Application of Enzyme-Linked Immunosorbent Assay to Diagnosis of Potato Leafroll Disease

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小島 誠*・滝沢 勤*・上田一郎**・四方英四郎** :
ジャガイモ葉巻病診断への酵素結合抗体法の応用

Abstract

Potato leafroll virus (PLRV) antigen was easily detected by enzyme-linked immunosorbent assay (ELISA) in potato foliage which had been collected in commercial fields or grown in a greenhouse. ELISA values were highest in newly expanded leaves and gradually declined with growth. When infected tubers were planted, viral antigen generally could be detected by ELISA before the development of symptoms on potato foliage. The pattern of results of ELISA tests using field-grown potato plants closely corresponded to the pattern of symptom development on potato plants. Since we were able to eliminate the non-specific background of the extracts from healthy green sprouts and tubers by heating for 10 min at 50 C, ELISA can be employed for PLRV-indexing using green sprouts or tubers. The ELISA values for leaf material desiccated over silica gel remained relatively constant throughout one month of storage and PLRV antigen was readily detected in dried samples. In comparative studies by ELISA and immunosorbent electron microscopy (ISEM), no obvious differences in ability to detect the PLRV antigen were observed. PLRV antigen (in purified preparations) could be accurately detected at even nanogram levels by both immunological methods.

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Key Words: diagnosis, potato leafroll, ELISA.

Introduction

The selection of virus-free seed potato planting stock is essential for successful potato production. For this purpose, many different field, greenhouse and laboratory methods have been used. Among them, serological methods are the most useful and convenient. Although we succeeded in producing antiserum against purified preparations of potato leafroll virus (PLRV) in 1974¹⁰⁾, the virus in the crude sap from infected potato plants could not be serologically detected until 1977¹⁾. In recent years, more sensitive serological methods such as immune electron microscopy^{4,9)} (or immunosorbent electron microscopy, ISEM)^{11,12)} and enzyme-linked immunosorbent assay (ELISA)²⁾ have been developed and applied for plant virus detection. Several workers have reported promising results with both methods using PLRV-infected foliage^{5,6,7,8,13,14)}, tubers^{3,16)}, roots¹⁾ of potato, and viruliferous aphids^{3,17)}. We discussed previously the

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optimum conditions for detection of PLRV antigen by ELISA, and pointed out some difficulties in eliminating non-specific background of the sprout extracts⁷⁾. In this paper, we examine the reliability of ELISA for detecting PLRV in foliage of field-grown potato plants and present data about some treatments for eliminating non-specific reactions in the extracts from tubers and sprouts; we also refer to the possibility of preservation of PLRV antigen for ELISA.

Materials and Methods

Plants. PLRV-infected and virus-free potato plants (cv. Danshaku) were grown from PLRV-infected and virus-free tubers, respectively, in a greenhouse unless otherwise mentioned. To examine the relationship between the results of ELISA tests and incidence of the symptoms appeared, about 100 potato plants were collected from commercial fields in Hokkaido during the summer of 1979, and stored at -40°C before use. To follow PLRV levels in potato foliage, ten progeny tubers from a PLRV-infected potato plant were planted on 4 June in a greenhouse. Upper leaves from these plants were detached at one week intervals and then stored as mentioned above. In preservation tests, potato leaves were cut up and aliquots were stored in vials and desiccated over silica gel. Samples were also fixed in 75 % ethanol or 5 % glutaraldehyde solutions.

ELISA. The procedures for ELISA followed those described previously⁷⁾. Leaf extracts from fresh, frozen or desiccated materials were prepared in 10 volumes (w/v) of PBS-Tween (0.02 M phosphate buffer, pH 7.4, containing 0.15 M NaCl and 0.05 % Tween 20). Extracts from tubers and green sprouts were prepared also in 10 volumes (w/v) of PBS-Tween, incubated at 50°C for 10 min, and briefly centrifuged before assaying. Gamma-globulin was obtained from newly prepared antiserum against PLRV⁷⁾.

