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Expression and functional validation of *Bombyx mori* nucleopolyhedrovirus ORF29, a conserved Nudix motif protein

HUI-QING CHEN[1], YA-JING ZHOU[1], KE-PING CHEN[1*], QIAN YU[2*]

[1]Institute of Life Sciences, Jiangsu University, 301 Xuefu Road, Zhenjiang, 212013, P. R. China; [2]Institute of Light Industry and Food Science, Zhongkai University of Agriculture and Engineering, Guangdong, 510225, P. R. China.

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Summary. – Our previous study showed that *Bombyx mori* nucleopolyhedrovirus (*Bm*NPV) *orf29* encodes a 26 kDa protein expressed in the early stage of infection cycle. *Bm*NPV ORF29, contains a conserved motif of Nudix (nucleotide diphosphate X) superfamily. It has the highest homology with ADP-ribose pyrophosphatase (ADPRase), a subfamily of Nudix pyrophosphatase. In this work, we purified the recombinant *Bm*NPV ORF29 in *Escherichia coli* by metal chelating affinity chromatography. The amino acid sequence of recombinant protein was confirmed by mass spectroscopic analysis and found that the purified protein could be able to catalyze the breakdown of ADP-ribose to AMP and ribose 5-phosphate, with K_m and K_{cat} values of 182 μmol/l and 5.3 s^{-1}respectively. The optimal activity was at alkaline pH (8.5) with Mg^{2+} (0.5-mmol/l) ions as the cofactor.

Keywords: Bombyx mori; Escherichia coli; BmNPV ORF29; Nudix pyrophosphatase; enzymatic activity

Introduction

The Nudix superfamily (InterPro IPR000086; Pfam PF00293) is widely distributed among eukaryotes, bacteria and viruses (McLennan, 2006). Nudix proteins are able to catalyze the hydrolysis of nucleoside diphosphates, linked to another moiety x (Bessman *et al*., 1996), hence the acronym "Nudix". They all possess the unique Nudix motif, Gx_5Ex_5[UA] $xREx_2EExGU$, where U is a bulky hydrophobic amino acid, and x can be any residue (McLennan, 2006). Nudix motif was first studied in 15 kDa MutT protein, hence the original name of the Nudix family – the MutT family (McLennan, 1999).

ADP-ribose is one substrate of the Nudix enzymes, which is produced intermediately as part of the turnover of NAD^+, mono- or poly-ADP-ribosylated proteins and cyclic ADP-ribose (Jacobson *et al*., 1994). High concentration of free ADP-ribose may lead to nonenzymatic ADP-ribosylation, which is a deleterious process that inactivates enzymes and could interfere with the recognition of enzymatic ADP-ribosylation (Just *et al*., 1994). ADP-ribose pyrophosphatase (ADPRase) belongs to a subfamily of Nudix proteins that catalyze the breakdown of ADP-ribose to AMP and ribose 5-phosphate to prevent ADP- ribose accumulation. (McLennan, 2006).

The *Baculoviridae* is a large family of diverse arthropod specific insect viruses with large circular, supercoiled, double stranded DNA genomes, and occluded, rod-shaped virions. *Bombyx mori* nucleopolyhedrovirus (*Bm*NPV) contains a covalently closed circular genome of 128 kbp, about 143 putative open reading frames based on the criterion that the ORF must be a single, continuous, non-overlapping coding region (Gomi *et al*., 1999; Jehle *et al*., 2006). Among these ORFs, *Bm*NPV ORF29 was found to belong to a unique Nudix superfamily. In a previous study, we found *Bm*NPV *orf29* encoded a 26 kDa protein in the early stage of infection cycle and was present in the budded virus (Chen *et al*., 2010). Here, we tried to elucidate the biochemical characterization of *Bm*NPV ORF29.

Materials and Methods

*Computer-assisted sequence analysis. Bm*NPV ORF29 was analyzed using ExPASy server (www.expasy.ch) for the prediction of motifs, domains, transmembrane regions and signal peptides (Appel *et al*.,

*Co-corresponding authors. E-mail: kpchen@ujs.edu.cn (K. Chen), yuqianchina@126.com (Q. Yu); phone: +86-511-8791923.

