Purification and Properties of Double-stranded RNA-degrading Nuclease, dsRNase, from the Digestive Juice of the Silkworm, Bombyx mori

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A nuclease which degrades double-stranded RNA was purified from the digestive juice of fifth-instar larvae of the silkworm, Bombyx mori, using gel filtration and affinity column chromatography. The enzyme was found to have a molecular weight of 41,000 as estimated by a gel filtration method and detected as a single band with the same molecular weight on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. It degraded double-stranded RNA of cytoplasmic polyhedrosis virus genome, synthetic Poly(I)/Poly(C), Poly(I) and Poly(C). It also digested copolymer Poly(AU), Poly(A) and Poly(U), but showed weak degradation of Poly(A)/Poly(U), Poly(C)/Poly(G), Poly(G) and natural DNA isolated from calf thymus. The pH range wherein the reaction occurred was greater than 7. The purified enzyme did not require Mg2+ to degrade CPV-dsRNA, whereas divalent cations including Mg2+, Zn2+, Mn2+ were needed to degrade synthetic Poly(I)/Poly(C). The enzyme activity was suppressed by Co2+, Zn2+ and Mn2+.

Key words: double-stranded RNA, digestive juice, Bombyx mori, alkaline nuclease

INTRODUCTION

Alkaline and acid nucleases such as RNase and DNase have been studied with respect to the growth and development of the silkworm, Bombyx mori. An acid RNase is distributed in the posterior silk gland and fat body in B. mori, and shows an increase in activity at the ecysis of each instar and in association with the histolysis of the silk gland during metamorphosis (Koga et al., 1969; Koga and Akune, 1972). A Mg2+-dependent alkaline nuclease, which degrades both DNA and RNA, was detected in the digestive juice and midgut tissues (Mukai, 1965; Funagumma et al., 1977). In addition, an Mg2+-dependent sugar-nonspecific nuclease which acts on both native and heat-denatured DNA and RNA has been found in pupae of the silkworm (Mukai, 1965). RNase III, specific for double-stranded RNA (dsRNA), has been isolated and purified from calf thymus (Ohtsuki et al., 1977), Escherichia coli (Robertson et al., 1968; Young and Steitz, 1978; Gotoh et al., 1974) and chick embryos (Hall and Crouch, 1977). This type of RNase appears to function in the processing of specific precursor RNA to activate such as tRNA, rRNA and mRNA, and has been shown to digest dsRNA in a step-wise manner: an initial phase of specific cleavage, followed by random degradation (Edy et al., 1976).

The midgut and digestive juice of the silkworm have been found to possess an RNase activity, which degrades dsRNA similar to synthetic Poly(I)/Poly(C) and the natural dsRNA genome of the cytoplasmic polyhedrosis virus (CPV) (Furusawa et al., 1993). This enzyme was tentatively called Bombyx dsRNase. However, the actual enzymatic characterization remains to be clarified. In this paper, we describe successful isolation of dsRNase from digestive juice of the silkworm, and characterization of its enzymatic properties such as substrate specificity, cofactor requirement and pH-activity profile in dsRNase activity.

MATERIALS AND METHODS

Preparation of digestive juice from silkworm larvae

Digestive juice was collected from larvae of Kinshu × Showa on the fourth day of the fifth instar by inducing vomiting with an electric shock of about 80 V. The digestive juice was collected and centrifuged at 10,000 g for 30 min in order to remove undigested mulberry debris. The supernatant was stored at −80°C until used for enzyme purification.

(NH4)2SO4 fractionation

The digestive juice was diluted with an equal volume of 50 mM phosphate buffer, pH 7.0. Solid (NH4)2SO4 was added to this solution, and was dissolved upon mixing to yield a saturation of 60%. After gentle stirring for 30 min,
the precipitate was separated by centrifugation at 10,000 g for 30 min and the supernatant was removed. The precipitate was dissolved in 50 mM phosphate buffer, pH 7.0, containing 1 mM PMSF.

**Gel filtration**

After dialysis, the solution was applied to a Superdex 75 column (Pharmacia Co. Ltd.) equilibrated with 50 mM phosphate buffer, pH 7.0, containing 0.5 M NaCl, 0.1% CHAPS and 1 mM PMSF. One ml each of eluant was collected after washing the column with 40 ml of the buffer. The following proteins with indicated molecular weight were used as markers, bovine serum albumin (BSA) (66,300), ovalbumin (43,000), chymotrypsinogen A (25,000) and RNase A (13,700).

