Acta Chemica Scandinavica, 1993: 47: 994–998
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ACTA
CHEMICA SCANDINAVICA
ISSN 0904–213X

Characterization of Antigenic Epitopes of the Coat Protein of Potato Virus X by Systematic Immunochemical Analysis of Synthetic Peptides

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Vuento, M., Vihinen-Ranta, M., Paananen, K., Blomqvist, S. and Kurppa, A., 1993. Characterization of Antigenic Epitopes of the Coat Protein of Potato Virus X by Systematic Immunochemical Analysis of Synthetic Peptides. – Acta Chem. Scand. 47: 994–998.

Analysis of the immunoreactivity of several polyclonal antisera to potato virus X (PVX) with short synthetic peptides covering the entire amino acid sequence of PVX coat protein revealed immunodominant regions at the *N*- and *C*-terminal ends of the coat protein. In addition antigenic epitopes were also found in the central part of the coat protein. The immunoreactivity to the synthetic peptides was found to correlate with surface characteristics, i.e., hydrophilicity, calculated polypeptide chain flexibility and with the vicinity of potential beta turns. Characterization of the antigenic properties of PVX will facilitate the development of detection methods for potexviruses.

Potato virus X (PVX) is the type member of potexviruses. It comprises flexible rod-like virions[1] containing a single-stranded RNA-genome.[2] The nucleic acid is encapsidated by copies of a single coat protein with a molecular weight of approximately 27 kDa.[3] The amino acid sequence of PVX coat protein (237 amino acids) has been deduced from the nucleotide sequence of the corresponding open reading frame of the PVX genome.[4] Potexviruses are important plant pathogens causing significant economic losses worldwide.[2] To date, the only effective way of reducing these losses is early detection of infection. Immunochemical methods for detection of these viruses are of great importance. Monoclonal antibodies have been raised to PVX and used for characterization of the antigenic properties of PVX.[5,6] However, to our knowledge, no systematic study of the antigenic epitopes of PVX has been carried out. A method for systematic immunochemical analysis of synthetic, overlapping peptides has been developed for epitope mapping purposes.[7] We have used this method to analyze the reactivity of anti-PVX antibodies with hexapeptides covering the entire amino acid sequence of the coat protein of PVX. In order to be able to reach general conclusions about antigenic sites on PVX coat protein, we have used polyclonal antisera, each produced by many lymphocyte clones during the immunization process, rather than monoclonal antibodies.

Materials and methods

Peptide synthesis. A complete set of overlapping (overlap 5 residues) hexapeptides covering the amino acid sequence[4] of PVX coat protein was synthesized by using the methodology of Geysen[7] with reagents (Epitope Mapping Kit) purchased from Cambridge Research Biochemicals, Cambridge, UK. In this method, pentafluorophenyl derivatives of Fmoc-protected amino acids are used to synthesize peptides on acrylic-coated polyethylene pins. The peptides remain, after acetylation of free *N*-termini and removal of the protecting groups, covalently attached to the pins, and are conveniently assayed for binding of antibody by using enzyme-linked immunosorbent methods.

Immunizations. Potato virus X was purified[8] from the leaves of tobacco (*Nicotiana tabacum* cv. Samsun NN) plants infected with PVX. Rabbits were immunized with 500 μg of purified PVX dissolved in 500 μl of phosphate buffered saline, pH 7.4 (PBS) and emulsified with Freund's complete adjuvant (Difco, USA). Injections were given subcutaneously on days 0, 30 and 60. Serum was collected on day 68.

Enzyme-linked immunosorbent assays (ELISA). Binding of anti-PVX antibodies to synthetic peptides was

analyzed by using ELISA procedures according to the instructions of Cambridge Research Biochemicals. Briefly, the peptides attached on polyethylene pins were allowed to react with diluted anti-PVX antisera. Bound antibodies were detected, after thorough washing of the pins, with peroxidase-labelled second antibody to rabbit immunoglobulins. Control experiments were run with sera from non-immunized animals. Signals given by the antisera and the low absorbance values obtained with control sera were used to calculate signal/background ratios for each peptide. To facilitate mathematical analysis of the results, the peptides were divided into three groups according to their signal/background ratios (>2, >4 and >8).

