Characterization of Potato Virus M epitopes with the use of synthetic peptides

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As a result of thermolysin hydrolysis of a coat protein (CP) of Potato Virus M Ukrainian Strain UJ (PVM), the heptapeptide $^{29}$AADFEGJC was found to be recognised by two PVM specific monoclonal antibodies (MAbs) M6D5 and M9G1. This heptapeptide represents the C-terminal part of tryptic tetradecapeptide $^{21}$EARPLPTAADFEGK\textsubscript{(P14)} which was also recognised by the same MAbs. The peptides represented sequences of tryptic (P14), thermolysin (P7) fragments and three heptapeptides containing alanine substitutions for Asp\textsubscript{31} and Glu\textsubscript{33} were synthesised to determine the contribution of dicarboxylic amino acids in the antigen-antibody interaction. All synthetic heptapeptides were recognised by both MAbs weakly in indirect ELISA. These peptides were also used as inhibitors of MAb-CP and MAb-P14 reactions in inhibition ELISA. The results of inhibition ELISA have shown the following: 1) the same concentrations of peptides were more effective to inhibit the interaction of MAbs with P14 than with CP; 2) substitutions of charged amino acids decreased noticeably the ability of peptides to inhibit the antigen-antibody interaction, especially the substitution of Asp\textsubscript{31}; 3) heptapeptides containing alanine substitutions suppressed more effectively the interaction of MAb M6D5 with antigens and were less effective to inhibit the reaction of MAb M9G1 with the same antigens. Thus the difference in Asp\textsubscript{31} and Glu\textsubscript{33} contributions to the antigen-antibody complex formation has been found.

Introduction. Previously the antigenic analysis of two Potato Virus M Ukrainian strains (PVM U1 and U7) was carried out with the use of three monoclonal antibodies (MAbs M6D5, M9G1 and M4C1) [1]. The PVM-specific epitopes were established to be located in the N-terminal region of the coat protein (CP) and sequentially overlapped each other [1]. In that study the tryptic fragments $^{22}$Glu-Lys\textsubscript{35} (PVM U1) and $^{22}$Gly-Lys\textsubscript{35} (PVM U7) were recognised by two MAbs M6D5 and M9G1. The amino acid substitution Glu\textsubscript{35} \textrightarrow Gly at the position 22 of PVM U7 CP did not affect the antibody binding of the tryptic peptide and PVM U7 CP. It was shown that MAb M9G1 interfered with MAb M4C1 for binding PVM CP sites in the dot immunobinding assay. The synthetic peptide P14, corresponding to the tryptic fragment $^{22}$Glu-Lys\textsubscript{35}, inhibited the MAbs M6D5 and M9G1 interaction with CP and peptide P14 with different level of ELISA inhibition. The modification of the side chain positive charge of Lys\textsubscript{35} to negative one in tryptic peptides $^{22}$Glu-Lys\textsubscript{35} and $^{22}$Gly-Lys\textsubscript{35} using citraconic anhydride, resulted in two-fold increase of the MAb M9G1 reaction and slightly reduced the MAb M6D5 interaction with both fragments. On the basis of these results, it was concluded that: 1) PVM-specific epitopes are located at the N-terminal region of PVM coat protein; 2) MAbs M6D5 and M9G1 recognise sequentially overlapping epitopes and the common part of both epitopes is presented in the $^{22}$GWGly-Lys\textsubscript{35} fragment; 3) MAbs M4C1 and M9G1 recognise either overlapping or conformational approximated epitopes. Also it was suggested that the region at position 34—49 of PVM CP is disposed to form the loop with $\pi$-turn [1]. In the present study the synthetic heptapeptides containing alanine substitutions of dicarboxylic amino acids were used for more detailed analysis of PVM epitopes.
Materials and Methods. Virus purification. PVM U1 strain was purified as described in [2], using 30 mM KH₂PO₄-KOH buffer with 5 mM EDTA and 0.2% 2-mercaptoethanol, pH 7.5, and 0.3 M glycine-KOH buffer, pH 8.5. All purification procedures were performed at 4 °C. The virus concentration was estimated assuming the extinction coefficient at 260 nm of 2.88 cm²·mg⁻¹. The A₂₆₀/A₃₆₀ = 1.21, A₅₆₀/₅₄₀ = 1.12 ratios [3] and SDS-electrophoresis [4] were used to analyse purity and quality of virus preparations. The purified virus were stored at −20 °C in 0.3 M glycine-KOH buffer, pH 8.5, containing 50% glycerol.