**Affinity chromatography on Poly(I)/Poly(C)-agarose**

The fractions containing dsRNase were collected after gel filtration, dialyzed against 50 mM phosphate buffer, pH 6.2, containing 1 mM PMSF and mixed with acetone in order to concentrate. The precipitate was solubilized in 50 mM phosphate buffer, pH 6.2, containing 1 mM PMSF and the solution was applied to a Poly(I)/Poly(C)-agarose column (6.4 × 31 mm, capacity: 1 ml) (Pharmacia Co. Ltd.) equilibrated with 50 mM phosphate buffer, pH 7.5, containing 1 mM PMSF and 0.1% CHAPS and mixed with acetone. The precipitate was separated by centrifugation at 10,000 g for 30 min and the supernatant was removed. The precipitate was dissolved in 50 mM phosphate buffer, pH 7.0, containing 0.1 M acetic acid in the positive reservoir, and 0.2 M NaCl and 0.1 M MgCl₂. This was incubated for 30 min at 37°C. An equal volume of 12% perchloric acid containing 20 mM lanthanum acetate was added to this solution. After centrifugation at 22,400 g for 20 min at 4°C, the absorbance of liberated nucleotides was detected at 260 nm and compared with a control solution that did not contain enzyme. One unit of activity was defined as the amount of enzyme required to solubilize 1 A₂₆₀ unit per ml of reaction mixture under the assay conditions described.

**Method 2:** Different kinds of RNA or DNA were dissolved in 0.1 M glycine-KOH buffer, pH 10.0, containing 8 mM MgCl₂, at a final concentration of 0.5 mg/ml. A 4 µl aliquot of this solution was mixed with 6 µl of enzyme solution and the mixture was incubated at 37°C for 3 h. Just after the reaction, one volume of the mixture was added to 2 µl of gel loading solution (10 mM Tris-HCl buffer, pH 7.0, containing 0.25% bromophenol blue, 40% sucrose, 30% glycerol and 1 mM EDTA), and then electrophoresed on a 1.5% agarose gel at a room temperature for about 20 min at a constant voltage of 100 V. A TAE buffer was used as the electrophoresis buffer, and the activity was estimated from the extent of nucleic acid degradation detected in the gel.

**Other procedures**

Protein samples were boiled in the presence of 1.25% sodium dodecyl sulfate (SDS) and 0.35 M 2-mercaptoethanol and electrophoresed through 12.5% polyacrylamide gel in the presence of 0.1% SDS (SDS-PAGE) for 2 h at a constant current of 20 mA. Gels were stained with a silver staining reagent (Daichi chem. Co., Ltd.). The following molecular markers with indicated molecular weight were used in SDS-PAGE: phosphorylase b (97,400), BSA (66,300), aldolase (42,400), carbonic anhydrase (30,000), trypsin inhibitor (20,100) and α-lysozyme (14,400). Protein concentration was measured by the methods of Lowry et al. (1951).

**RESULTS**

**Purification of double-stranded RNase**

A summary of purification of *Bombyx* dsRNase is presented in Table 1. In the final step of purification, 11 µg of dsRNase with a specific activity of 12,459 U/mg proteins was obtained (0.01% recovery) from 93.5 mg protein in digestive juice. By 60% (NH₄)₂SO₄ fractionation, a moderate purification (2.7-fold) was obtained. The precipitate was fractionated by gel filtration using Superdex 75. The dsRNase activity was observed as a single peak with a specific activity of 4,700 U/mg protein (Fig. 1). When Sephadex G-75 was used instead of Superdex 75, a peak of activity with a specific activity of 3,000 U/mg protein was obtained (data not shown). Affinity chromatography
The precipitate from digestive juice by 60% (NH₄)₂SO₄ saturation was fractionated through gel filtration (Superdex 75) with elution buffer, pH 7.0, containing 0.5 M NaCl, 0.1% CHAPS and 1 mM PMSF. The pooled enzyme fraction was concentrated by adding of 80% acetone. The solubilized sample was applied to affinity column chromatography. To adsorb the sample to Poly(I)/Poly(C) agarose, 50 mM phosphate buffer containing 1 M KCl, 0.1% CHAPS, 1 mM PMSF was used. The activity of dsRNase was measured using Method 1 as described in MATERIALS AND METHODS, with Poly(I)/Poly(C) as a substrate. Enzyme unit (1 U) was defined as A₂₆₀ = 1.0 which was given in 1 ml of reaction mixture containing 1 μl of dsRNase fraction.