Abbreviations: ADPRase = ADP-ribose pyrophosphatase; *Bm*NPV = *Bombyx mori* nucleopolyhedrovirus

1994). Homologues were explored by using BLASTP searching tool in the updated GenBank/EMBL and SWISS-PROT databases (Altschul *et al*., 1997; Pearson, 1990). The sequence alignment was carried out with ClustalW (http://www.ebi.ac.uk/clustalw) and edited with Genedoc software (Ver.2.04) (Free Software Foundation, Inc., MA, USA).

*Expression and purification of recombinant protein. Bm*NPV *orf29* coding sequence was amplified as described previously (Chen *et al*., 2010). Briefly, *Bm*NPV *orf29* coding sequence was amplified from the *Bm*NPV genomic DNA and subcloned into the pET30a (+) expression vector in frame with the N-terminal 6 × His tag. To express recombinant protein, a freshly transformed colony was cultured in LB medium supplemented with kanamycin (50 μg/ml) at 37°C overnight. This overnight culture was inoculated into fresh LB medium and cultured at 37°C with vigorous shaking. When A600 reached 0.6, the expression of *Bm*NPV *orf29* was induced with IPTG (final concentration 0.1–2 mmol/l during optimization) and further cultured at 28°C for 6 hrs. Cells were harvest by centrifugation (4500 x g, 4°C, 15 mins) and SDS-PAGE analysis on a 12% gel was performed to estimate the expression level of *Bm*NPV ORF29. The cell pellet was resuspended in buffer A (50 mmol/l sodium phosphate, 300 mmol/l NaCl, 1 mmol/l EDTA, 0.5 mmol/l PMSF, pH 8.0), then the cell suspension was lysed by sonication. The supernatant was loaded onto a Ni-NTA affinity column (Qiagen). Purification conditions were standardized by optimizing pH, the concentration of salt and imidazole. After washing the captured column with 20 mmol/l and 40 mmol/l imidazole, the fusion protein was eluted with 250 mmol/l imidazole. The eluted protein was dialyzed against buffer B (50 mmol/l sodium phosphate, 150 mmol/l NaCl, pH 7.5) at 4°C. The concentration of protein was determined by the Bradford method using bovine serum albumin as the standard (Bradford, 1976).

Western blot analysis. Protein samples were separated by SDS-PAGE and transferred onto a PVDF membrane in cold Towbin buffer (0.025mol/l Tris, 0.19 mol/l glycine, 20% methanol). The membranes were blocked in 3% skimmed milk powder in PBST for 1 hr followed by incubation with the mouse anti-His monoclonal antibody (Santa Cruz Biotechnology) as a primary antibody for 1 hr at room temperature. Subsequently, the membrane was incubated with a goat anti-rabbit IgG conjugated to HRP diluted 1:5,000 for 1 hr at room temperature. The signal was detected with a DAB substrate solution.

In gel digestion and peptide analysis by MALDI-TOF. The proteins located in the band visualized by the Coomassie Blue staining were digested with trypsin and analyzed by matrix-assisted laser desorption ionization-time-of flight mass spectrometry (MALDI-TOF) (Bruker Daltonics, Germany), which generated the peptide sequence in addition to peptide mass information. Masses of tryptic peptides obtained by MALDI-TOF were used as inputs to search corresponding proteins against the database NCBInr and MSDB, via the program MASCOT.

Enzyme assay. Enzyme velocities were assayed by measuring the conversion of a phosphatase-insensitive ADP-ribose to the phosphatase sensitive products, AMP and ribose 5-phosphate. The liberated inorganic orthophosphate from both AMP and ribose 5-phosphate was measured according to a method described previously (Ooga *et al*., 2005). The amount of free phosphate was then quantified to represent the activity of the enzyme. The standard incubation mixture (50 μl) contained 50 mmol/l Tris-Cl (pH 8.0), 2 mmol/l $MgCl_2$, 2 mmol/l ADP-ribose, 0.1–1 milliunits of the purified *Bm*NPV ORF29, and 2 units of alkaline intestinal phosphatase. After 15 min at 37°C, the reaction was terminated by the addition of EDTA, and inorganic orthophosphate was measured. Blanks without enzyme and/or substrate were run in parallel. The kinetic parameters were determined by fitting the initial rates of the reaction to the Michaelis–Menten equation. An unrelated recombinant protein, NUDT5 (ProSpec, China), was used as a control to exclude the possibility of contamination from bacteria.