To test the binding of affinity-purified anti-peptide antibodies (below) to PVX, polystyrene microtiter plates (Maxisorp F96, Nunc, Denmark) were coated with purified PVX (20 µg/ml) in PBS overnight at +4°C. Remaining binding sites were blocked by incubating with a solution containing 1% (w/v) bovine serum albumin, 1% (w/v) chicken ovalbumin and 0.1% (w/v) sodium azide in PBS (PBS-BSA) for 4 h at 22°C. The plates were then washed four times with PBS. Into the wells were pipetted 100 µl of PBS-BSA and, with mixing, 10 µl aliquots of urea-eluted anti-peptide antibodies (below), to give a final urea concentration of 0.7 M. The plates were incubated for 4 h at 22°C and then washed four times with PBS. Bound immunoglobulins were quantitated by incubation with peroxidase-conjugated antibody to rabbit immunoglobulins (DAKO, Denmark) diluted in PBS-BSA (prepared without azide) for 2 h at 22°C. After thorough washing with PBS, peroxidase activity was measured with a Multiskan Plus microtiter plate reader (Labsystems, Helsinki, Finland) equipped with a 405 nm filter. The peroxidase substrate used was 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (Sigma, St. Louis, USA).

Purification of antibodies reacting with synthetic peptides. The synthetic peptides found to bind antibodies from anti-PVX antisera were used for affinity purification of antibodies. Equal aliquots of the four anti-PVX antisera used in the above experiments were pooled, the pooled serum was diluted 1 : 50 in PBS-BSA and pipetted in 200 µl aliquots into the wells of microtiter plates. The peptide-containing tips of polyethylene pins were immersed in the antibody solution and incubated with gentle agitation for 1 h at 22°C. The pins were then washed four times with PBS and the tips were eluted with 200 µl of 8 M urea in PBS for 1 h at 22°C with gentle agitation. The eluted antibodies were immediately diluted with PBS-BSA and tested for binding to PVX as described above. The assay was run with duplicate samples. As a control, pins containing hexapeptides from the coat protein of potato virus Y were allowed to react with anti-PVX antiserum. The control pins were eluted with urea and eluates were analyzed by ELISA as described above. An antibody giving an ELISA signal at least two times higher than signal given by control was identified as binding to PVX.

Calculation of secondary structure. Calculation of the putative secondary structure of PVX coat protein was carried out by the method of Fasman.[9] Autocorrelation analysis for calculation of the probable helical content was carried out by using the method of Horne.[10]

Calculation of hydrophilicity and flexibility. The moving average calculation of local hydrophilicity was carried out by using the parameters of Parker *et al.*[11] with a window of six amino acid residues. Predicted chain flexibility was calculated by using the method of Karplus and Schulz.[12]

Calculation of correlation of immunoreactivity with hydrophilicity, flexibility or with predicted secondary structure. These calculations were carried out by using the methods published earlier.[13]

Results and discussion

The binding patterns of antibodies from four individual polyclonal anti-PVX antisera, from different experimental animals, are shown in Fig. 1 scans (a)–(d). The binding patterns were superficially very similar, each identifying a cluster of reactive peptides near the *N*-terminus of PVX coat potein, a few reactive peptides near the *C*-terminus and some reactive peptides evenly distributed in the central part [scan (a) is an exception] of the coat protein. Fig. 1(f) shows all the peptides that were reactive with at least one of the four sera tested. The immunodominance of the *N*- and *C*-termini was evident, but reactive peptides were also seen fairly evenly distributed along the entire amino acid sequence. Individual variations between the sera tested are strikingly evident from Fig. 1(e), which shows only those few peptides that reacted with all four polyclonal antisera. Thus, Fig. 1 demonstrates a significant heterogeneity in the individual polyclonal response to the immunogen. It also demonstrates the need to use several polyclonal antisera in order to reach valid conclusions about the location of immunoreactive peptides.

Fig. 1(g) shows the location of those 26 peptides binding antibodies that also bound to PVX. Many peptides identified as reactive by scans (a)–(d), especially those from the central region, were not included in this group. However, this group did include some peptides from the central region, notably the peptides 115, 128, 130 and 139–142. Fig. 1(g) also shows several peptides from the *N*- and *C*-terminal regions. This result supports the immunodominance of the *N*- and *C*-termini of the polypeptide chain suggested by scans (a)–(d).

