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Oligomerization of the Potato Virus X 25-kD Movement Protein

A. D. Leshchiner[1,2], E. A. Minina[3], D. V. Rakitina[1], V. K. Vishnichenko[2], A. G. Solovyev[1,2], S. Yu. Morozov[1], and N. O. Kalinina[1]*

[1]*Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, 119992 Moscow, Russia; fax: (495) 939-3181; E-mail: kalinina@genebee.msu.ru*
[2]*Institute of Agricultural Biotechnology, Russian Academy of Agricultural Sciences, ul. Timiryazevskaya 42, 127550 Moscow, Russia; fax: (495) 977-0947; E-mail: vish@iab.ac.ru*
[3]*Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, ul. Miklukho-Maklaya 16/10, 117997 Moscow, Russia; fax: (495) 336-4511; E-mail: alyona.minina@gmail.com*

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Abstract—A 25-kD movement protein (25K protein) encoded by the first gene of the potexvirus *Potato virus X* triple gene block of transport genes is essential for the viral movement in infected plants. The 25K protein belongs to superfamily 1 of NTPase/helicases and exhibits *in vitro* RNA helicase, Mg^{2+}-dependent NTPase, and RNA-binding activities. In the present work, the ability of 25K protein for homologous interactions was studied using the yeast two-hybrid system, protein chemical cross-linking in the presence of glutaraldehyde, far-Western blotting, and ultracentrifugation in sucrose density gradients. The 25K protein was shown to form homodimers and homooligomers. Sites of homologous protein–protein interactions were found in both the N- and C-terminal portions of the protein.

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The 25K protein encoded by the first gene of the triple gene block (TGB1 protein) of the potato virus X (PVX) transport genes is a multifunctional protein manifesting *in vitro* some biochemical activities and being responsible for different functions *in vivo* during the viral life cycle [1, 2]. This protein belongs to superfamily 1 of NTPase/helicases and has seven conservative motifs specific for this superfamily [3]. 25K protein demonstrates *in vitro* RNA helicase activity and can unwind RNA duplexes in both directions [4]. 25K protein is also a Mg^{2+}-dependent NTPase [5, 6] and can nonspecifically and cooperatively interact with RNA [7]. Moreover, 25K protein specifically interacts with an end of PVX virions and thus activates translation of the genomic RNA in cell-free systems and induces structural rearrangement of the virion *in vitro* [8, 9]. In infected plants, 25K protein facilitates cell-to-cell transport of the PVX genome through the plasmodesmata. This is realized with involvement of two other movement proteins, TGB2 and TGB3, and also the virus coat protein [1]. 25K protein and a homologous TGB1 protein of white clover mosaic potexvirus can associate with plasmodesmata and increase their effective pore size and even move cell-to-cell in the plant in the absence of other components of the viral movement system [10-12]. 25K protein is also a suppressor of post-transcriptional gene silencing and a determinant of pathogenicity [13-15].

In this work, we have characterized the ability of 25K protein of potato virus X to oligomerize and shown that it can form both homodimers and homooligomers.

MATERIALS AND METHODS

Materials used were as follows: the Matchmaker System 3 two-hybrid yeast system (Clontech, USA); Taq-polymerase and restrictases (Fermentas, Estonia); Ni-NTA agarose (Qiagen, USA); secondary antibody conjugate with alkaline phosphatase and glutaraldehyde (Merck, Germany); a BCIP/NBT substrate and protein kinase A (Sigma, USA); X-ray film (Kodak, USA); Hibond-C extra nitrocellulose membrane with pore diameter of 0.45 μ (Amersham, USA).

Yeast two-hybrid system. The Matchmaker System 3 two-hybrid yeast system (Clontech) was used. The gene of

Abbreviations: AD, BD) activator and DNA-binding domains of the yeast Gal4 protein, respectively; GA) glutaraldehyde; PVX) potato virus X; TGB(1-3) proteins) proteins encoded by the genes (1-3) of the triple gene block.

* To whom correspondence should be addressed.

the PVX 25K protein was amplified using primers 2H-25K-N (CGAATTCATGGATATTCTCATCAGTAG) and Y2H-25K-C (CGGATCCTATGGCCCTGCGCGGACA) on the pRT103-PVX25K template. The amplification product and vectors pGAD-T7 and pGBK-T7 (Clontech) were treated with restrictases *Eco*RI/*Bam*HI and ligated. Yeast cells of the AH 109 strain (Clontech) were transformed using lithium acetate according to the Clontech protocol (Clontech Laboratories, Inc., Protocol No. PT3247-1, version No. PR94575).

