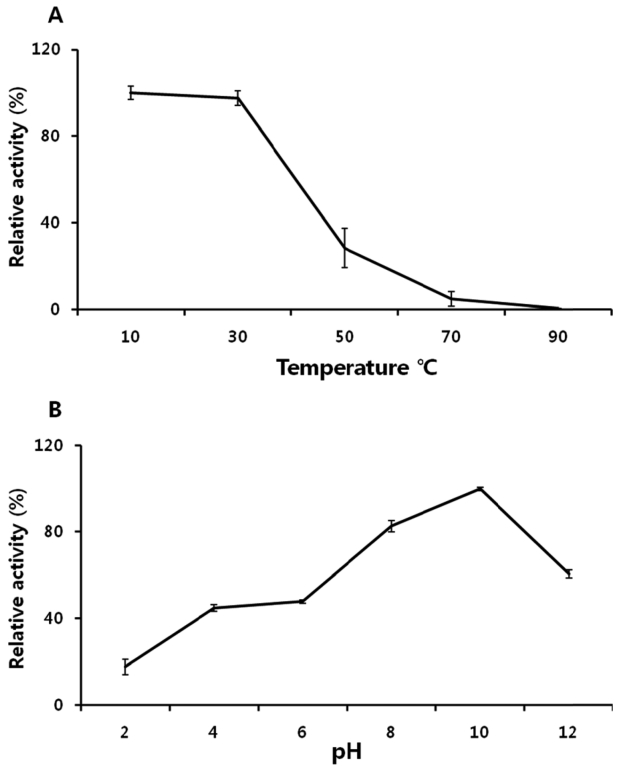

Fig. 3. Effects of temperature (A) and pH (B) on the activity of the recombinant Hi-SP1 protease assay. The effect of temperature on the stability was examined by pre-incubating the enzyme at different temperatures (10-90°C) for 1 hr and the effect of pH on the stability was examined by pre-incubating the enzyme at different pH values (2-12) for 1 hr. Three replicates of each treatment were prepared.

30°C. Further increases in temperature altered protease activity, and any further increase in the temperature decreased the enzyme activity; the lowest activity was detected at 90°C, mainly due to thermal denaturation (Klomklao *et al*., 2007). As shown in Fig. 3B, when recombinant Hi-SP1 was pre-incubated at pH of values 2–12, the optimum pH was found to be 10.0 (Fig. 3B). The total proteolytic activity of the recombinant Hi-SP1 for azocasein hydrolysis increased when the pH was increased from 6.0 to 10.0. The relatively low activity at an acidic pH was probably due to changes in charge distribution and conformation, which prevented the enzyme from proper substrate binding (Simpson and Haard, 1984). Unlike mammalian enzymes, the low stability of the enzyme in buffers with acidic pH values (<4.0) is typical for insect chymotrypsins (Terra and Ferreira, 1994; Terra *et al*., 1996). Recombinant Hi-SP1 was active at a broad range of pH, with the maximum activity detected at pH 10.0. This property of the enzyme is important when considering its industrial applications.

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