The three-dimensional structure of PVX is not known, but there is evidence that the *N*- and *C*-termini of the coat protein are located on the surface of the virion. Thus, the *N*- and *C*-terminal portions of the coat protein can easily be cleaved off by proteases.[14] As predicted from the amino acid sequence, the termini do not contain alpha helices

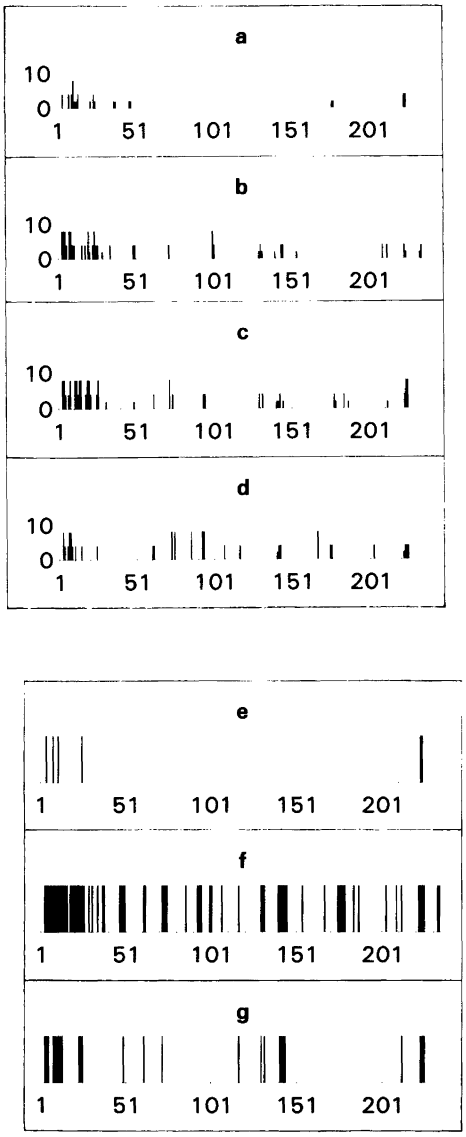

Fig. 1. Binding of polyclonal anti-PVX antibodies to synthetic, overlapping hexapeptides covering the sequence of coat protein of PVX. PVX coat protein peptides synthesized on acrylic coated polyethylene rods were allowed to react with antibodies to native PVX raised in four individual rabbits (a)–(d). Bound rabbit antibodies were quantitated with peroxidase-labeled second antibody to rabbit immunoglobulins. The vertical axis shows signal/background (the background was determined with non-immune rabbit serum) ratio obtained in enzyme-linked immunosorbent assay: values 8, 4 and 2 indicate that this ratio was >8, >4 and >2, respectively. Value 0 on the vertical axis indicates that signal/background ratio was <2. Positions of peptides recognized as immunoreactive with all four antisera are shown in (e). Positions of peptides recognized as antigenic by at least one of the four antisera are shown in (f). Positions of peptides reacting with antibodies that also bind to PVX are shown in (g). These positions were determined by affinity-purifying antibodies with synthetic peptides and by testing for binding of the purified antibodies to PVX, as described in the Materials and Methods section. The vertical scale in (e)–(g) is arbitrary. For clarity of presentation, the positions (p) of the peptides shown in this Figure are $p=n+2$, n being the number of the first residue of the peptide.

(Ref. 15, see also below). Hydrophilicity and flexibility profiles calculated for PVX coat protein showed maxima at both termini of the polypeptide chain (not shown). Thus, these portions of the coat protein should be easily accessible to antigen-presenting cells during immunization, which could explain their immunodominance observed in the present study. However, for the same reason, these portions of the coat protein may not be optimal for raising antibodies to be used in virus detection, since these portions may be lost from the virions during processing of plant samples. The epitopes found in the central portion of the coat protein could be more interesting in this respect. The presence of antibodies in anti-PVX antisera reacting with peptides from the central part was probably to a great deal related to antigen processing during the immune response (reviewed in Ref. 16). This idea was supported by the findings that many of the antibodies reacting with peptides did not bind to PVX [Fig. 1(f)–(g)]. However, some peptides from the central region did bind antibodies that also reacted with PVX [Fig. 1(g)]. These peptides may represent structures located at the surface of the central region, or they could represent regions unfolding during the immunoassay procedure.