Coating γ -globulin was used at concentration of 1–2 $\mu\text{g}/\text{ml}$ and the enzyme conjugate (alkaline phosphatase, Boehringer grade I) at 1/400–1/800. Incubation with γ -globulin and the enzyme-conjugate was at 37°C for 4 hr. The plates filled with antigens to be tested were incubated overnight at 4°C . We usually took readings of the ELISA values one hr after adding the substrate by a spectrophotometer (Hitachi model 624 or 100–10) using 1 cm light-path length cuvettes at 405 nm.

ISEM. Fresh carbon-coated Formvar supporting films were coated with γ -globulin at 2 $\mu\text{g}/\text{ml}$ and incubated for one hour at 37°C . These EM-grids on which diluted, purified PLRV preparations had been placed, were incubated overnight at 4°C . After washing with water, specimens were stained with 2 % uranyl acetate and viewed under an electron microscope (JEOL 100 B) at 80 KV. The mean number of the particles on EM negative films ($5.9 \times 8.2 \text{ cm}$) taken at magnification of 20,000 at each dilution were converted to the numbers per standard area (SA, $1,000 \mu\text{m}^2$)¹¹⁾.

Results

Detection of PLRV antigen in potato foliage

PLRV antigen was easily detected by ELISA in potato foliage which had been collected in the fields or grown in a greenhouse. No obvious differences among ELISA values from different plant organs tested, such as leaf, stem and petiole or their

Table 1. Detection of PLRV antigen in potato foliage by ELISA

Experiment ^{a)}	Organ	Part	A ₄₀₅ value ^{b)}
I	leaf	tip	0.46
		middle	0.28
		low	0.29
	petiole	tip	0.32
		middle	0.34
		low	0.06 ^{c)}
II	leaf	tip	0.19
		low	0.45
III	leaf	tip	0.25
		middle	0.21
		low	0.18
	stem	tip	0.19
		middle	0.25
		low	0.30
IV	leaf	tip	0.46
		middle	0.29
		low	0.30
	petiole	tip	0.08 ^{c)}
		middle	0.34
		low	0.32
V	leaf	tip	0.22
		middle	0.22
		low	0.18
	stem	middle	0.34

a) Potato plants (cv. Norin-ichigo) grown in a greenhouse were used in experiments I and II and field-grown potato plants (cv. Danshaku) were used in III-V.

b) Levels of A₄₀₅ values in healthy samples were between 0.02 and 0.05.

c) These low values may be due to the uneven distribution of the viral antigen within petioles.

positions (low, middle and tip) were obtained except 2 cases (Table 1).

Although two petioles at low or tip position showed inconsistent ELISA values, which might be derived from the uneven distribution of the viral antigen, all middle petioles could be reliably used.

Variation in antigen levels of PLRV infected potato foliage

Ten tubers from a PLRV-diseased potato plant were planted after breaking dormancy in June in a greenhouse. Upper leaves were harvested for the assay at one week intervals for PLRV detection. ELISA values were highest in the newly expanded leaves (June 25) and gradually declined as the leaves aged (Fig. 1). As shown in Fig. 1, the viral antigen could be detected before symptoms developed in all cases.

Relationship between ELISA values and visual indexing

To examine the relationship between ELISA values and visual indexing, we collected about 100 samples from field-grown potato plants during the summer. As shown in Table 2, the results of ELISA tests closely corresponded to those of visual indexing. The virus antigen was detected in eighteen out of nineteen plants with PLRV-symptoms, and was

also detected in three out of 14 plants with leafroll-like, questionable symptoms. These numbers, three and one (asterisks in Table 2) might be due to primary infection of PLRV; the disease was detected by ELISA before it had time to express symptoms.

PLRV-indexing using green sprouts by ELISA

PLRV-indexing by ELISA was conducted using green sprouts from potato tubers which had been collected at random from naturally infected, field-grown potato plants. Green sprouts from the distal (rose end) of tubers were used for ELISA and those from the middle were planted for observation of symptoms in a greenhouse. The

Fig. 1. Variation of PLRV antigen contents in potato foliage with growth. Ten tubers obtained from a diseased parental potato plant were planted on 4th June, and were assayed for viral contents. The results from 4 individuals out of 10 were illustrated. Secondary symptoms on the tested plants were confirmed on the day indicated with S. Corresponding ELISA values of healthy plants (○.....○) were also shown.