Coat protein preparation. PVM U1 coat protein (CP U1) was prepared according to [5]. The purified virus at the concentration of 3—5 mg/ml in the 0.3 M glycine-KOH buffer, pH 8.5, was treated with the mixture of guanidine-HCl and LiCl at the final concentration of 4 M and 2 M, respectively, and followed by freezing at −70 °C for 3 h and thawing at room temperature. The precipitated virus RNA was collected by centrifugation at 5000 g for 30 min. The supernatant containing coat protein was dialysed against ammoniac water, pH 8.0, and lyophilised. The protein concentration was estimated according to Lowry et al. [6].

Proteolytic cleavage of PVM coat protein. CP U1 was digested with thermolysin («Sigma», USA) in 0.1 M ammonium bicarbonate, pH 8.0, at 37 °C for 4 h at the enzyme-substrate ratio 1:100 (w/w). The reaction was stopped by adding 0.1% acetic acid to pH 2.5. The thermolysin-treated proteins were stored at −20 °C.

Fractionation of thermolysin peptides and sequencing. CP U1 thermolysin mixture was fractionated by High Performance Liquid Chromatography (HPLC) on a C18 LiChrosorb RP-18 column (5 μm, 4.6 mm x 250 mm, «LKB», Sweden) using the 2—60% linear gradient of acetonitrile («Fluka», Switzerland) in 0.1% trifluoroacetic acid (TFA) at the flow rate of 1.0 ml/min. Optical density was monitored at 206 nm and 280 nm. The collected fractions were dried and peptide material of each fraction was dissolved in an appropriate buffer before using in further experiments.

The thermolysin fractions found to be recognised by MAbs were repeatedly fractionated using the same column and combination of linear gradient (2—40%) and isocratic acetoniitrile elution in 0.1% TFA. The amino acid sequence analysis was performed in a gas sequenator, model 816 («Knauer», Germany), according to the manufacturer protocol.

Peptide synthesis. Synthetic peptides P14 (H₂N-22EARPLTAADFEGK⁻²⁵-COOH), P7 (H₂N-²⁹AADFEGK⁻²⁰-COOH), P1 (H₂N-²⁹AAAFEGK⁻²⁰-COOH), P2 (H₂N-²⁹AADFAGK⁻²⁰-COOH), P3 (H₂N-⁻⁹⁹KDASSVF⁻¹⁰⁰-COOH) and peptide H₂N⁻¹⁴⁴KDASSVF⁻¹⁵⁴-COOH as a negative control were synthesised (NPO Verta, St.-Petersburg, Russia) by the solid phase method using Boc-protected amino acids, according to [7].

ELISAs. Lyophilised protein and vacuum dried peptide samples were dissolved in phosphate buffered saline: 10 mM Na₂HPO₄-NaH₂PO₄, pH 7.3, containing 0.14 M NaCl (PBS). Microtiter plates («Nunk», Denmark) were coated with antigens by evaporating PBS at 37 °C overnight. Antigen-coated wells were rinsed with PBS and blocked with 1% BSA in PBS for 4 h at 37 °C. MAbs were diluted in PBS containing 0.05% Tween-20 (PBST) and 0.3% BSA and incubated with antigens for 2 h at 37 °C. After this the plates were washed five times with PBST, and the rabbit anti-mouse IgG-horse radish peroxidase conjugate (1:2000) in PBST with 0.3% BSA was added. The plates were incubated at 37 °C for 1.5 h and washed five times with PBST. A colour reaction was developed by adding substrate buffer (0.1 M citrate-0.15 M Na₂HPO₄, pH 4.5), containing 0.6 mg/ml o-phenylenediamine («Fluka», Switzerland) and 0.01% H₂O₂. ELISA optical density was measured at 450 nm using Kinetic Microplate Reader (Molecular Devices, «Fisher», USA).

In the inhibition ELISA experiments, MAbs were incubated with two-fold decreasing concentrations of synthetic peptides P14, P7, P1, P2 and P3 for 1 h at room temperature. Then these mixtures were added to the precoated antigens CP U1 — 500 ng (14 pM) per well or peptide P14 — 50 ng (33 pM) per well with following incubation for 1 h at 37 °C. The other steps of inhibition ELISA were performed as described above in ELISA procedure.

Further in the text all concentrations of antigens and inhibitors mean the amount of the material per well.