Results obtained with the methods of Fasman[9] and Horne[10] suggested a helical content of 32.5% and 23.3%, respectively, for the PVX coat protein. This agrees well with the prediction (33% alpha helix) published by Sawyer *et al.*[15] The distribution of putative alpha helices along the polypeptide chain was studied both with the method of Fasman and with the method of Horne. These methods gave for residues 1–70 helical contents of 40.0% and 9.3%, respectively. For residues 71–170 the corresponding values were 32.0% and 39.5%, and for residues 171–237 25.8% and 5.9%, respectively. These results support the idea of a compactly folded central portion and less folded *N*- and *C*-termini. With the Fasman method, we identified 15 potential beta turns (25.3%) in the PVX coat protein. The value for beta-sheet structure was 30.8%. Thus, 88.6% of the 237 amino acid residues were assigned to a secondary structure. The predicted distribution of secondary structure elements along the amino acid sequence of PVX coat protein is shown in Fig. 2. This distribution is interesting since a striking correlation with predicted beta-turn structures was found (Table 1). In contrast, no correlation between the location of epitopes and the alpha-helical or beta-sheet structures was found (not shown).

As shown in Table 1, 87.5% of the immunoreactive hexapeptides displaying a signal/background ratio of >8 were located at the sites of calculated beta turns or in their immediate vicinity. Of the peptides showing a lower signal/background ratio of >4, 74% had a similar location. The group of peptides reacting with antibodies binding to PVX also showed a high probability (73%) of being located close to potential beta turns. For comparison, similar calculations with the complete set of overlapping hexapeptides of PVX coat protein showed

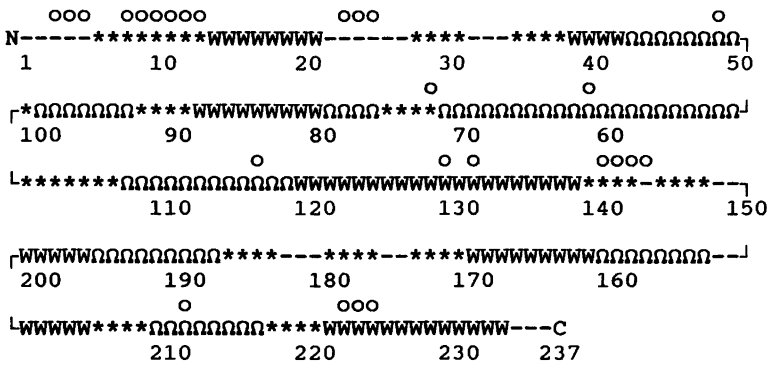

Fig. 2. Predicted secondary structure of PVX coat protein and the distribution of immunoreactive peptides. The calculations were carried out by the method of Fasman.[11] Beta strands have not been aligned. Ω, alpha helix; W, beta sheet; *, beta turn; –, no predicted secondary structure. The distribution of antigenic peptides (the group of peptides reacting with antibodies which also bind to PVX is shown) along the amino acid sequence is indicated with symbols 'o' (the symbol 'o' above the position of an amino acid residue indicates that the residue is the third residue of an antigenic hexapeptide). Residue numbers are shown below the line.