Table 2. Relationship between ELISA values and incidence of symptom on potato foliage

Symptom-ratings ^{a)}	ELISA ^{b)}	No. plants
positive (19)	+	18
	-	1
positive? (14)	+	3*
	±	4
	-	7
negative (70)	+	1*
	±	15
	-	54
total		103

a) Plants with typical leafroll symptoms and with questionable leafroll symptoms were referred as "positive" and "positive?", respectively. The numbers following the ratings are the numbers of samples tested.

b) +: more than $\times 4$ of healthy ELISA value
 ±: $\times 2-4$ of healthy ELISA value
 -: less than $\times 2$ of healthy ELISA value

Table 3. PLRV-indexing by ELISA using green potato sprouts

Results of ELISA tests ^{a)}	No. plants	Symptom development
positive	33	33
positive?	2	0
negative	13	15

a) positive: over 0.2 in A_{405} values
 negative: below 0.1 in A_{405} values
 positive?: between 0.1 and 0.2 in A_{405} values

results of ELISA tests also corresponded almost exactly to the symptoms that developed on potato foliage (Table 3). However, PLRV indexing using green sprouts was rather difficult because healthy sprouts gave relatively high ELISA values⁷⁾. As shown in Table 3, the threshold value (0.1) was calculated from non-infected potato samples. Preliminary experiments, however, revealed that heat treatment of sap at 50 C for 10 min was effective in eliminating the non-specific background of the samples. Since the non-specific ELISA absorbance values in the heat-treated healthy material averaged 0.05 (0.04–0.06), PLRV-indexing by ELISA became much easier even in green sprouts were used as the source (Fig. 2).

Fig. 2. Detection of PLRV antigen by ELISA in green sprouts from naturally infected tubers collected at random from field. Extracts from green sprouts were heated to 50 C for 10 minutes. ELISA values varied from 0.13 to 0.36, when 24 sprout samples were individually tested. □: diseased; ■: healthy; ▨: control (PBS-Tween).

Fig. 3. Detection of PLRV antigen by ELISA in tubers from naturally infected potato plants. Extracts from tubers were treated to 50 C for 10 minutes. ELISA values varied from 0.12 to 0.45, when 18 tuber samples were individually tested. □: diseased; ■: healthy; ▨: control (PBS-Tween).

PLRV-indexing using tubers by ELISA

Similarly, heat treatment of sap (50 C for 10 min) also made it possible to do tuber indexing using tubers collected from naturally infected, field-grown potato plants. As shown in Fig. 3, the ELISA absorbance values for healthy tubers ranged around 0.05, and those for infected tubers (rose end) that had been stored for 6 months in a cellar ranged between 0.1 and 0.45.

Preservation of PLRV antigen for ELISA tests

The possibility of antigen preservation and the effect upon ELISA values of the length of time during which antigens were preserved, were tested with dried, PLRV-infected potato foliage. Potato leaves with typical symptoms were cut up with a razor

Fig. 4. Preservation of PLRV antigen in potato leaf material dried over silica gel (●—●). Results of storage of leaf material in 75 % ethanol (○····○) were also represented.

Fig. 5. Comparison of ISEM (●—●) and ELISA (○—○) in efficiency to detect PLRV antigen in diluted, purified preparations. Microplates and EM-grids were coated with γ -globulin at the concentration of 2 μ g/ml.

blade and the aliquots were stored in vials and desiccated over silica gel. Fig. 4 shows the level of antigenicity of the virus in dried leaf material stored at room temperature for one month. Fresh samples gave the highest ELISA absorbance values, and then the level declined by 1/3 after 5 days. Thereafter, the values for dried leaf material remained relatively constant throughout one month of storage. ELISA values of samples fixed in ethanol declined gradually in 20 days (Fig. 4). Similar decline was observed in samples fixed in glutaraldehyde.

Comparison of ISEM and ELISA in efficiency for detecting PLRV antigen

To compare the efficiency of ISEM and ELISA, some experiments were conducted using purified PLRV preparations. Gamma-globulin (2 μ g/ml) was used for coating the

ELISA plates and EM-grids. No obvious differences in ability to detect the PLRV antigen were observed, since PLRV antigen could be accurately detected at even nanogram levels by both methods (Fig. 5).

