PREPARATION OF RECOMBINANT COAT PROTEIN OF PRUNUS NECROTIC RINGSPOT VIRUS

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Summary. – The coat protein (CP) gene of Prunus necrotic ringspot virus (PNRSV) was cloned into pET16b vector and expressed in *Escherichia coli*. CP-enriched fractions were prepared from whole cell lysate by differential centrifugation. The fraction sedimenting at 20,000 x g for 30 mins was used for preparation of a rabbit antiserum to CP. This antiserum had a titer of 1:2048 and reacted in a double-antibody sandwich ELISA (DAS-ELISA).

Key words: Escherichia coli; Prunus necrotic ringspot virus; recombinant coat protein

Introduction

PNRSV is a worldwide spread pathogen of many deciduous trees (sweet and sour cherry, plum, peach, apricot, almond and apple trees), hop and roses. Symptoms caused by this virus vary widely – from no obvious change to serious damage (Uyemoto and Scott, 1992). The indexing on sweet cherry discriminate rugose mosaic disease-causing strains and strains inducing mild mottle or none symptoms. Typing of American strains of PNRSV has revealed mostly three serotypes: C3, C9, and C30 (Crosslin and Mink, 1992).

Recently, the sequence variability of PNRSV CP has been intensively studied and many new isolates have been described (Hammond and Crosslin, 1998; Scott *et al.*, 1998; Aparicio *et al.*, 1999; Vašková *et al.*, 2000). Molecular phylogenetic analyses clustered the PNRSV isolates into three groups, (I, II and III), which correlate with the pathotype and/or serotype (Vašková *et al.*, 2000; Hammond and Crosslin, 1998). However, only a few European isolates of PNRSV were pathotyped and/or serotyped.

Two length variants of PNRSV CP have been detected, a shorter CP with 224 amino acids and a longer CP with 226 amino acids. The isolates with the shorter CP are disseminated in all three phylogenetic groups, while those with the longer CP occur only in the phylogenetic group I, which contains the isolates from trees with wrinkled leaves.

Here we describe the preparation of a recombinant PNRSV CP in *E. coli*, its partial purification and preparation of an antiserum suitable for PNRSV detection.

Materials and Methods

Virus. PNRSV (*Bromoviridae* family, *Ilarvirus* genus (subgroup 3), *Prunus necrotic ringspot virus* species) isolate 1/13 (Acc. No. AF170156) originated from a cherry growing in the northern Czech Republic and caused very strong disease symptoms on this host. CP of this isolate consists of 226 amino acids.

Cloning of CP gene. Nucleic acid from 0.2 g of fresh infected tissue was isolated by a phenol/chloroform method and precipitated by isopropanol. The complete CP gene corresponding to nt 952–1657 on the complete PNRSV RNA4 (the numbering according to that used for Acc. No. AF013287) was reverse transcribed and amplified by the Access reverse transcription–PCR (RT-PCR) Kit (Promega) under conditions described by Petrzik and Svoboda

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Abbreviations: CP = coat protein; DAS-ELISA = double-antibody sandwich ELISA; PNRSV = Prunus necrotic ringspot virus; RT-PCR = reverse-transcription–PCR; SDS-PAGE = polyacrylamide gel electrophoresis in the presence of SDS

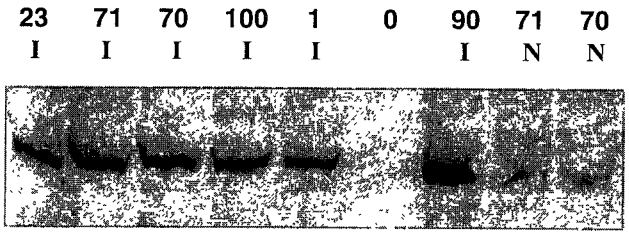

Fig. 1
Western blot analysis of various clones of *E. coli* for the presence of recombinant PNRSV CP
Induced (I) and non-induced (N) clones. Control *E. coli* culture without recombinant vector (0).
The polyclonal PNRSV antiserum used as primary antibody and the anti-rabbit alkaline phosphatase-conjugated IgG (Fc) used as secondary antibody.

(1997). The upstream primer with a *Nde*I restriction site 5′-ataCATATGGTTTGCCGAATTTGC-3′ and the downstream primer with a *Bam*HI site 5′-aaGGATCCTTCGGAGAAATTC GAGTGTGC-3′ (the sites underlined, non-viral nucleotides shown in lower case) were used. The amplified product of 705 bp was cleaved by appropriate enzymes and inserted into the pET16b expression vector with T7 bacteriophage promoter (Novagen). The recombinant vector was cloned in *E. coli* BL21 using ampicillin as selection marker.

Expression of CP gene was induced with IPTG (Serva) in final concentration of 0.1 mmol/l and the culture was incubated for 16 hrs.