Results and Discussion

Sequence analysis

Querying *Bm*NPV ORF29 amino acid sequence against the NCBI Conserved Domain Database (Marchler-Bauer *et al*., 2005) showed a potential Nudix motif in the N-terminal part. The Nudix signature sequences of this ORF protein, together with human MTH, *E. coli* ADP-ribose and other characterized Nudix hydrolases with unique substrate specificity are shown in Fig.1. Multiple alignments revealed

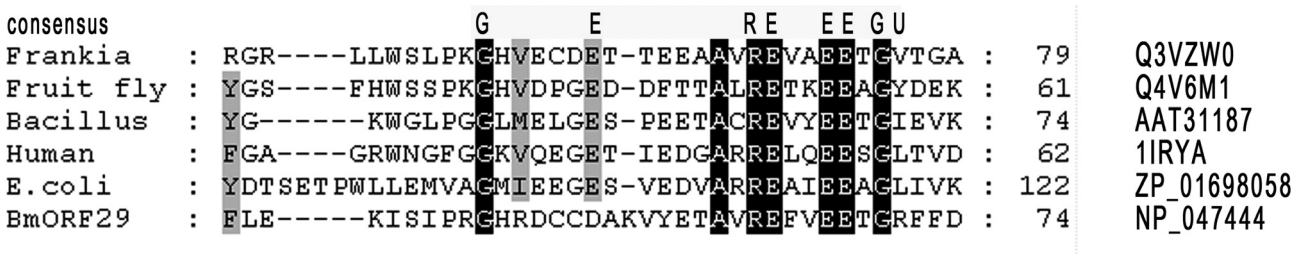

Fig. 1

Sequence comparisons in the Nudix hydrolases family

The numbers to the right of sequences correspond to the number of residues. Each sequence is accompanied by a GenBank Acc. No.

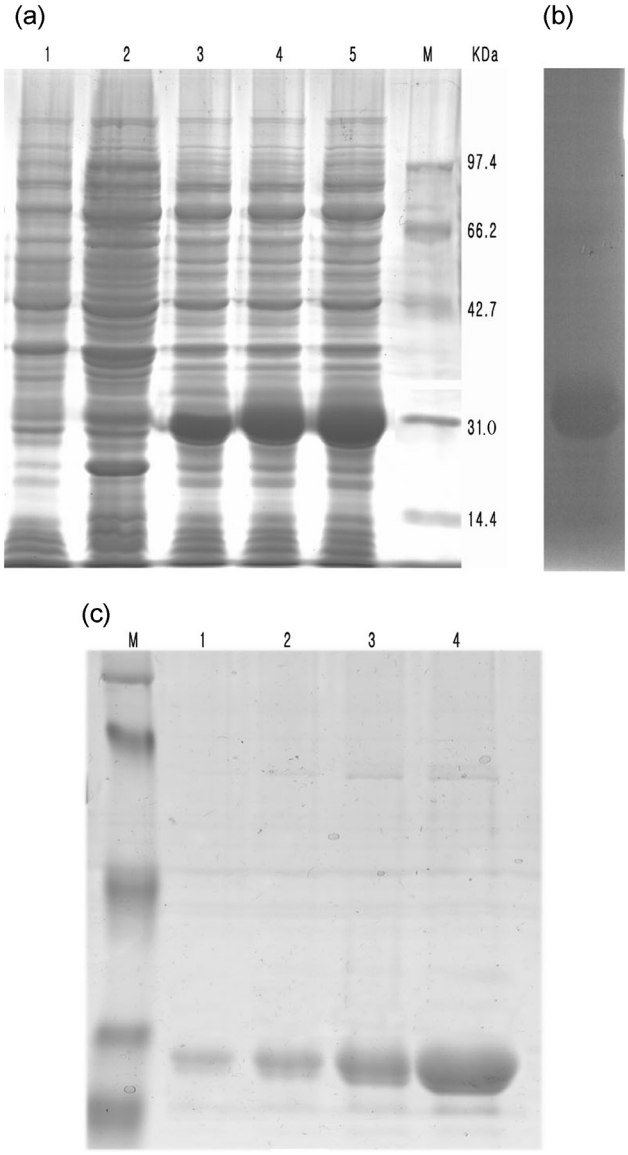

Fig. 2

SDS–PAGE stained with Commassie blue and Western blot analysis of recombinant *Bm*NPV ORF29