**Properties of dsRNase**

The molecular weight of the present dsRNase was determined to be about 41,000 by the filtration with a Superdex 75 column, in which the elution rate was compared with those of marker proteins described in MATERIALS AND METHODS (detailed patterns not shown). Similar value was obtained by SDS-PAGE (Fig. 3), indicating that the dsRNase is a monomer enzyme. The effect of pH on the activity of the purified dsRNase was examined (Fig. 4). The enzyme showed the activity when the pH of the buffer was larger than 7.0. At the moment, the largest activity was obtained when the pH of the given buffer was 11.0 (no experiment was done with a buffer of pH 12.0). Isoelectric focusing data showed that the pI of dsRNase was 8.5.

The extent of degradation of the different synthetic substrates by the purified dsRNase was investigated (Table 2); the activity against dsRNAs, Poly(C)/Poly(G) and Poly(A)/Poly(U), was lower than that against another dsRNA, Poly(I)/Poly(C). However, the activity against single-stranded RNA (ssRNA), Poly(A), Poly(C), Poly(I), Poly(U) and Poly(AU), was very high. No marked degradation was observed when Poly(G) and calf thymus DNA were used as substrates.

Fig. 5 shows the results of incubation of synthetic dsRNA, CPV-dsRNA, DNA or yeast single-stranded RNA.
with the purified dsRNase in buffer either with or without 8 mM Mg$^{2+}$. The enzyme solution was used after being diluted 12, 14 or 72 times. After the incubation, each sample was electrophoresed on an agarose gel. It was found that the purified dsRNase did not degrade either yeast ssRNA or calf thymus DNA, but degraded CPV-dsRNA and Poly(I)/Poly(C). In the buffer lacking Mg$^{2+}$, Poly(I)/Poly(C) was not degraded, whereas CPV-dsRNA was degraded to same extend.

The effect of divalent cations on the activity of dsRNase was examined (Fig. 6). Ten mM Mg$^{2+}$ and 10 mM Ca$^{2+}$ increased the dsRNase activity by 210% and 148%, respectively. However, 10 mM Mn$^{2+}$, 10 mM Zn$^{2+}$ and 20 mM Co$^{2+}$ suppressed the activity to 45%, 12% and 31%, respectively.

**DISCUSSION**

*Bombyx* dsRNase, a monomer enzyme with a molecular weight of 41,000, is active under high alkaline conditions with a buffer of pH 11.0, and has a pl of about 8.5. Divalent cations such as Mg$^{2+}$ and Ca$^{2+}$ activated this enzyme, whereas other cations including Mn$^{2+}$, Zn$^{2+}$ and Co$^{2+}$ suppressed the activity. There are a number of nucleases with alkaline pl values such as RNase A with an optimum pH of 7.5, a molecular weight of 13,000 and a pl of 9.6, bovine thymus endoribonuclease H with an optimum pH of about 8 and a molecular weight of 70,000-90,000 (Stavrianopoulos and Chargaff, 1973), and *Shizosaccharomyces pombe* Pac I with an optimum pH of 8.5 and a molecular weight of 45,500 (Rotondo and Frendewey, 1996); this has a similar amino acid sequence to RNase III (March et al., 1985). In addition, there is a base-non-specific nuclease, extracellularly secreted by *Staphylococcus aureus*, with an optimum pH of 9.0-10.0 and a molecular weight of 17,000 (Heins et al., 1967). *Bombyx* dsRNase was ob-

### Table 2. Substrate specificity of purified dsRNase

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific activity (U/mg protein)</th>
<th>Degradation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly(I)/Poly(C)</td>
<td>5,486</td>
<td>100</td>
</tr>
<tr>
<td>Poly(C)/Poly(G)</td>
<td>2,743</td>
<td>50</td>
</tr>
<tr>
<td>Poly(A)/Poly(U)</td>
<td>1,524</td>
<td>28</td>
</tr>
<tr>
<td>Poly(A)</td>
<td>14,019</td>
<td>256</td>
</tr>
<tr>
<td>Poly(C)</td>
<td>10,324</td>
<td>188</td>
</tr>
<tr>
<td>Poly(G)</td>
<td>762</td>
<td>14</td>
</tr>
<tr>
<td>Poly(I)</td>
<td>11,010</td>
<td>201</td>
</tr>
<tr>
<td>Poly(U)</td>
<td>19,086</td>
<td>348</td>
</tr>
<tr>
<td>Poly(AU)</td>
<td>8,076</td>
<td>147</td>
</tr>
<tr>
<td>DNA</td>
<td>457</td>
<td>8</td>
</tr>
</tbody>
</table>

The substrate was dissolved at a final concentration in 0.1 M glycine-KOH buffer, pH 10, containing 0.1 M NaCl and 1 mM MgCl$_2$. The dsRNase fraction from affinity column chromatography was added to above reaction mixture, and then incubated at 37°C for 30 min.