Expression of recombinant protein genes in *E. coli* cells and purification of recombinant proteins by chromatography on Ni-NTA agarose. The *E. coli* strain M15 cells containing a high-copy repressor plasmid pRep-4 were transformed using the previously prepared recombinant plasmids [5-7]. The clones were grown overnight at 37°C on the standard 2× YT medium in the presence of ampicillin (100 μg/ml) and kanamycin (25 μg/ml). The overnight culture was diluted tenfold, grown at 37°C up to optical density of 0.8 at 600 nm. The protein expression was induced with isopropyl β-D-thiogalactopyranoside (final concentration 1-2 mM) followed by growth for 2-4 h at 37°C. The cells were separated from the culture liquid by centrifugation at 6000 rpm for 10 min using a Beckman J-21 centrifuge (Beckman, USA). Chromatography of $(His)_6$-proteins was performed under denaturing conditions as described in the isolation protocol of The QIAexpressionist (Qiagen). The resulting fractions were analyzed by SDS electrophoresis in 15% or 20% polyacrylamide gels according to Laemmli [16]. The gels were stained with Coomassie Brilliant Blue R-250.

Immunoblotting. The nitrocellulose membrane with the applied proteins was incubated with 5% defatted milk in buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.05% Tween-20 and then with a polyclonal rabbit antiserum to 25K protein. The sample was developed using a conjugate of secondary antibodies with alkaline phosphatase and the BCIP/NBT substrate solution.

Protein cross-linking in the presence of glutaraldehyde. Recombinant proteins were dialyzed against tri-distilled water. A 20-μl sample was supplemented with 0.5-1 μg protein in buffer containing 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 100 mM NaCl, and glutaraldehyde (GA) was added to the final concentration of 0.001, 0.01, 0.025, and 0.05%. The samples were incubated in the dark for 30 min at room temperature and analyzed by SDS-PAGE with subsequent immunoblotting.

Far-Western blotting. The nitrocellulose membrane with the applied proteins was incubated in blocking buffer containing 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.05% Tween-20, and 5% BSA, and then placed into buffer of the same composition but containing 1% BSA. The membrane was incubated for 3-4 h at room temperature in solution of ^{32}P-labeled 25K protein phosphorylated with protein kinase A as described in the producer's protocol (Sigma) and then washed free from the unbound protein and subjected to radioautography.

North-Western blotting. Proteins fractionated by SDS-PAGE were applied onto a nitrocellulose membrane. The membrane was incubated in buffer containing 20 mM Tris-HCl (pH 7.5), 6 M urea, and 0.05% Tween-20 and then placed into buffer containing 20 mM Tris-HCl (pH 7.5), BSA (0.2 g/liter), Ficoll (0.2 g/liter), and polyvinylpyrrolidone (0.2 g/liter), and the proteins were renatured for 1.5 h. The membrane was incubated for 1 h with ^{32}P-labeled RNA prepared on the linearized plasmid pXT7-25 by RNA polymerase T-7 transcription as described in [5] in a renaturing buffer containing 100 mM NaCl. The membrane was washed clean in this buffer, dried, and subjected to radioautography.

Ultracentrifugation in sucrose density gradient. Dialyzed protein preparations were layered onto 10-30% sucrose concentration gradient prepared in buffer containing 50 mM Tris-HCl (pH 7.8), 100 mM NaCl, 5 mM $MgCl_2$, and 2 mM dithiothreitol and centrifuged at 4°C for 21 h at 36,000 rpm in an SW41 rotor on a Beckman L-2 ultracentrifuge. Proteins from the collected fractions were precipitated with 12% TCA. The precipitates were collected by centrifugation, washed in acetone, dried, and dissolved in the Laemmli sample buffer. The proteins of the fractions were analyzed by SDS-PAGE and immunoblotting. The developed membranes were scanned, and the band intensities were calculated with the GelPro computer program. The data processed with the Excel program were plotted as the protein quantity (the manifestation intensity) in the gradient fractions.