Monoclonal antibodies. MAbs M6D5, M4C1 and M9G1 were raised against native virus particles of PVM-Russian strain and were characterised earlier [8]. Professor M. Saarma, Institute of Chemical Physics and Biophysics (Estonia) has kindly provided the antibodies for this study.

Results and Discussion. For the further study of PVM antigenic structure the thermolysin hydrolysis of PVM CP was carried out. The products of proteolytic cleavage were fractionated by HPLC using linear acetonitrile gradient. The fractions collected were tested by indirect ELISA using three MAbs, M6D5, M4C1 and M9G1. MAb M4C1 did not re-
recognise any thermolysin fraction while MAbs M6D5 and M9G1 reacted with fraction 17. After additional HPLC purification using a combination of linear gradient and isocratic acetonitrile elution it has been shown, that fraction 17 consists of three subfractions: 17, 17, and 17. Only subfraction 17, was recognised by MAbs M6D5 and M9G1 in ELISA. The primary structure of 17, subfraction peptide AADFEGK was established on a gas sequenator. The sequence of this heptapeptide corresponds to the fragment of PVM Russian strain CP [9] at the positions 29–35 and represents the C-terminal part of tryptic tetrapeptide EARPLPTAADFEGK that was also recognised by MAbs M6D5 and M9G1 [1].

In previous work [1] we pointed out that lack of reaction between MAb M9G1 and lengthened peptides Glu/Gly-Arg or may be caused by structural changes in the C-terminal region of this fragment after protein cleavage by trypsin. These structural changes did not affect the recognition of Glu/Gly-Arg peptides by MAb M6D5. The similar phenomenon, when the antibody recognised the short peptide and did not recognise the lengthened peptide, have been observed by other investigators [10, 11] and had no explanation until present. In our case we suggested that region Gly-Asp formed the loop with π-turn [12] and some of M9G1 epitope’s amino acids were involved in the formation of this structure. The enzymatic destruction of PVM CP resulted in drastic distortions of the π-turn structure and abolished the recognition of M9G1 epitope in peptides Glu/Gly-Arg by corresponding MAb, whereas the main portion of M6D5 epitope located out of the π-turn structure and both peptides Glu/Gly-Lys and Glu/Gly-Arg were recognised by MAb M6D5. Thus, most likely, the M6D5 epitope is located at the N-terminal part while M9G1 epitope is shifted to the C-terminal part of Glu/Gly-Arg fragment [1].

On the basis of ELISA of thermolysin fragments, the linear heptapeptide AADFEGK (P7) was synthesised. Additionally, the following peptides, containing alanine substitutions of charged amino acid were synthesised: 1) peptide AADFEGK (P1) with Asp → Ala substitution; 2) peptide AADFAGK (P2) with Glu → Ala substitution; 3) peptide AADFAGK (P3) with Asp → Ala and Glu → Ala simultaneous substitutions. The alanine substitutions can help to find out the contribution of negatively charged amino acids in the MAb-antigen interaction. Generally accepted, that in most cases one amino acid substitution on the protein molecule surface resulted only in local changes, although the mutations are known that alter the distant regions conformation and thus have an indirect effect on antigenicity [13–16].

In indirect ELISA MAbs M6D5 and M9G1 reacted with 500 ng (14 pM) of CP equally \( E_{450} = 2.5 \), while the reaction of MAb M6D5 with synthetic peptide P14 (50 ng or 33 pM) \( E_{450} = 2.6 \) was significantly higher then of MAb M9G1 \( E_{450} = 1.4 \). The difference in reactions between MAbs and peptide P14 shows that only the part of M9G1 epitope is presented in peptide P14, whereas this peptide contains the most part of M6D5 epitope. Above mentioned concentrations of CP and peptide P14 were used as coating concentrations in inhibition ELISA.

Synthetic peptides (P7, P1, P2 and P3) were analysed in indirect and inhibition ELISA formats. In indirect ELISA MAbs M6D5 and M9G1 detected the peptide P7 at the minimal concentrations of 10 pM (7 ng) and 1 nM (700 ng), respectively, while peptides P1, P2 and P3 were not recognised by the antibodies at the concentration of 100 nM. The results of analysis of synthetic peptides P7, P1, P2 and P3 as coating antigens were highly contradictory and the level of MAbs reaction did not correlate with peptide concentrations.

