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ScienceDirect
Insect Biochemistry and Molecular Biology 37 (2007) 176–183
Insect Biochemistry and Molecular Biology
www.elsevier.com/locate/ibmb

Molecular characterization of a cDNA encoding extracellular dsRNase and its expression in the silkworm, *Bombyx mori*

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Received 3 July 2006; received in revised form 8 November 2006; accepted 9 November 2006

Abstract

A double-stranded ribonuclease (Bm-dsRNase) was separated from the digestive juice of the silkworm larvae, *Bombyx mori*. The full-length cDNA was produced and sequenced using a 20mer primer designed from the N-terminal sequence of the Bm-dsRNase. The cDNA had an ORF encoding 51 kDa precursor protein which can be divided into three domains: a signal peptide, an N-terminal propeptide and a mature Bm-dsRNase. The precursor has an Arg–Ser cleavage site, which produces the 43 kDa mature protein by post-translational processing. The 43 kDa protein had conserved catalytic amino acid residues which are also found in the active site of the *Serratia marcescens* dsRNase. Expression of the precursor occurred in the middle and posterior midgut tissues, starting from Day 1 of the fifth instar larvae. The 43 kDa protein was produced in this tissue from Day 2, and coincidentally secreted into the lumen containing digestive juice. This was supported by the immunohistochemical observation that the mature proteins were localized in the apical side of midgut cells for extracellular secretion.

Keywords: *Bombyx mori*; dsRNase; Midgut; Digestive juice; Precursor; Propeptide

1. Introduction

Double-stranded ribonuclease (dsRNase) has been used for molecular biological studies on the replication of RNA-containing viruses and nucleic acid conformation (Robertson et al., 1968; Nestle and Roberts, 1969). Apart from the usefulness of dsRNase as a tool, studies on *in vivo* functions of RNaseIII, a dsRNase family, revealed that the enzyme is involved in production of putative guide RNAs which are related to RNA interference (Bernstein et al., 2001). Cellular functions of RNase III include processing of rRNA precursors (Belasco and Higgins, 1988), cleavage in the 5′-termini of cellular mRNAs (Court, 1993) and cleavage within polycistronic transcripts produced by bacteriophages (Hajnsdorf et al., 1994). The activity of RNase III is governed by the dsRNA-binding domain (dsRBD) with an unique sequence composed of 65–70 amino acid residues.

Another family of dsRNase was found from various organisms such as bacteria, insects and mammals, and has a relatively high similarity in amino acids involving their activities. The most striking characteristic of dsRNases belonging to this family is its broad substrate specificity: dsRNA is the most preferred substarte, but other nucleic acids, ssRNA, ssDNA and dsDNA, can also be degraded to a lesser extent (Meiss et al., 1999). Thus, the family is often categorized into DNA/RNA non-specific nuclease. The best-studied representative of this family is the dsRNase found in extracellular fluids of *Serratia marcescens* and was reported to have a simple function related to nutritional digestion (Nestle and Roberts, 1969). The enzyme characterization indicated the presence of five catalytic amino acids (Arg78, Arg108, His110, Asn140 and Glu148) in an active site. Although all of the enzymes have a catalytic mechanism similar to that of *Serratia* dsRNase, their physiological functions are apparently different: mitochondrial DNA replication (Cote and Ruiz-Carrillo,

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doi:10.1016/j.ibmb.2006.11.004

1993), DNA repair (Dake et al., 1988) and nutrition (Beliaeva et al., 1976; Meiss et al., 1998).

The digestive juice of the silkworm, *Bombyx mori*, contains various hydrases such as protease (Kotani et al., 1999, Jiang et al., 2000), lipase (Ponnuvel et al., 2003) and dsRNase (Furusawa et al., 1993; Arimatsu et al., 2007). The proteases serve to digest ingested food, while the lipase acts as an anti-viral element against nucleopolyhedrovirus. Successful purification of dsRNase was reported using Poly(I)·Poly(C) affinity column chromatography (Arimatsu et al., 2007); however, little information is available concerning physiological significance *in vivo* of either family of these dsRNases. In this paper, we describe (1) complete nucleotide sequence encoding the precursor protein of dsRNase, and comparisons with dsRNases from different organisms, (2) the production of mature dsRNase by post-translational processing and (3) a possible function of the dsRNase in relation to the timing of gene expression.