RESULTS AND DISCUSSION

Detection of homologous protein–protein interactions using the yeast two-hybrid system. The yeast two-hybrid system was used to show the homologous interaction of 25K protein. The gene of PVX 25K protein was cloned into the yeast expression vectors pGAD-T7 and pGBK-T7. The resulting constructions encoded 25K protein fused with an activator domain (AD) and DNA-binding domain (BD) of the yeast protein Gal4, respectively. The vectors pGAD-T7-T and pGBK-T7-p53, encoding, respectively, a large T-antigen of the SV40 virus fused with AD and a region of the mouse protein p53 fused with BD, were used as a positive control. The p53 protein and SV40 T-antigen were earlier shown to interact with each other under conditions of the yeast two-hybrid system [17]. The vector pair of pGAD-T7-T and pLam-C (the latter encoding the lamin C region fused with BD) was used as a negative control. Under conditions of the yeast two-hybrid system, lamin C fails to produce complexes and interact with other proteins [18].

These constructions were used to transform the strain AH 109 yeast cells possessing the reporter genes of

adenine and histidine enzyme synthesis controlled by the GAL4 promoter. The co-transformed cells were planted onto medium SD-2 and the cells containing both vectors were selected (Fig. 1a). Selective medium SD-2 is free of leucine and tryptophan necessary for yeast cell growth, and the genes of enzymes responsible for synthesis of these amino acids are encoded by the pGAD-T7 and pGBK-T7 vectors, respectively. The protein–protein interaction was revealed by detecting the Gal4-dependent expression of the reporter genes. To do this, the co-transformed cells were planted onto selective adenine- and histidine-free medium SD-4. As expected, the cell co-transformation with the pGAD-T7-T and pGBK-T7-p53 vectors was associated with an active cell growth on the selective medium SD-4, whereas cells co-transformed with the pGAD-T7-T and pLamC constructions were unable to grow on the medium lacking adenine and histidine (Fig. 1b). The presence of colonies upon cell co-transformation with the pGAD-T7-25K and pGBK-T7-25K vectors suggested that 25K protein was capable of homologous interaction under conditions of the yeast two-hybrid system (Fig. 1b). Similar results were earlier obtained for the Potato mop-top pomovirus TGB1 protein [19]. This protein was detected by immunoblotting in extracts from infected plants only as a monomer, but its ability to form dimers was shown using the yeast two-hybrid system [19].

Oligomerization of 25K protein preparations in the presence of glutaraldehyde (GA). Under standard conditions of SDS-PAGE and on staining with Coomassie, the recombinant 25K protein was revealed as a single band with a molecular weight of about 30 kD [5]. By immunoblotting, 25K protein was also detected as a monomer in extracts of PVX-infected tobacco plants [20]. However, the cooperative binding of the protein with RNA [7] and data obtained under conditions of the yeast two-hybrid system suggested its ability for homogeneous interaction. This ability of the protein was analyzed using chemical cross-linking in the presence of GA, which is a short self-polymerizing reagent mainly interacting with ε-amino groups of lysine. Treatment of 25K protein preparation with increasing concentrations of GA and the subsequent development by immunoblotting indicated that the protein could produce dimers, trimers, and tetramers (Fig. 2). Minor amounts of dimer and trimer were also found in the initial preparation of 25K protein (Fig. 2, control). Production of dimers was earlier observed in preparations of the Bamboo mosaic potexvirus recombinant TGB1 protein [21, 22]. The efficiency of GA cross-linking and character of resulting 25K protein oligomers did not change with increase in the NaCl concentration in solution to 500 mM (Fig. 2). Therefore, the interaction should not be electrostatic.

Regions involved in homologous protein–protein interactions in 25K protein. To reveal the protein regions responsible for homologous interactions, we used the previously described 25K protein mutants: a protein with the deleted C-terminal portion ($25K_{I-II}$) and a protein with the deleted N-terminal portion (ΔN25K), which represented, respectively, the N- and C-portion of 25K protein [6, 7]. By SDS-PAGE and staining the gel with Coomassie, the recombinant mutant $25K_{I-II}$ protein was visualized as two bands, one of which corresponded by molecular weight to the recombinant protein monomer and the other (with the lower electrophoretic mobility) to its dimer (Fig. 3a, lane *1*). The dimer and monomer amounts were comparable. Thus, the mutant protein representing the N-terminal portion of 25K protein (consisting of 96 amino acid residues) can form stable dimers resistant to SDS. Such enormously stable oligomers detectable by denaturing SDS-PAGE have been described for some proteins [23-25]. Treatment of $25K_{I-II}$ protein preparations with GA resulted in appearance of an additional oligomer (possibly a hexamer) (Fig. 3a, lane *3*). However, the ΔN25K mutant always gave only one band in the gel on staining with Coomassie (Fig. 3b, lane