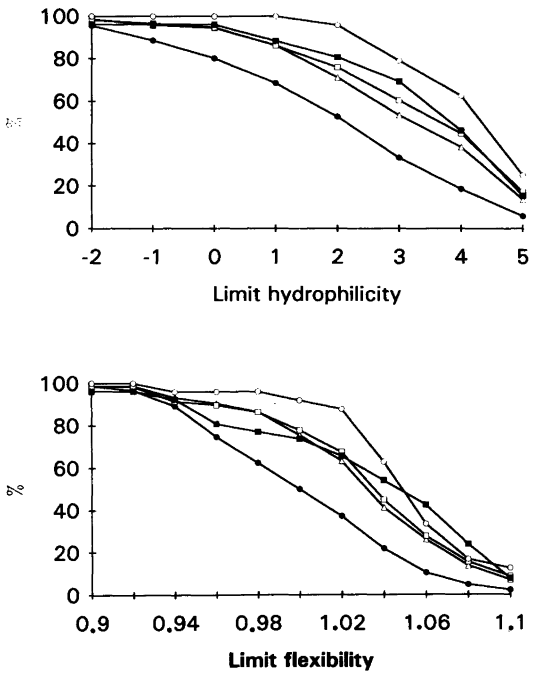

Fig. 3. Hydrophilicity and calculated flexibility of the immunoreactive hexapeptides of PVX coat protein. Hexapeptides identified as immunoreactive with any of the four antisera [Fig. 1 (a)–(d)] were divided into three categories according to the signal/background ratio they showed in ELISA assay. The figure shows the proportion (as a percentage) of the hexapeptides in each peptide group having average hydrophilicities [Fig. 3(a)] or calculated flexibilities [Fig. 3(b)] higher than the limit values shown on the hydrophilicity or flexibility scale (vertical axis). The figure also shows similar data calculated for the group of peptides that react with antibodies which also bind to PVX. ○, immunoreactive peptides, signal/background >8; □, immunoreactive peptides, signal/background >4; △, immunoreactive peptides, signal/background >2; ■, immunoreactive peptides, antibody binds to PVX; ●, the total group of the 232 peptides studied.

that only 52% of these peptides were located at or adjacent to the positions of calculated beta turns.

As beta turns are considered to be located on the surface of proteins,[17] it was interesting to analyze whether the antigenicity of the peptides correlated with other surface characteristics such as hydrophilicity and flexibility. Such a correlation was indeed found (Fig. 3). The immunoreactive peptides were found to have higher

Table 1. Correlation of immunoreactive epitopes of PVX coat protein with predicted beta turn structures.[a]

	Group of peptides				
	Immunoreactive signal strength			Immunoreactive antibody binds to PVX	All
	>2	>4	>8		
Number (N)[b] of peptides in the group	73	58	24	26	232
$N1$[c]	48	38	19	16	114
$N2$[d]	5	5	2	3	17
$100(N1+N2)/N$	72.6	74.1	87.5	73.1	52.2

[a]The immunoreactive hexapeptides identified by scans (a)–(d) (Fig. 1) were divided in three groups according to the signal/background ratio given by these peptides in ELISA (signal determined with antibodies against native PVX, background determined with non-immune rabbit serum). The fourth column represents peptides binding antibodies which also react with PVX. [b]N = number of peptides in the group. [c]$N1$ = the number of peptides with one or more residues overlapping with predicted beta turns. [d]$N2$ = the number of peptides directly adjacent to predicted beta turns. When a peptide was found both in overlapping and in an adjacent position, it was counted in $N1$ only. Similar data calculated with the complete set of overlapping hexapeptides of PVX coat protein is shown for comparison (last column on the right).

average hydrophilicities than the parent group of all peptides studied [Fig. 3(a)]. The immunoreactive peptides were also found to represent areas of higher flexibility than the parent group of all peptides studied [Fig. 3(b)]. In both cases, the correlation was found to improve with increasing signal strength observed for the enzyme-linked immunosorbent assay [Fig. 3(a,b)]. However, even the most reactive peptides usually contained one or two hydrophobic residues. As shown in Fig. 3, the group of peptides binding antibodies that also bound to PVX had average flexibilities and hydrophilicities higher than the peptide groups with signal/background ratios of >2 and >4.

The results suggest that the polyclonal response to PVX coat protein, as analyzed with overlapping hexapeptides covering the entire amino acid sequence, gave preference to surface structures. The results also suggest that synthetic peptides derived both from the *N*- and *C*-termini and from the central region are able to mimic structures that are both immunogenic and accessible to antibody in the virus particles. We have recently obtained similar results with potato virus Y.[13] These results are rather unexpected since previous work employing the same method of analysis in the study of several polyclonal anti-protein antibodies has revealed no such selection.[18,19] While further studies are needed to clarify the reason for this discrepancy, it seems probable that the hydrophilic peptides derived from the central portion of PVX coat protein could be used to design immunochemical methods for detection of PVX. Attempts in this direction are currently in progress in our laboratory.

Acknowledgements. This work was supported by the Council for Agricultural Sciences, Academy of Finland. We thank Ms. Anne Makkonen and Ms. Pirjo Kauppinen for skilful technical assistance.

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Received January 29, 1993.