![Fig. 3. SDS-PAGE (12.5%) analysis of dsRNase fractions obtained using gel filtration and affinity column chromatography. A, Superdex 75 fractions containing dsRNase; B, unabsorbed protein from Poly(I)/Poly(C)-agarose column; C, fractions obtained from Poly(I)/Poly(C)-agarose containing dsRNase. The gel was stained with a silver staining reagent. M: molecular weight markers (see MATERIALS AND METHODS).](image)

![Fig. 4. Effect of pH on the activity of purified dsRNase activity. Poly(I)/Poly(C) was dissolved at a final concentration of 50 mg/ml in the following buffers: 0.1 M sodium acetate/acetate acid (acetate buffer) (pH 4.0 and 5.0), 0.1 M phosphate buffer (pH 6.0 and 7.0), 0.1 M Tris-HCl buffer (pH 8.0 and 9.0) and 0.1 M glycine-KOH buffer (pH 10.0 and 11.0), all of which contained 0.1 M NaCl and 1 mM MgCl$_2$. The solution (495 µl) was mixed with 5 µl of dsRNase fraction from affinity chromatography or 5 µl of the solution which was prepared from 60% (NH$_4$)$_2$SO$_4$ precipitate (see Table 1). The activity was measured using Method 1 described in MATERIALS AND METHODS.](image)
Purification of Bombyx dsRNase

served to have common properties with RNase III and endoribonuclease H in that the enzymatic activity was enhanced by adding Mg\(^{2+}\). However, Bombyx dsRNase whose activity is suppressed by either Mn\(^{2+}\) or Co\(^{2+}\) differs from both RNase III and endoribonuclease H, which are stimulated by these ions.

The nuclease of the larval digestive juice and midgut tissues cited in the INTRODUCTION was reported to have a molecular weight of 22,000, and does not have specificity against sugars or bases for endonucleic cleavage of DNA or ssRNA (Mukai, 1965; Funaguma et al., 1977). In addition, it is activated by 1 mM Mg\(^{2+}\) (Mukai, 1965). Another nuclease with a molecular weight of 85,000 and an optimum pH of 9.5 exists in the larval midgut of the silkworm (Funaguma et al., 1977). This nuclease requires Mg\(^{2+}\) and cleavages both DNA and ssRNA and is thought to be a proenzyme for digestive nuclease, based on results obtained from an immunological assay (Funaguma et al., 1977). Moreover, silkworm pupae have a sugar-non-specific nuclease, which is slightly different from a larval alkaline nuclease (Himeno et al., 1968). Thus, the current Bombyx dsRNase has a different molecular weight and divalent cation requirement from those of other dsRNase and Mg\(^{2+}\)-dependent alkaline silkworm nucleases reported before.

Bombyx dsRNase is an endonuclease that recognized the structure and bases of synthetic dsRNA substrates. Showing lower degradation of Poly(C)/Poly(G) and Poly(G), the enzyme was suggested to be quite unreactive to the guanine substrate. It also appeared to exhibit slow degradation of synthetic double-stranded Poly(A)/Poly(U), whereas being very reactive to Poly(A) and Poly(U). Thus, Bombyx dsRNase exhibits a difference in the extent of degradation by discriminating between single- and double-stranded RNAs.

Furthermore, Bombyx dsRNase showed very little activity to yeast ssRNA and calf thymus DNA, while it was able to degrade both CPV-dsRNA and Poly(I)/Poly(C). Even in the absence of Mg\(^{2+}\), it was able to degrade CPV-dsRNA. Consequently, Bombyx dsRNase is considered to be a base-recognition type nuclease. Preliminary experiments showed that the dsRNase is synthesized in the midgut cells and secreted into the midgut lumen. Upon virus infection in the midgut cells, the enzyme potentially attacks the viral dsRNA \textit{in vivo}. The secreted dsRNase may also have a degradative function for nutrients. Virological and physiologica roles of function are not known, and are to be investigated.

E. coli RNase III, reactive to dsRNA, has an optimum pH of 7.6, a molecular weight of 45,000-55,000 and an Mg\(^{2+}\) requirement. Additionally, its activity is enhanced by Co\(^{2+}\) and Mn\(^{2+}\) cations (Robertson et al., 1968). S. pombe Pac I (also reactive to dsRNA) requires Mg\(^{2+}\), Co\(^{2+}\) and Mn\(^{2+}\) (Rotondo and Frendewey, 1996). These enzymes are known to have double-stranded binding domains (dsRBD) at the C-terminal end. For example, RNase III has catalytic domain 150 residues from the N-terminal end of the amino acid chain, and dsRBD 79 residues from the C-terminal end (Kharrat et al., 1995). Such structural features...
of *Bombyx* dsRNase are now under investigation in our laboratories.

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