Mapping antigenic epitopes of potato virus Y with antibodies affinity-purified by using overlapping synthetic peptides

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Synthetic, overlapping peptides representing the entire amino acid sequence of potato virus Y (PVY) coat protein were used to affinity-purify antibodies from polyclonal antisera to PVY. In testing the binding of the purified antibodies to PVY particles, antigenic epitopes were identified. The N-terminal and C-terminal regions of the PVY coat protein were found to contain most of the antigenic epitopes. The results will facilitate the development of detection methods for PVY based on synthetic peptides.

Key words: coat protein, Pepscan, plant virus, PVY

Introduction

Mapping of antigenic epitopes of viral coat proteins by systematic immunochemical analysis of synthetic, overlapping peptides (Pepscan) can provide information useful in developing immunochemical detection methods for viruses (GEYSEN et al. 1984, SHUKLA et al. 1989). In its standard form, this technique involves synthesis of a number of peptides on acrylic-coated polyethylene pins and testing of these peptides for binding of anti-viral antibodies. While this method is extremely well suited for identification of sequence-dependent linear epitopes that are targets of diagnostic reagents, it has been argued that the antibodies binding to short peptides may not necessarily bind to whole antigen molecules (reviewed by TRIBBICK et al. 1991) and thus may not in all cases represent epitopes present on intact antigen. A recently published modification (TRIBBICK et al. 1991) of the standard technique overcomes this problem. In this modified method, synthetic peptides are used to affinity-purify antibodies which can then be tested for binding to intact antigen. We have been interested in the antigenic epitopes of potato virus Y, an important plant pathogen. In our previous study carried out by using the standard Pepscan procedure, we found the antigenic epitopes of PVY coat protein to be distributed along the entire amino acid sequence, including the C-terminal part of the coat protein (VUENTO et al. 1993). However, in an earlier study, antibodies to potyvirus particles were found to bind to peptides derived mainly from the N-terminus (residues 1–70, SHUKLA et al. 1989). Therefore, it was of interest to study whether antibodies binding to synthetic peptides also would bind to PVY.

Abbreviations used: BSA = Bovine serum albumin, OA = Ovalbumin, PBS = Phosphate buffered saline, PVY = Potato virus Y.

Material and methods

Purification of PVY (N strain) and production of polyclonal antibodies against PVY have been described earlier (VUENTO et al. 1993). The complete set of overlapping hexapeptides (overlap 5 residues) covering the amino acid sequence (SHUKLA et al. 1986) of PVY coat protein was synthesized by using the methodology of Geysen (GEYSEN et al. 1984) with reagents (Epitope mapping kit) purchased from Cambridge Research Biochemicals (Cambridge, UK). This methodology uses pentafluorophenyl derivatives of N-fluorenylmethoxycarbonyl (Fmoc)-protected amino acids (ATHERTON and SHEPPARD 1985). The peptides, 262 peptides in all, were synthesized on acrylic-coated polyethylene rods attached to polyethylene racks, each holding 96 rods. The dimensions of the racks were such that all the 96 rods could be fitted in the wells of a 96-well microtiter plate.

The binding of anti-PVY antibodies to these peptides was tested as described by VUENTO et al. (1993). Affinity purification of antibodies from polyclonal anti-PVY antisera was carried out by using the method of TRIBBICK et al. (1991) except that instead of using buffers of extremely acidic or basic pH, we eluted the antibodies from the peptide pins with urea, a reversible protein denaturant (CREIGHTON 1993). The procedure was as follows: antiserum samples were diluted 1:50 in phosphate-buffered saline, pH 7.4, containing 1% w/v bovine serum albumin, 1% w/v ovalbumin and 0.1% (v/v) Tween 20. The diluted antiserum was pipetted in aliquots of 200 µl onto 96-well microtiter plates (ImmunoPlate, Nunc, Denmark). The peptide-containing rods were inserted into these wells so that the globular tips of the rods were fully covered by the solution. The peptide-containing rods were incubated in the antibody solution for 1 hour at 22°C and then washed 3 times with an excess of phosphate-buffered saline (PBS). To achieve elution, 200 µl of a solution of 8 M urea in PBS was pipetted into the wells of similar 96-well microtiter plates. The peptide-containing rods were inserted into the wells and incubated for 1 h at 22°C, carefully shaking the plate occasionally. After this time, the rods were removed, washed with an excess of PBS, distilled water and methanol, air dried and stored at room temperature until used again. The urea solutions containing eluted antibodies were immediately diluted to neutralize the denaturing effect of concentrated urea, and analyzed by using the enzyme-linked immunosorbent procedures described below.