This effect can be referred to very poor adsorption of short peptides to the plate material that leads to decrease or lack of ELISA reaction. Usually, to avoid such problem, the short peptides are used as inhibitors of the interaction between antibodies and antigens. In our study we used this approach to analyse the abilities of synthetic peptides P7, P1, P2 and P3 to inhibit the reaction of MAbs M6D5 and M9G1 with PVM CP and synthetic peptide P14. Synthetic peptide VASDDSVESTH was used as a negative control and displayed any inhibition capability in MAb-CP and MAb-P14 reactions.

The inhibition ELISA results showed that peptides P7, P1, P2 and P3 inhibited the interaction between MAbs and CP or P14 with various effectiveness. 50 % inhibition of the M6D5-P14 and M9G1-P14 interactions was achieved using following concentrations of inhibitors \( C_{50} \): 17 and 22 pM of P7 and 24 pM and 12 nM of P14, respectively (Fig. 1, 2). Maximum 95—96 % inhibition activity of peptide P7 was observed at concentration 390 pM \( C_{max} \). In the range of 1.5—100 pM concentrations of peptide P7 inhibited the reaction M9G1-P14 weaker, than the reaction M6D5-P14 (Fig. 1, lines 2 and 1, respectively). The reaction M9G1-P14 was more effectively inhibited with peptide P7 (Fig. 1, line 2) than with peptide P14 (Fig. 2, line 3): \( C_{50} = 22 \) pM and 12 nM, respectively. Peptide P7 at the concentration of 390 pM caused the 96 % suppression of M9G1-P14 interaction (Fig. 1, line 2), while the same molar concentration of peptide P14 suppressed only 8 % of this reaction (Fig. 2, line 3). 95 % of P14
Inhibition of M6D5-P14 and M9G1-P14 interactions with the use of synthetic peptides P7 and P14 as inhibitors. Lines: 1 — inhibition of M6D5-P14 interaction by peptide P7; 2 — inhibition of M9G1-P14 interaction by peptide P7; 3 — inhibition of M6D5-P14 interaction by peptide P14. Coating concentration of peptide P14 was 50 ng (33 pM). MAbs were used at the next dilutions: M6D5 — 1:1000, M9G1 — 1:500. The vertical dotted lines (—) mark the inhibitor concentrations required for 50 % inhibition of antibody-antigen interaction. Negative control data are not shown.

Inhibition of MAbs-CP and MAbs-P14 interactions with the use of peptides P7 and P14 as inhibitors. Lines: 1 — inhibitor P14, interaction M6D5-CP; 2 — inhibitor P7, interaction M6D5-CP; 3 — inhibitor P14, interaction M9G1-P14; 4 — inhibitor P14, interaction M9G1-CP. Coating concentration of CP was 500 ng (13 pM), of peptide P14 — 50 ng (33 pM). MAbs were used at the next dilutions: M6D5 — 1:1000, M9G1 — 1:500. Vertical dotted lines (—) mark the inhibitor concentrations required for 50 % inhibition of the antibody-antigen interaction.

The inhibition effect was achieved at the concentration of 200 nM (Fig. 2).

The higher concentrations of peptide P7 were required to inhibit the MAbs-CP interaction in comparison with MAbs-P14. 50 % inhibition of M6D5-CP reaction was achieved in the presence of 2.5 and 2.2 nM of peptides P7 and P14, respectively, and C_max of both peptides were 200 nM (Fig. 2, lines 1, 2). The interaction M9G1-CP was suppressed by 50 % with 136 nM of peptide P14 (Fig. 2, line 4). These results showed that conformation of peptide P7 was more appropriate for MAb M9G1 paratope while the peptide P14 was stronger inhibitor of reactions M6D5-CP/P14.

The results of inhibition ELISA of MAbs-CP and MAbs-P14 interactions using peptides P1, P2 and P3 as inhibitors are presented in Fig. 3 and 4. 50 % inhibition of M6D5-P14 reaction (Fig. 3) was achieved at the concentrations of peptide P1 10.5 nM (line 2), P2 — 5 nM (line 1), P3 — 100 nM (line 5). The same inhibition effect of M6D5-CP reaction was observed at the following peptide concentrations: P1 — 36 nM (line 4), P2 — 17.5 nM (line 3); peptide P3 (line 6) did not show 50 % inhibition at the used concentration range.