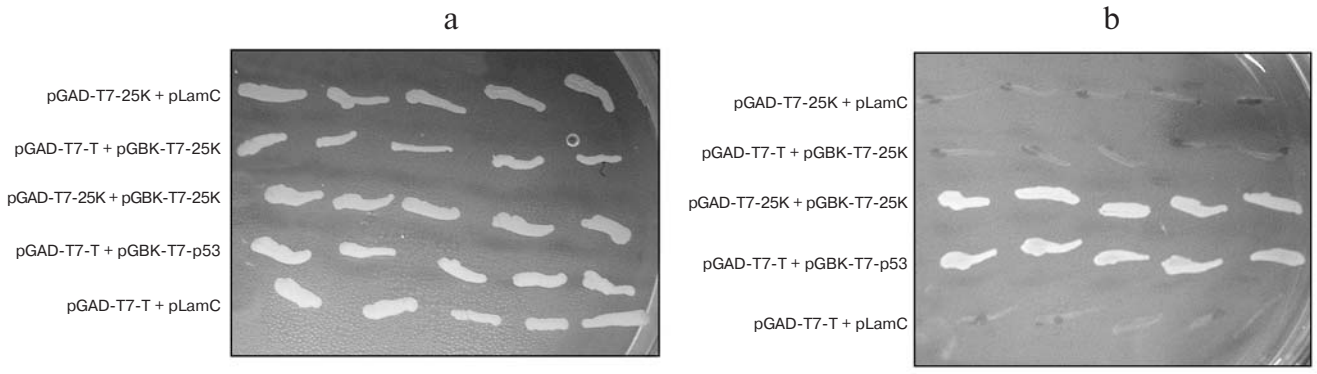

Fig. 1. Ability of 25K protein for homologous interaction under conditions of the yeast two-hybrid system. Growth of transformed yeast cells on the selective mediums SD-2 (a) and SD-4 (b). pGAD-T7-T + pGBK-T7-53, positive control; pGAD-T7-T + pLamC, negative control; pGAD-T7-T + pGBK-T7-25K and pGAD-T7-25K + pLamC, controls of self-activity of 25K protein fused with the activator domain (AD) and DNA-binding domain (BD) of the yeast protein Gal4, respectively.

1). Nevertheless, by immunoblotting dimers were revealed in the ΔN25K protein preparation (Fig. 3b, lane *2*), and treatment with GA increased the quantity of dimers but no oligomers appeared (Fig. 3b, lane *3*). Thus, both portions of 25K protein contain regions capable of homologous interaction.

These findings were also confirmed by another experimental approach. Upon SDS-PAGE, 25K protein and its mutants were applied onto a nitrocellulose membrane, which was incubated with ^{32}P-labeled 25K protein. The 25K protein used in this experiment was a modified polypeptide carrying next to the sequence of six histidine residues a site of phosphorylation by protein kinase A. This protein was expressed in *E. coli*, isolated by affinity chromatography, and treated *in vitro* with protein kinase A in the presence of [γ-^{32}P]ATP. Figure 3c shows that both the full-length protein and the two mutants interacted with the ^{32}P-labeled 25K protein in the presence of 100 mM NaCl.

The cooperative RNA–protein interaction suggested that placing of the first protein molecule on RNA should facilitate the binding of other molecules. The N-terminal region of 25K protein (the 25K$_{I-II}$ mutant protein) interacted with RNA in solution with the same cooperativity (Hill coefficient values) as the full-length 25K protein [7]. The N-terminal subdomain of 25K protein (the 25K$_{I-II}$ mutant protein) is the least structure sufficient for manifestation of the ATPase and RNA-binding activities of the protein [7]. The data presented here indicate that this minimal subdomain is also responsible for the protein oligomerization. Moreover, by North-Western immunoblotting natural dimers and oligomers of the 25K$_{I-II}$ protein were shown to effectively interact with labeled RNA (Fig. 3a, lane *4*). These findings not only reveal the functional activity of oligomers but also suggest that the sites responsible for binding with RNA and the protein