Discussion

PLRV antigen was easily detected by ELISA in all tested parts of potato foliage i. e., leaf, petiole and stem. Tamada and Harrison (1980)¹⁵⁾ reported that PLRV antigen could be detected by ELISA in young leaves of potato plants with primary infections before symptom development. Therefore ELISA tests may be useful for detection of PLRV in potato foliage with primary infections, and to distinguish the disease caused by PLRV from other diseases with similar symptoms but different causes. The validity of use of ELISA may also expand to the investigation of weeds as potential reservoirs of PLRV. Another value of ELISA as a diagnostic tool of PLRV is that detection of the viral antigen in tubers or sprouts of potato plants becomes possible as shown in Figs. 2 and 3. In ELISA tests using tubers or sprouts, non-specific background of the samples has been a limiting factor^{7,8,13)}. However, we were able to overcome such a problem in this experiment by heating the extracts for 10 min at 50°C. Tamada and Harrison (1980)¹⁶⁾ also pointed out the same problem and suggested that pre-incubation for 3–6 hr at room temperature was effective in eliminating such background. Under our condition, tuber indexing was successful using ELISA. However, Tamada and Harrison reported that the levels of PLRV antigen in each part of the tuber varies depending on the length of time and the conditions of tuber storage after lifting¹⁶⁾. Thus, in order to replace visual tuber-indexing in a greenhouse by ELISA tests, it is necessary to take a suitable part of the tuber to be tested according to the storage period.

Preservation of PLRV antigen also seems to be a critical point for practical use of ELISA tests. Desiccation of leaf material over silica gel in small vials¹⁴⁾ was confirmed to be also useful for PLRV. Thus, such vials with dried leaves, can be mailed easily for ELISA diagnosis. This system might be beneficial for large scale diagnosis of potato leafroll disease in wide areas.

Although we usually took ELISA absorbance readings one hour after addition of substrate, more reliable results could be obtained by prolonged incubation with substrate especially when PLRV contents were rather low (Kojima, unpublished data). For this purpose, microplate photometers, such as, MTP-12 (Corona Electric, Japan), by which ELISA absorbance values can be rapidly read within few minutes for each plate without addition of sodium hydroxide, will be helpful.

Kojima *et al.*⁶⁾ previously showed that ISEM can be employed for the diagnosis of PLRV from potato sprouts and foliage. And in this paper, we demonstrated that the sensitivity for detecting PLRV antigen by ELISA and ISEM was about the same. Based on these results, both methods can be readily employed for diagnosis of PLRV, with certain advantages for each method: ELISA tests make it possible to handle a large number of samples and ISEM needs only small volumes (<200 µl) of extracts for assay.

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和 文 摘 要

ジャガイモ葉巻病診断への酵素結合抗体法の応用

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酵素結合抗体法 (ELISA) により、一般ほ場あるいは温室で育成したジャガイモの茎葉から容易にジャガイモ葉巻ウイルス (PLRV) が検出された。罹病塊茎を植え、生じてくる地上部の PLRV 抗原量を経時的に調べた結果、萌芽後展開した葉で最も高く、生育が進むにつれて漸減した。その際、病徴発現以前に抗原の検出が可能であった。一般ほ場から採集してきた葉巻症状株、疑似株、無病徴株103株につき ELISA による診断と病徴の度合との関係を調べたところ、ほぼ一致した。しかし、疑似株、無病徴株から各々3株、1株が陽性と判定された (ほ場における1次感染株と考えられる)。一方、ジャガイモ塊茎、萌芽を用いての ELISA による診断は非特異反応が高く困難であったが⁷⁾、それらの汁液を加熱処理 (50°C, 10分間) することにより低下させることができたので、塊茎等を用いた診断も可能となった。さらに、病葉を小片にしてシリカゲル上で乾燥保存しても十分抗原を検出できることから、抗原の保存、郵送も可能となった。また、ELISA と免疫電顕法 (ISEM) につき PLRV 抗原検出感度につき比較したところ、ほとんど差がなく、両検定法とも ng 単位のウイルス抗原を正確に検出できることから、ジャガイモ葉巻病の診断には有用であると考ええる。