(a) Expression of recombinant *Bm*NPV ORF29 on the 12% SDS-PAGE. Lane 1: BL21 (DE3); lane 2: BL21 (DE3) harboring empty vector; lanes 3–5: BL21 (DE3) harboring pET-28a(+)-*Bm*NPV ORF29 induced at 30, 28, and 16°C; lane M, pre-stained protein marker. (b) Western blot analysis of recombinant *Bm*NPV ORF29 with anti-His antibody. (c) Different elution fractions from Ni-NTA beads harboring *Bm*NPV ORF29 protein with the elution buffer containing imidazole (lanes 1–4, soluble protein eluted with 5,100, 200, 800 mmol/l imidazole, respectively).

a conserved domain $GX_5EX_7REUXEEX_2U$ aligned with confirmed conserved domain positions within other Nudix hydrolase, which was also designated as NUDT5. One feature in NUDT5 Nudix sequence is Tyr-119, which is not a bulk amino acid like Ile, Leu or Val as in the majority of other Nudix signature sequences.

Over-expression and purification of BmNPV ORF29

The pET-30a(+)-*Bm*NPV *orf29* and the negative empty vector pET30a(+) were transformed into *E. coli* BL21 (DE3). At 37°C, a band corresponding to *Bm*NPV ORF29 protein with the expected molecular weight was shown. However, the majority of *Bm*NPV ORF29 was accumulated in insoluble form and the minority in soluble form. Generally, lower temperatures for culturing and expressing are beneficial to expression of soluble protein (Swalley *et al.*, 2006; Vasina and Baneyx, 1997). The *Bm*NPV ORF29 proteins were induced at 30, 28, and 16°C (Fig. 2a). The soluble fraction was remarkably increased, especially at 16°C. The soluble fusion protein was purified using the Ni-NTA column (Novagen) (Fig. 2c). 1 liter cell culture yielded 2.0 mg of highly pure protein after purification as measured by the Bradford method with bovine serum albumin as the standard. The expression of recombinant 6 × His-tagged *Bm*NPV ORF29 was also confirmed by anti-6 × His monoclonal antibody (Fig. 2b).

Mass spectrometry

To further determine if the purified protein obtained after SDS-PAGE was *Bm*NPV ORF29, the corresponding protein band was digested with trypsin and analyzed by MALDI-TOF mass spectrometry (Fig. 3). The Mascot search was performed with carbamidomethyl as the fixed modification of cysteine and variable N-terminal Gln-pyroGlu. Thirteen peptide fragments were identified in mass spectra. By comparing the masses of identified peptides to the hypothetical tryptic peptides for proteins in non-redundant NCBI database using the MASCOT search engine, *Bm*NPV ORF29 was obviously identified with MOWSE score of 186. The identified 13 peptide fragments were matched against the deduced amino acid sequence of *Bm*NPV ORF29 with 68% sequence coverage.

Enzymatic activity assay

Most of the characterized Nudix hydrolases require an alkaline pH and the presence of divalent ions to become fully active. The *Bm*NPV ORF29 has its optimal activity to ADP ribose at the expected alkaline pH around 8.5 (Fig. 4). *Bm*NPV ORF29 requires the presence of Mg^{2+} to achieve optimal activity (0.5 mmol/l Mg^{2+}), while the activity was decreased at high concentration of Mg^{2+} (Fig. 5). Mn^{2+} and Zn^{2+} could partially substitute for Mg^{2+} (data not shown). The kinetic parameters of *Bm*NPV ORF29 were estimated with ADP-ribose at concentrations between 0.1 to 3 mmol/l.