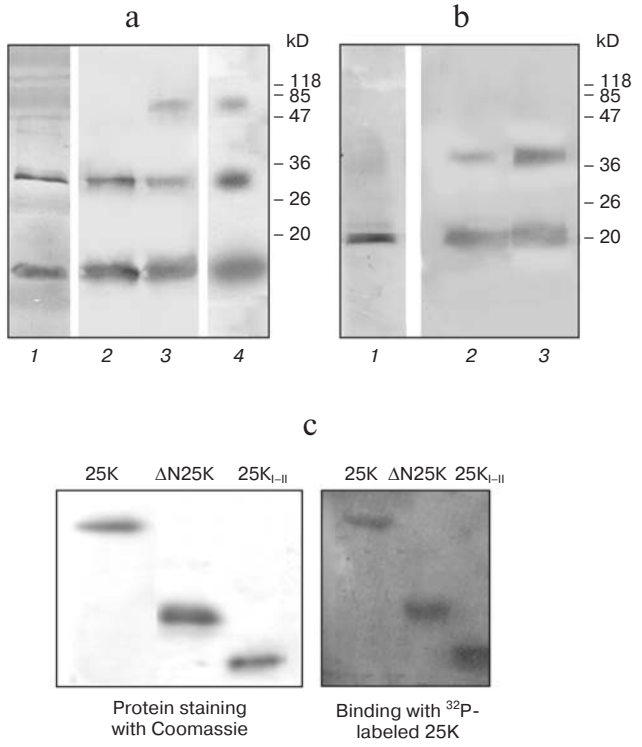

Fig. 3. Detection of the ability of 25K protein mutants for homologous interaction. The recombinant proteins with the deleted C-terminal portion (25K$_{I-II}$) (a) and N-terminal portion (ΔN25K) (b) were analyzed by SDS-PAGE and immunoblotting. Lanes: *1*) gel staining with Coomassie; *2*) immunoblotting of the GA-untreated protein; *3*) immunoblotting of the protein treated with 0.01% GA; *4*) North-Western blotting of the RNA-binding activity of 25K$_{I-II}$ mutant protein and its oligomers. A nitrocellulose membrane with the 25K$_{I-II}$ protein applied on it was incubated with ^{32}P-labeled RNA-transcript and subjected to radioautography. To the right, positions of marker proteins are shown. c) Far-Western blotting of interactions of 25K protein and its deletion mutants with the ^{32}P-labeled full-length 25K protein (staining with Coomassie and radioautograph).

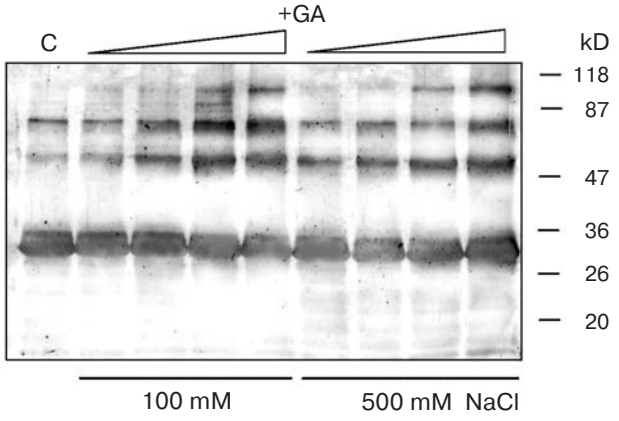

Fig. 2. Oligomerization of 25K protein. Analysis of the recombinant 25K protein preparation by SDS-PAGE and immunoblotting without GA treatment and in the presence of different concentrations of GA (from 0.001 to 0.05%) and NaCl (100 and 500 mM). C, control.

homooligomerization are not overlapping because they are available for RNA inside oligomers.

The C-terminal portion of the protein does not interact with RNA [6, 7]. The findings of the present work indicate that this region of 25K protein also contains a site for homologous protein–protein interactions. The C-terminal portion in the full-length 25K protein containing two cysteine residues is likely to be involved in the protein dimerization providing for stabilization of oligomers produced by the N-terminal region.

Analysis by ultracentrifugation in sucrose density gradient of oligomeric complexes produced by 25K protein and its N-terminal region (the 25K$_{I-II}$ protein). Preparations of 25K and 25K$_{I-II}$ recombinant proteins were fractionated by ultracentrifugation in sucrose density gradient (10-30%), and the resulting gradient fractions were analyzed by SDS-PAGE and immunoblotting. The protein under analysis was not detected in the gradients as monomers