Peptides P1, P2 and P3 revealed poor inhibition abilities in M9G1-CP and M9G1-P14 reactions (Fig. 4), and only peptide P2 suppressed reaction M9G1-P14 at C_50 = 38 nM (Fig. 4, line 1). Peptide P7 with an original sequence almost completely suppressed the interaction MAbs-P14 at C_max = 390 pM (Fig. 1, lines 1, 2), while peptide P2 at the concentration of 200 nM showed 90 % and 97 % inhibition of M9G1-P14 (Fig. 4, line 1) and M6D5-P14 (Fig. 3, line 1) reactions, respectively. 200 nM of peptide P2 suppressed by 92 % and 35 % reactions M6D5-CP (Fig. 3, line 3) and M6D5-CP (Fig. 3, line 5), respectively. Peptide P7 inhibited by 97 % the M6D5-CP reaction (Fig. 2, line 2).

200 nM of peptide P1 containing the alanine substitution of aspartic acid inhibited by 94 % and 10 % the interactions M6D5-P14 (Fig. 3, line 1) and M9G1-P14 (Fig. 4, line 4), respectively. The same peptide inhibited by 83 % and 2 % the interaction M6D5-CP (Fig. 3, line 4) and M9G1-CP (Fig. 4, line 5), respectively.

Simultaneous substitution of Asp^{31} and Glu^{33} in peptide P3 decreased drastically the ability of this peptide to inhibit the reaction of MAbs with both antigens. At the concentration 200 nM peptide P3 did not suppress completely any reaction, displaying 72 % and 33 % of inhibition in M6D5-P14 (Fig. 3, line 5) and M6D5-CP (Fig. 3, line 6) interactions, respectively. The reactions of MAb M9G1 with CP and P14 were inhibited with 200 nM of peptide P3 by 1 % (Fig. 4, line 6) and 18 % (Fig. 4, line 3), respectively.

Thus, the comparison of inhibition abilities of
peptides P7, P1, P2 and P3 showed that peptide P3 (Asp$^{31}$ → Ala and Glu$^{33}$ → Ala) was the weakest. Peptide P2 (Glu$^{33}$ → Ala) maintained the highest residual inhibition activity in comparison with peptides P1 and P3. Peptide P1 (Asp$^{31}$ → Ala) also significantly affected the reactions of MAb s with both antigens. Peptides P1, P2 and P3 were more effective inhibitors of MAb M6D5-antigen reactions (Fig. 3) than MAb M9G1-antigen reactions (Fig. 4). The same inhibitor (peptides P14, P7, P1, P2, P3) concentrations more effectively suppressed the interactions MAb s-P14, than MAb s-CP. These data indicate that the native conformation and environment of this region in the protein molecule are very important for the interaction between MAb s and PVM epitopes, even if all critical residues of the epitopes are assumed to be located in the Glu-Lys region (peptide P14).

In present study the contribution of individual residues (Asp$^{31}$ and Glu$^{33}$) in the MAb-antigen complex formation has been evaluated using the alanine substitutions. It has been established that both residues Asp$^{31}$ and Glu$^{33}$ are involved in the M6D5 and M9G1 epitopes and contribute differently to the formation of the antigen-antibody complexes. Asp$^{31}$ is more crucial residue of both epitopes than Glu$^{33}$. Both amino acids Asp$^{31}$ and Glu$^{33}$ in M9G1 epitope are more important for recognition by corresponding MAb than the same residues in the structure of M6D5 epitope. The simultaneous substitution of Asp$^{31}$ and Glu$^{33}$ in the M9G1 epitope drastically decreases the recognition of this epitope by the antibody that shows the key role of these residues in the epitope, while M6D5 epitope in the absence of Asp$^{31}$ and Glu$^{33}$ residues partially reserves the ability to be recognised by the corresponding antibody. It is obvious that neighbour amino acids in the M6D5 epitope considerably contribute to the antigen-antibody complex formation.

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Стаття описує аналіз епітопів М-віруса картоплі (PVM) термолізином виділено фрагмент MJ^ M9G1 с теми же антигенами. Таким чином, Asp та Glu по-різному впливають на формування комплексу антиген—антитіло; 3) гептапептиди, що містять заміни Асп та Глу на аланин. Все синтетичні гептапептиди слабко взаємодіяли з обою MKA в непрямому ІФА. Результати інгібування ІФА показали, що: 1) гептапептиди зі структурою триптичного тетрадекапептида 22EARPLPTAADFEG35, що розпізнавався двома моноклональними антителами (МКА) M6D5 та М9G1, специфічними до PVM, розглядалися як ініціатори взаємодії МКА-М9G1 з такими самими антигенами. Таким чином, Asp та Glu вносять вклад в формування комплексу антиген—антитіло.

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