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Functional expression and structural characterization of ORF cDNA encoding chitinase of the beet armyworm, *Spodoptera exigua* (Lepidoptera: Noctuidae)

Hee Yun Park, Aron Paek, Seong Eun Jeong *

Department of Biological Sciences, Hannam University, Daejeon 306-791, Republic of Korea

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ABSTRACT

Chitin is an important component of the exoskeleton and peritrophic matrix in insects. Its bio-degradation is initiated by the endo-splitting chitinase. We cloned an ORF cDNA encoding chitinase from the last instar larva of the beet armyworm, *Spodoptera exigua* (Lepidoptera: Noctuidae), into *E. coli* to confirm its functionality. Its amino acid sequence was compared with previously described lepidopteran chitinases. *S. exigua* chitinase expression enhanced cell growth approx. 1.5 fold in transformed *E. coli* than in the wild strain in a 1% colloidal chitin-containing medium with insufficient regular nutrients. Compared with the wild strain, the two intracellular chitin degradation derivatives, glucosamine and *N*-acetylglucosamine, increased approx. 5.8 and 1.5 fold, respectively, and extracellular chitinase activity in the transformed strain was about 1.6 fold higher. The ORF of *S. exigua* chitinase-encoding cDNA including stop codon was composed of 1674 bp nucleotides and the calculated molecular weight of the deduced 557 amino acid residues was about 62.6 kDa. The ORF consisted of an *N*-terminal leading signal peptide (AA 1–20), a catalytic domain (AA 21–392), a linker region (AA 393–493), and a *C*-terminal chitin-binding domain (AA 494–557) showing a typical molting fluid chitinase structure. Phylogenetic analysis determined that all 5 noctuid chitinases were grouped together, while two bombycid enzymes and one tortricid enzyme mapped together in one lineage. In the noctuid group, the sub-lineages reflected their taxonomic relationships at the Genus level.

Introduction

Chitin is a very abundant amino-sugar compound (Cohen-Kupiec and Chet, 1998; Adams, 2004). It is the main component in arthropod exoskeletons, fungal cell walls, and nematode eggshells (Kramer and Koga, 1986; Cohen, 1987; Kuranda and Robbins, 1991; Bakkers et al., 1999). It has strong β-(1,4)-glycosidic linkages between its *N*-acetyl-D-glucosamine and glucosamine residues (Kramer et al., 1995) which are broken down by two chitinolytic enzymes. Chitinase (EC 3.2.1.14) is a rate-limiting, endo-splitting enzyme that plays an initial role in the bio-degradation of chitin, which is followed by the exo-splitting enzyme β-*N*-acetylglucosaminidase (Chernin et al., 1998; Cohen-Kupiec and Chet, 1998). Chitinase is very widespread in both chitin-containing organisms and non-chitin containing organisms including vertebrates, plants, bacteria, and baculoviruses (Collinge et al., 1993; Hawtin et al., 1997; Cohen-Kupiec and Chet, 1998).

All described chitinases have a multidomain structure consisted of at least one unit of each of the two major functional domains (a catalytic domain and a substrate-binding domain) and a linker region connecting the two domains (Morimoto et al., 1997; Arakane et al., 2003; Merzendorfer and Zimoch, 2003). The catalytic domains of various chitinases have an active site common to family 18 glycosylhydrolase, but the substrate-binding domains vary (Suetake et al., 2000; Tjoelker et al., 2000; Gaines et al., 2003).

Insect chitinase plays a role in digestion of old cuticle during post-embryogenesis (Girard and Jouanin, 1999; Feix et al., 2000; Yan et al., 2002). Molting fluid chitinases have a theoretical molecular mass range of 40–85 kDa, and consist of a signal peptide, a catalytic domain, a linker region, and a chitin-binding type II domain (Kramer et al., 1993; Choi et al., 1997; Koga et al., 1997; Kramer and Muthukrishnan, 1997; Nielsen et al., 1997; Shen and Jacobs-Lorena, 1997; Kim et al., 1998; Mikitani et al., 2000; Royer et al., 2002). Since chitinase-encoding cDNA was first isolated from the tobacco hornworm, *Manduca sexta* (Kramer et al., 1993), most molecular biological investigations on insect chitinases have been on serious lepidopteran

* Corresponding author. Tel.: +82 42 629 8756; fax: +82 42 629 8751.
E-mail address: sej@hau.kr (S.E. Jeong).