To test the binding of antibodies to PVY, microtiter plates (ImmunoPlate, Nunc, Denmark) were incubated with purified PVY (100 µl of a solution containing 20 µg/ml of PVY in PBS) overnight at +4°C. Excessive binding sites were blocked by incubation with PBS containing 1% bovine serum albumin and 1% ovalbumin (PBS-BSA-OA) for 4 hours at 22°C. After thorough washing with PBS, aliquots of PBS-BSA-OA (100 µl) were pipetted into the microtiter wells. Aliquots (10 µl) of antibodies eluted with 8 M urea were added by mixing to wells containing the PBS-BSA-OA solution. The microtiter plates were incubated for 4 hours at 22°C and then washed thoroughly with PBS. Bound antibodies were quantitated by incubation with peroxidase-labelled anti-rabbit IgG antibodies (DAKO, Denmark) diluted in PBS-BSA-OA for 2 hours at 22°C. After thorough washing with PBS, peroxidase activity was measured by using a Multiskan Plus microtiter plate reader (Labsystems, Helsinki, Finland) equipped with a 405-nm filter with 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) as a substrate. For controls, nonimmune rabbit serum was allowed to react with synthetic peptides, and the material eluted from the peptides was assayed as described above.

Results and discussion

The binding to PVY of antibodies eluted from synthetic overlapping hexapeptides derived from PVY coat protein is shown in Fig. 1a. The immunodominance of the N-terminal and C-terminal parts of the amino acid sequence is indicated by the frequency of reacting antibodies (Fig. 1a). The frequency of reacting antibodies was higher

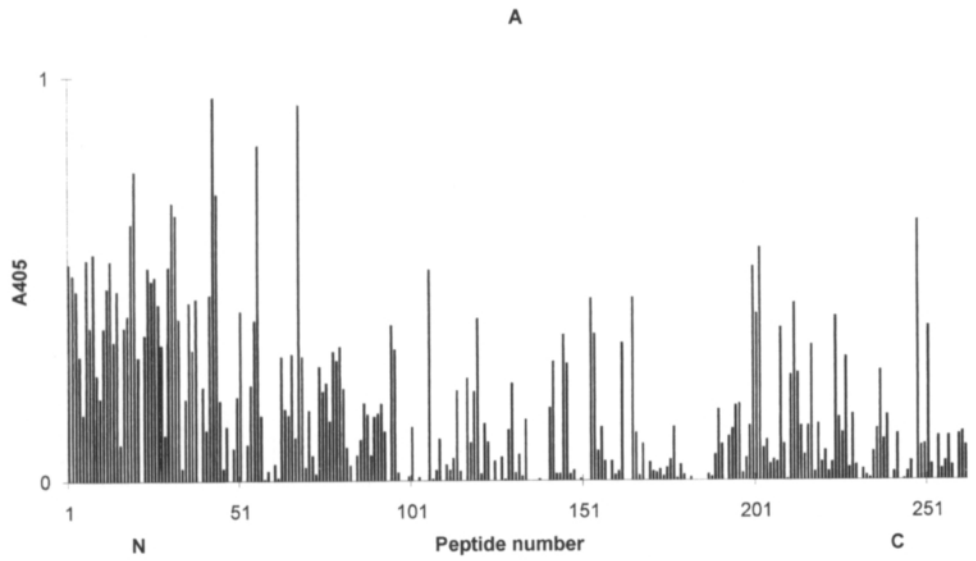

Fig. 1a. Binding of affinity-purified antibodies to PVY. Antibodies were purified from anti-PVY antisera by using a complete set of synthetic, overlapping hexapeptides covering the entire amino acid sequence of PVY coat protein. The purified antibodies were assayed for binding to PVY with the enzyme-linked immunosorbent assay (ELISA). Signals (A_{405}) obtained in ELISA are shown on the vertical axis. Signals given by non-immune rabbit serum have been subtracted from the data shown. Peptides are identified by the number (counted from the N-terminus of the amino acid sequence) of their first amino acid residue.

at the N-terminus than at the C-terminus. However, antibodies binding to PVY were also eluted from peptides derived from the central part of the amino acid sequence. The present results reflect the distribution of linear epitopes only since possible non-linear epitopes would not show up by this approach. We also recognize that binding of viral particles to plastic surfaces may result in unfolding of the particles (LAVER et al. 1990) and in modification of epitopes of native antigens (STEVENS et al. 1986). Despite these limitations, the results strongly support the model of SHUKLA et al. (1988), suggesting that the N-terminus and the C-terminus of the amino acid sequence are located at the surface of the PVY coat protein structure and induce antibodies during immunization. This suggestion is also supported by findings that immunization with peptides derived from N-terminal and C-terminal regions has given rise to antibodies that react with PVY (OHSHIMA et al. 1992, VUENTO et al. 1993). It appears from the present data that some parts of the central region may also have similar properties.