Preparation of CP-enriched fractions. PNRSV CP was partially purified from one liter of the grown bacterial culture. The cells were centrifuged at 15,000 x g for 15 mins and the pellets were resuspended in 20 ml of 50 mmol/l Tris-HCl pH 8.0, 1 mmol/l EDTA, 100 mmol/l NaCl, 0.2 mg/ml lysozyme, 1% Triton X-100, 100 U/ml RNase I, 5 mmol/l benzamidine, and 100 µg/ml DNase I and incubated at 20°C for 1 hr. The bacterial lysate was centrifuged at 2,300 x g for 10 mins in Ti 50.2 rotor (Beckman). The pellet was saved and the supernatant further centrifuged at 5,000 x g for 20 mins. The process was then repeated at 10,000 x g for 30 mins, at 20,000 x g for 30 mins, at 50,000 x g for 45 mins, and at 100,000 x g for 1 hr. All these centrifugations were carried out at 4°C. Each pellet from these centrifugations, resuspended in small volume of PBS buffer, represented individual CP-enriched fraction and was subjected to further testing.

Protein concentration in each fraction was assayed spectrophotometrically at 280 nm (A_{280} = 1.0 for 1 mg/ml).

Polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE). The grown bacterial cells were pelleted by 2 mins centrifugation, resuspended in 100 µl of the Laemmli buffer, boiled for 2 mins and a 40 µl aliquot was loaded on 15% polyacrylamide gel containing SDS (Laemmli, 1970). Also individual CP-enriched fractions from the differential centrifugation were subjected to SDS-PAGE. For visualization of the separated proteins Coomasie Brilliant Blue was employed.

Western blot analysis. For this purpose, the proteins separated by SDS-PAGE were electroblotted to a nitrocellulose membrane (0.45 µm, Sigma) in a Mini Trans-Blot apparatus (Bio-Rad) at 90 mA for 16 hrs using a buffer consisting of 25 mmol/l Tris pH 8.3, 192 mmol/l glycine, 0.04% SDS, and 20% methanol. A polyclonal PNRSV antiserum (Loewe) diluted 1:2000 was used as primary antibody and an anti-rabbit alkaline phosphatase-conjugated IgG (Fc) (Promega) diluted 1:2000 was used as secondary antibody. The bands of interest were visualized by reaction with a substrate (BCIP/NBT, Serva) according to Sambrook *et al.* (1989).

DAS-ELISA. Individual CP-enriched fractions from differential centrifugation were subjected to DAS-ELISA using a polyclonal PNRSV antiserum (Loewe), according to Clark and Adams (1977).

Preparation of PNRSV CP antibodies. Three subcutaneous injections of 1, 2 and 2 mg of total protein of the CP-enriched fraction, obtained from the centrifugation at 20,000 x g for 30 mins, were given at 1st, 7th, and 14th day, respectively, to a rabbit. Five days after the last injection the rabbit was bled for serum preparation.

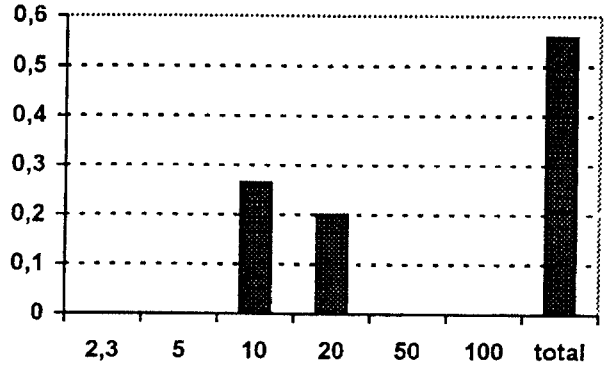

Fig. 2
DAS-ELISA of various CP-enriched fractions from differential centrifugation
Ordinate: A_{405} values. Abscissa: CP-enriched fractions from centrifugation at various g (x 10^{-1}). Total = unfractionated lysate.

Results and Discussion

Several bacterial clones containing the recombinant vector were isolated and tested by Western blot analysis for expression of PNRSV CP (Fig. 1). Non-induced clones produced much lower but visible amounts of this protein, while in non-transformed *E. coli* no such protein was detected. SDS-PAGE revealed one strong band at position corresponding to the M_r of about 25 K (data not shown). This is the expected M_r of the complete CP of the 1/13 isolate of PNRSV.

There are several methods for isolation of a desired protein from bacterial lysate. We chose differential centrifugation for its easy handling, simplicity and high yields of CP.

The recombinant CP from clone 90 was detected by DAS-ELISA in the fractions sedimenting at 10,000 x g and 20,000 x g (Fig. 2). Scanning of electrophoretic profiles of all the fractions showed the highest relative amount of proteins of M_r of about 25 K in fractions sedimenting at 10,000 x g and 20,000 x g too (data not shown). This analysis revealed that about 7 mg of total protein in PNRSV CP-enriched fractions was isolated from 1 liter of induced bacterial culture in total.

The titer of the prepared rabbit PNRSV CP antiserum was 1:2048. Although CP monomers were used for the immunization, the antiserum reacted in DAS-ELISA with positive PNRSV control as well as with naturally PNRSV-infected samples. The A_{405} value for the positive control was about 0.147 ± 0.045 (average from five experiments), which was about 5 times higher than that for the non-infected cucumber plant (0.031 ± 0.011), commonly used for propagation of PNRSV. The antiserum did not react with the lysate of non-transformed *E. coli* cells (A_{405} was 0.005 ± 0.005).

Here we demonstrated that a simple preparation of recombinant PNRSV CP-enriched fractions by differential centrifugation can be used for the PNRSV antiserum preparation.

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