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pest species because cuticle formation and degradation are good potential targets for pest management (Sakuda et al., 1986; Cohen, 1993; Krishnan et al., 1994; Gopalakrishnan et al., 1995; Wang et al., 1996; Ding et al., 1998).

The beet armyworm (*Spodoptera exigua*) is a serious polyphagous lepidopteran pest. Although two chitinase-encoding cDNA sequences were registered in GenBank (AY678531, GU371868), their functionality has not been demonstrated. We isolated a cDNA encoding chitinase from *S. exigua* which has different amino acid residues from the previous registrations. We cloned the ORF into *E. coli* to elucidate its functional expression and compared the amino acid composition to previously described lepidopteran chitinases.

Materials and methods

Insect and cDNA synthesis

Beet armyworm, *Spodoptera exigua*, larvae were reared at 25 ± 1 °C, 60% RH, under 16:8 (L:D) on cabbage. Last instar larvae were chopped with dissecting scissors and immediately immersed in TRIzol reagent (GIBCO/BRL, Cincinnati, USA). Dissected tissue (100 mg) was homogenized in 1 ml of TRIzol using a micro-pestle and total RNA was extracted according to the manufacturer's instructions. Double stranded cDNA was synthesized from total RNA using the Universal Riboclone cDNA Synthesis System (Promega, Madison, USA) according to the manufacturer's instructions.

Construction of full-length chitinase-encoding cDNA

PCR experiments were performed in a Model 480 Thermal Cycler (Perkin-Elmer, Norwalk, USA). Double stranded cDNA was used as template to amplify the 1674 bp of chitinase-encoding cDNA. A gene-specific primer pair starting with the initiation codon (ATG) and the stop codon (TAG) (5′ primer, ATGAGAGCGATACTGGCG; 3′ primer, CTAAGGCTCGCAGTCTTGACG) was designed to amplify the full-length ORF according to a previously registered *S. exigua* chitinase-encoding cDNA sequence. The 50 μl PCR reaction mixture contained 100 ng cDNA template, 0.25 mM dNTPs, 0.2 mM $MgCl_2$, and 10 μM each of 5′ and 3′ primers. A hot start (5 min pre-incubation at 95 °C, followed by the addition of 2 U Taq polymerase at 80 °C) was followed by 39 PCR cycles (95 °C, 1 min; 52 °C, 1 min; 72 °C, 2 min). PCR products were separated by electrophoresis in an agarose gel. The product was excised and eluted from gels, ligated into plasmid pCR2.1, cloned into *E. coli* using a TA cloning kit (Invitrogen, Carlsbad, USA), and sequenced.

Real-time PCR

A 20 μl of reaction mixture containing 0.2 μg transformed *E. coli* total cDNA template, 10 pmol each of 5′ and 3′ primers (5′ primer, GTGGCGATGAAAACCCTC; 3′ primer, CGATCGGTCCTCCCTCGG), and 10 μl PCR Master Mix (Power SYBR® Green; Applied Biosystems, Foster City, USA) was used to confirm the introduction of insect chitinase cDNA. The PCR reaction was performed with a preheating step at 95 °C for 5 min, followed by 40 cycles (95 °C for 1 min, 53 °C for 1 min, and 72 °C for 2 min) using a Rotor-Gene 3000 (Corbett Research, Mortlake, Australia). Specificity of PCR product was confirmed by agarose gel electrophoresis.

Measurement of intracellular amino sugars

The transformed *E. coli* strain (XL1) with *S. exigua* chitinase was cultured in a 10X diluted media (10^{-1} LB liquid media) containing 1% colloidal chitin, 1 mM IPTG, and 0.1 mg/ml Amp. The inoculated cells (10^4 cells/ml) were incubated for 24–48 h at 37 °C with shaking (225 rpm) and then counted to compare the proliferation rate with wild strain.