2. Materials and methods

2.1. Experimental animals and tissue sampling

The larvae of the silkworm (*B. mori*), 'Kinshu' × 'Showa' race, were reared on artificial diet (Nihon-nosanko, Japan). For purification of Bm-dsRNase, the digestive juice from Day 3 larvae of the fifth instar stage was collected by electric shock. The yield of digestive juice was approximately 10 ml from 50 larvae. For cDNA production, midgut tissue was dissected from Day 3 larvae of the fifth instar stage. For Northern blotting analysis, we used various tissue samples as follows: midgut (anterior, middle and posterior tissues separately collected), silkgland, fat body and Malpighian tubules which were collected on Day 3 of the fifth instar stage; middle midgut tissues from the fourth molting stage to the end of the fifth instar stage. For Western blotting analysis, the middle midgut tissue and digestive juice were collected daily throughout the fifth instar stage.

2.2. Bm-dsRNase purification

The purification of Bm-dsRNase from digestive juice was carried out by a previous method (Arimatsu et al., 2007) with modifications: approximately 10 ml of digestive juice was centrifuged at 1000*g* at 4 °C for 20 min. To the supernatant, a 10-fold volumes of 50 mM phosphate buffer (pH 8.0) was added and then applied on a CM-Sepharose column (∅ 28 mm × 30 mm; Pharmacia Co. Ltd.). After elution with 50 mM phosphate buffer containing 1 M NaCl, the elutant was concentrated by centrifugation in a Centricon (Ultrafree 15, Millipore Co. Ltd.), and loaded on a Superdex 75 column (∅ 16 mm × 540 mm; Pharmacia Co. Ltd.). After fractionation with 50 mM phosphate buffer (pH 6.0) containing 1 M NaCl, all fractions having dsRNase activity were pooled, followed to dilute with an equal volume of 50 mM phosphate buffer (pH 6.0). The diluted fraction was then loaded on a Poly(I)·Poly(C)-agarose column (∅ 14 mm × 25 mm; Pharmacia Co. Ltd.). Elution was carried out with 50 mM phosphate buffer (pH 6.0) containing 2 M NaCl. For purity evaluation, dsRNase fractions obtained from each step were subjected to SDS-PAGE analysis.

2.3. Bm-dsRNase assay

The reaction mixture (500 µl) was composed of 0.1 M glycine-KOH buffer (pH 10), 0.1 M NaCl, 1 mM $MgCl_2$ and 25 µg Poly(I)·Poly(C). After incubation for 30 min at 37 °C, the reaction was stopped by addition of an equal volume of 20 mM lanthanum acetate dissolved in 12% perchloric acid, and then cooled on ice for 40 min. The precipitate was removed by centrifugation at 22,000*g* for 20 min. Absorbance of the supernatant was measured spectrophotometrically at 260 nm. One unit of activity was defined as the amount of enzyme producing an absorbance of 1.0 at 260 nm from the degradation of 50 µg/ml Poly(I)·Poly(C) for 30 min at 37 °C (Siwecka, 1997). Protein content was determined by the method of Lowry et al. (1951).

2.4. N-terminal sequencing

The dsRNase fraction purified by chromatographical separation was run on SDS-PAGE (10% gel). Electroblotting was performed on a polyvinylidene difluoride (PVDF) membrane in 20% methanol containing 48 mM Tris at 108 V for 30 min. The membrane was stained with 0.1% Comassie Brilliant Blue R-250. The Bm-dsRNase band was cut and analyzed by an ABI Model 476A for the N-terminal sequencing.

2.5. cDNA cloning and sequencing

The total RNA was isolated by a sequential treatment of acid guanidinium thiocyanate, phenol and chloroform (AGPC method) described by Sambrook and Russell (2001a). Based on the seven amino acid sequence near the N-terminal of Bm-dsRNase, the degenerate primer (5′-ACITTYMGIGTIAAYGGIGA-3′) was designed for 3′RACE. The reaction was performed according to the supplier's instructions of the 3′RACE and 5′RACE kit (Gibco BRL Co. Ltd.). First strand cDNA was obtained by reaction of Superscript II reverse transcriptase (Gibco BRL Co. Ltd.) with heat-denatured total RNA and an oligo (dT) adapter primer with a 22-base clamp (5′-GGCCACGCGTCGACTAGTACT-polyT-3′). PCR was performed with the degenerate primer and reverse primer (5′-TGACTAGTACTTTTTTTTTTT-3′). The product was cloned into a TA cloning vector (Novagen Co. Ltd.). After sequencing, gene-specific primer #1 (5′-AGTCAGTCT-TAGCAGCAAG-3′) was prepared for 5′RACE. The cDNA was purified with spin cartridge purification kit (Gibco BRL Co. Ltd.) and tailed with poly(C) at the 5′ end