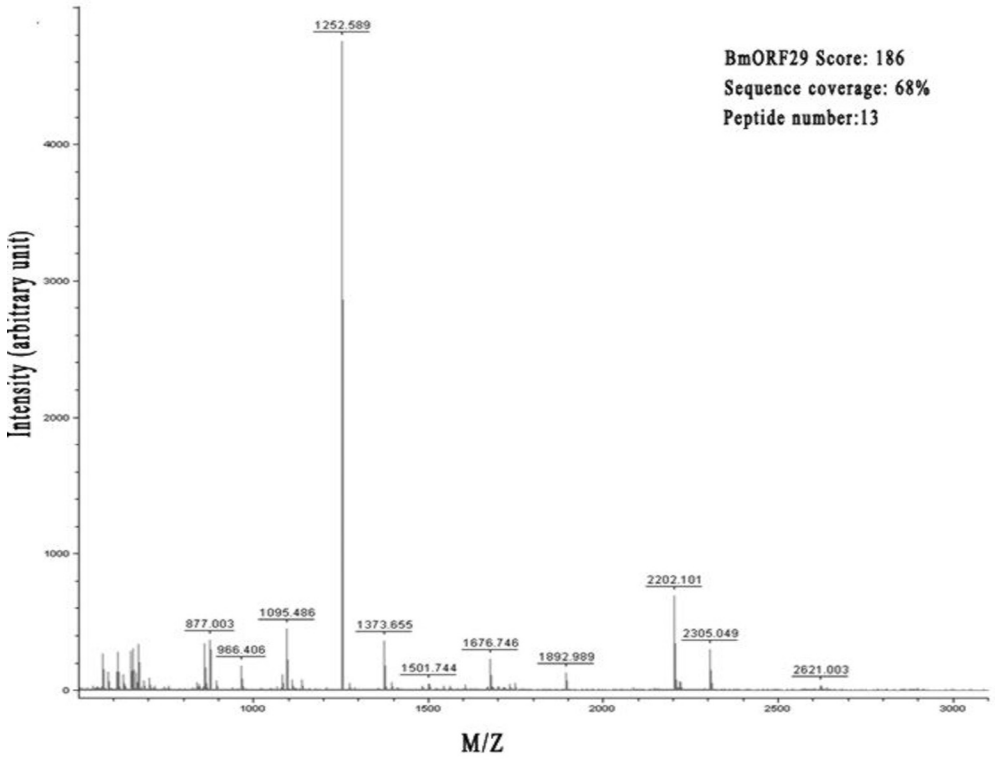

Fig. 3

MALDI spectra of tryptic digest of recombinant *Bm*NPV ORF29

The identified protein, score, amino acid sequence coverage and the number of identified peptides are shown in the right corner. The sequences of identified peptides, shown in bold and underlined, covered 68% of *Bm*NPV ORF29 amino acid sequence.

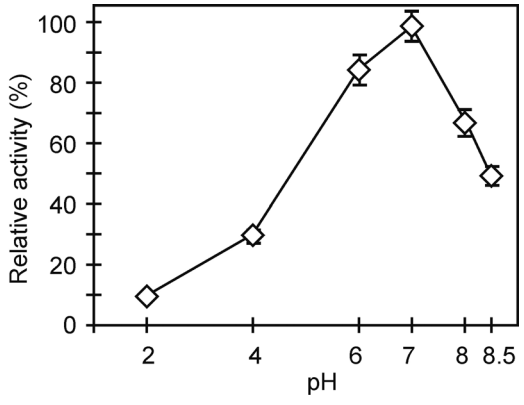

Fig. 4

Effect of pH on ADPRase activity of *Bm*NPV ORF29

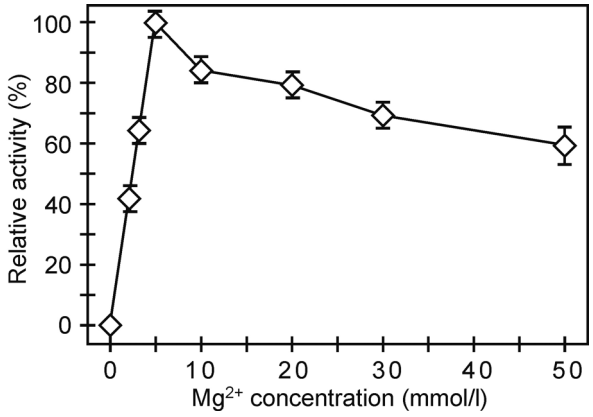

Fig. 5

Effect of Mg^{2+} on ADPRase activity of *Bm*NPV ORF29

In the conditions of pH 8.5, the K_m value for the hydrolysis of ADP-ribose was 182 ± 0.12 μmol/l, and K_{cat} value was 5.3 ± 0.2 s^{-1}. The specific activity of isolated recombinant *Bm*NPV ORF29 was calculated to be 7.8 ± 0.06 units mg^{-1}. These data are the mean of three independent determinations ± S.D.

Conclusion. This report confirms our preliminary results and describes the purification and detailed biochemical characterization of the *Bm*NPV ORF29 protein, a member of the Nudix family of proteins, which catalyses breakdown of ADP-ribose to AMP and ribose 5-phosphate.

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