E. coli cells were collected by centrifugation (7000 × *g*, 10 min) and crushed in 1 ml DW using a Sonifier 450 ultrasonicator (Branson, Danbury, USA) for 3 min in an ice chamber. After removing cell debris by centrifugation (10,000 × *g*, 10 min), the cell lysate was subjected to HPLC (600 Controller and 486 Tunable Absorbance Detector — 210 nm filter) (Waters, Milford, USA) for the analysis of intracellular D-glucosamine and *N*-acetyl-D-glucosamine levels. A 50 μl aliquot was injected onto an ODS column (5 μm, 4.6 mm × 150 mm) equipped with a C18 Brownlee lab guard column. It was eluted with acetonitrile (solvent A) and H_2O (solvent B) at 1 ml/min flow rate using the following gradient program: 0–2 min, 0% A–100% B; 2–10 min, linear gradient to 40% A–60% B; 10–12 min, linear to initial condition.

Assay of extracellular chitinase activity

E. coli cells were cultured as described above for 72 h at 37 °C, 225 rpm. 0.5 ml of culture filtrate containing 10% CC-RBB (colloidal chitin dyed with Remazol Brilliant Blue in 0.2 M sodium phosphate buffer, pH 7.0) solution was incubated for 1 h at 50 °C and then boiled for 5 min. The remaining colloidal chitin was precipitated by centrifugation (5000 × *g*, 5 min). The supernatant containing the released Remazol Brilliant Blue by the secreted chitinase action was measured at 595 nm. 1 U of enzyme activity equals an increase of 0.01 OD from the control level (Gomez Ramirez et al., 2004).

Analysis of amino acid sequence of S. exigua chitinase cDNA

Deduced amino acid sequences were analyzed using McVector 7.0 software suite (Oxford Molecular Ltd, Palo Alto, USA). Hydropathy plots (Kyte and Doolittle, 1982) obtained with the McVector protein analysis toolbox were scaled with Adobe Illustrator 9.0 to allow direct comparisons. Multiple sequence alignments of the deduced amino acid sequences and bootstrap tree were obtained using the ClustalW (1.4) algorithm (Thompson et al., 1994).

Results and discussion

Cloning and functional expression of ORF cDNA encoding S. exigua chitinase

Insect chitinases have a 40–85 kDa molecular weight range (Kramer and Muthukrishnan, 1997), and previously reported lepidopteran chitinases range from 52 to 63.4 kDa (Kramer et al., 1993; Kim et al., 1998; Goo et al., 1999; Shinoda et al., 2001). The ORF of chitinase-encoding cDNA amplified from *S. exigua* was composed of 1674 bp nucleotides (GenBank JN558351), including the stop codon. Its deduced 557 amino acid residues had 62.6 kDa of calculated mass (Fig. 2). Two reliable *S. exigua* chitinase-encoding cDNA nucleotide sequences have been registered (GenBank AY678531 and GU371868). One is not available because of its impaired chitin-binding domain (GenBank AY51557). The cDNA from this study has 35 and 38 different nucleotides from the two previous registrations, respectively, resulting in 4 and 2 different amino acid residues, respectively, in its ORF. The variations in amino acid sequence, however, do not seem to affect the protein structure or function considerably because the positions of the 4 altered amino acids are not in the two major functional units (catalytic domain and chitin-binding domain). One (AA 7) is in the signal peptide and the other three (AA 478, 481, and 492) are in the linker region which do not cause dramatic changes in their hydropathy features. All substituted residues are small apolar, except for the large apolar valine in AA 481 of AY678531.

To confirm functionality, we transformed the XL1 *E. coli* strain with the *S. exigua* chitinase-encoding ORF cDNA inserted in the pCR2.1 vector. The 40 cycles of real-time PCR amplified 803 ng/μl of a single PCR