Comparison of the present data with results obtained by the standard approach of measuring the binding of anti-PVY-antibodies to peptides (Fig. 1b) revealed a few interesting differences. In a few cases, antibodies binding to peptides gave only weak signals when eluted and tested for binding to PVY. These cases included the peptides 85–89, 185–186, 241–247, 256 and 261–262. The antibodies binding to these peptides apparently did not recognize the respective epitopes in the viral particles. The amino acid sequence (PVY strain D, SHUKLA et al. 1986) used for synthesis of hexapeptides varies in a few amino acid residues from the corresponding amino acid sequence of the viral strain (PVY strain N) used for immunization. The number of variant amino acid residues has been reported to be 9 (ROBAGLIA

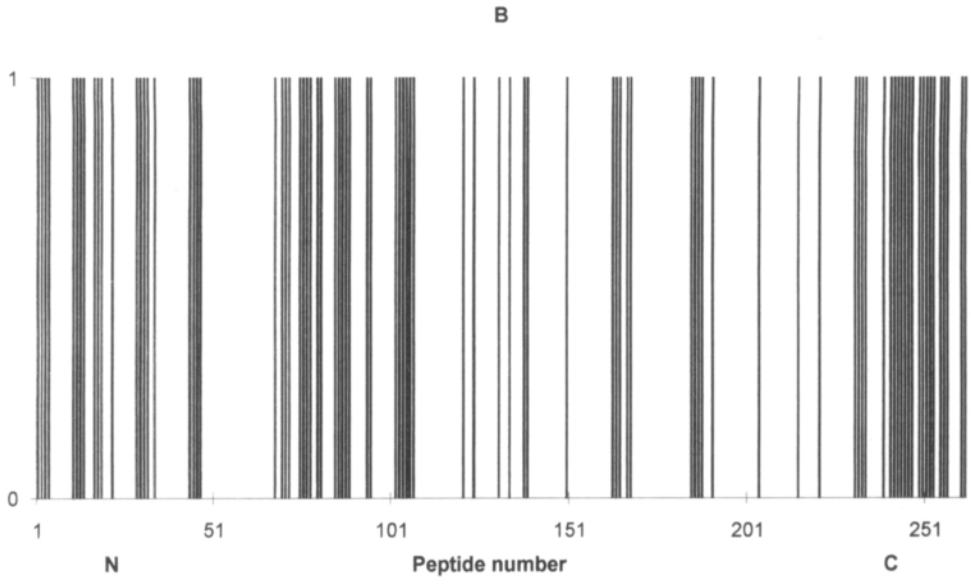

Fig. 1b. Pepscan analysis of binding of anti-PVY antibodies to synthetic peptides. The set of peptides used and the polyclonal anti-PVY antiserum were the same as in Fig. 1a. The peptides binding antibodies from the anti-PVY antisera are identified as vertical columns (the scale is arbitrary). All peptides giving an ELISA signal at least twice the size of the background signal (background signals were determined by using non-immune rabbit serum) have been included.

et al. 1989) or 20 (VAN DER VLUGT et al. 1989), apparently depending on the virus isolate used for analysis. However, this variation could not explain the above results since the variant sites were not included in the above mentioned peptides.

On the other hand, the present experiments identified as epitopic several peptides not detected by the standard method of testing binding of anti-PVY antibodies to peptides. Thus the peptides 23–28, 36–38, 42–43, 55–56 and 200–202 did not give signals in the direct assay (Fig. 1b); yet antibodies reacting with PVY were eluted from these peptides (Fig. 1a). It has been suggested, in connection with a similar case (TRIBBICK et al. 1991), that even low amounts of antibody, escaping detection when bound to peptide, may have after elution sufficient affinity to show significant binding to viral antigens. In the case of peptides 23–28, 36 and 55–56, but not in the case of peptides 37–38, 42–43 and 200–202, the amino acid substitutions (at positions 24, 26, 29, 31, 36 and 58; ROBAGLIA et al. 1989, VAN DER VLUGT et al. 1989) between peptides and the virus may have contributed to the weakness of binding of an anti-PVY antibody to a peptide. Taken together, these results suggest that in order to obtain a reliable epitope map of a protein antigen, one should test both for antibodies bound to peptides and for antigen-binding antibodies eluted from the peptides. The mapping of antigenic epitopes of PVY coat protein will facilitate the development of detection methods for PVY, based on synthetic peptides and antibodies to synthetic peptides. This work is currently in progress in our laboratory.

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SELOSTUS

Perunavirus Y:n kapsidiproteiinin antigeenisten epitooppien kartoitus käyttäen synteettisten peptidien avulla affiniteettipuhdistettuja vasta-aineita

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Työssä syntetisoitiin limittäisten heksapeptidien sarja, joka kattoi perunavirus Y:n (PVY) kapsidiproteiinin koko aminohapposekvenssin. Peptidien avulla affiniteettipuhdistettiin anti-PVY vasta-aineita polyklonaalisista anti-PVY antiseerumeista, ja vasta-aineiden sitoutuminen PVY-partikkeleihin tutkittiin entsyymi-immunoassaymenetelmillä. Näin saatiin identifioitua PVY:n kapsidiproteiinin antigeenisia alueita, joita löydettiin erityisesti kapsidiproteiinin polypeptidiketjun päistä. Vertailu menetelmään, jossa mitattiin anti-PVY-vasta-aineiden sitoutumista ko. peptidisarjan peptideihin, paljasti useita eroavaisuuksia.

Tulokset osoittavat, että luotettavan epitooppikartan saamiseksi on tutkittava sekä vasta-aineiden sitoutumista peptideihin että peptideistä eluoitujen vasta-aineiden sitoutumista virusantigeeniin. Saadut tutkimustulokset auttavat synteettisiin peptideihin perustuvien määritysmenetelmien kehittämisessä kasviviruksille.