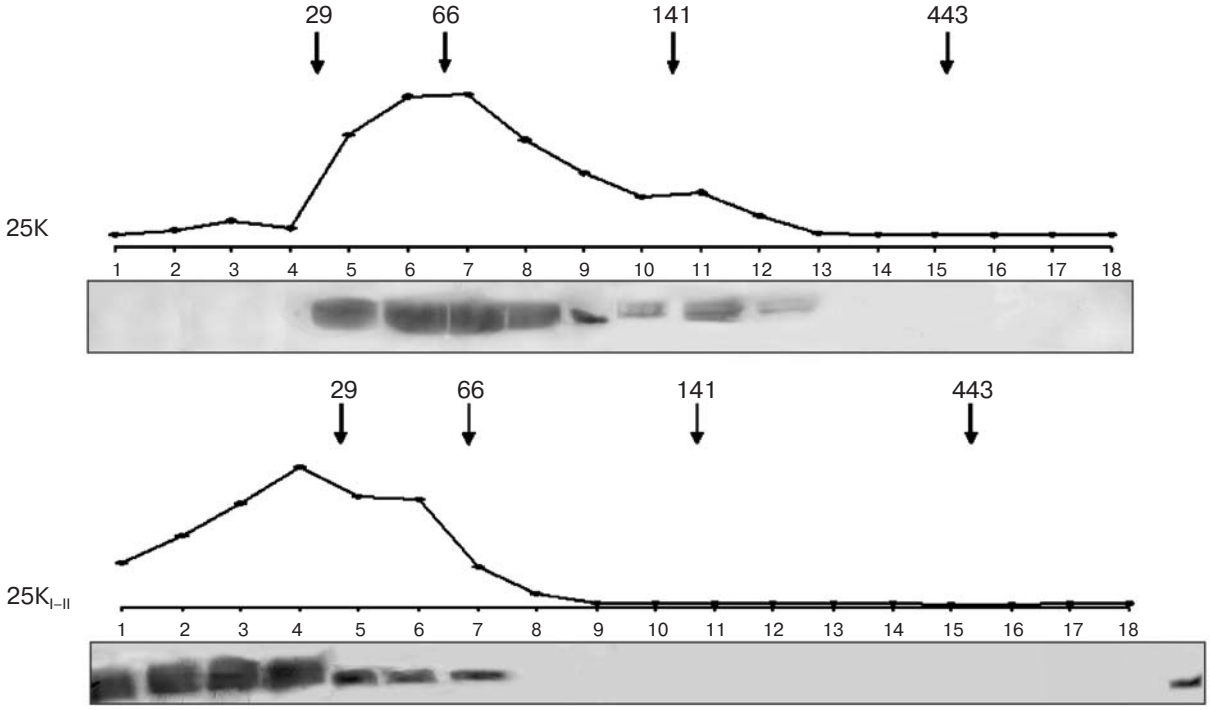

Fig. 4. Fractionation of the full-length 25K protein and the $25K_{I-II}$ mutant protein by ultracentrifugation in sucrose density gradient. Preparations of the recombinant proteins were centrifuged in 10-30% sucrose density gradient. The proteins of the gradient fractions were analyzed by SDS-PAGE and immunoblotting. At the top, the distribution of the band density (intensities of the protein immunodetection) in the fractions is presented. The arrows show positions of marker proteins (with the molecular weight indicated), which were fractionated in parallel tubes: alkaline anhydrase (29 kD), BSA (66 kD), alcohol dehydrogenase (141 kD), and apoferritin (443 kD).

(Fig. 4): all proteins were revealed as different oligomers. The major part of 25K protein was recognized as a dimer, and the minor fraction seemed to be a hexamer. The major part of $25K_{I-II}$ protein also was detected as a dimer.

Thus, we demonstrate the ability of the PVX 25K movement protein to form homodimers and homooligomers. In solutions, 25K protein is present, at least, as a dimer. We have also shown that dimers and oligomers produced by the protein N-terminal region interact with RNA.

Oligomerization is known to play an important role in functioning of different proteins. 25K protein is an RNA helicase [4]. Various helicases functioning as dimers and oligomers are prone to homologous interactions [3, 26]. Thus, the enzymatic activity of the tobacco mosaic virus replicase helicase domain with the amino acid sequence closely related to that of TGB1 proteins directly depends on the ability of the protein to form homohexamers [27]. PVX virions are suggested to be destabilized during translocation through plasmodesmata *in vivo*, that results in transport of translatable viral RNA in the adjacent cells [9, 28]. One cannot exclude that 25K takes part in this process as an homooligomer. We have earlier shown that 25K protein forms complexes with both high-molecular-weight viral RNAs and short RNA oligonucleotides [7]. Thus, it could also appear that the revealed ability of 25K protein to oligomerize is necessary for producing ribonucleoprotein complexes with short interfering RNA during its functioning as a suppressor of post-transcriptional gene silencing.

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