Specific Serological Detection of the Transmissible Virus in Pea Seed Infected by Pea Seed-borne Mosaic Virus

K. Masmoudi, M. Suhas, R. K. Khetarpal, and Y. Maury

First, second, and fourth authors: Département de Pathologie végétale, Institut National de la Recherche Agronomique, 78026, Versailles, France; and third author: National Bureau of Plant Genetic Resources, PUSA Campus, New Delhi, 110012, India.
Partially funded by the European Union (RC 8001-CT90-007).
Accepted for publication 16 March 1994.

ABSTRACT

Masmoudi, K., Suhas, M., Khetarpal, R. K., and Maury, Y. 1994. Specific serological detection of the transmissible virus in pea seed infected by pea seed-borne mosaic virus. Phytopathology 84:756-760.

The capsid protein of pea seed-borne mosaic virus (PSbMV), analyzed in mature pea seed, had a molecular mass of 33 kDa in infected embryos but a molecular mass of only 29–27 kDa in seed testas. A proteinase activity, induced in pea seed testas, efficiently cleaved the capsid protein in vitro as did a mild trypsin treatment. The occurrence of this proteinase activity was independent of the infection; its physiological induction in testas occurred at a development stage that corresponds to the active growth of the embryo. The use of an antiserum specific to the deleted part of the protein enabled detection of PSbMV only in embryos. This serological differentiation between the seed-transmitted and seedborne virus may have a direct application in the routine screening of pea seed lots.

RESUME

La protéine capside du pea seed-borne mosaic virus (PSbMV), analysée à partir de graine de pois à maturité, a une masse moléculaire de 33 kDa lorsqu'on l'extrait des embryons infectés et de 27–29 kDa seulement à partir des téguments. Une activité protéolytique, induite dans les téguments, peut opérer efficacement ce clivage in vitro, comme le fait aussi la trypsine. La présence de cette protéase est indépendante de l'infection; son induction physiologique dans les téguments se situe à un stade de développement qui correspond à la croissance active de l'embryon. L'utilisation d'un serum spécifique de la partie clivée ne permet de détecter le virus qu'au niveau des embryons. Cette différenciation sérologique du virus transmis par la graine et du virus nontransmissible peut trouver une application pratique intéressante dans une sélection sanitaire des semences de pois basée sur le taux de transmission du PSbMV.

Pea seed-borne mosaic virus (PSbMV) is a potyvirus of pea. The flexuous, rod-shaped virus particle is composed of a 33-kDa capsid protein and a positive-sense, single-stranded RNA molecule 9,924 nucleotides long (7). PSbMV is seed-transmitted at high frequencies, and its economical importance is linked to this biological property (14). Seed transmission is linked to the invasion of the embryo sac before or just after fertilization (23). The virus retains its infectivity in the embryo during seed maturation and storage, but the virus from mature seed testas is not infectious (9). However, the virus can be detected by serological techniques in both mature embryos and testas (13).

The purpose of the present work was to compare the stability of the capsid protein in both seed components, with the hope of serologically differentiating the seed-transmitted virus of infected embryos from the nontransmitted virus of infected testas. As generally observed with the potyviruses, the N- and C-termini of the capsid protein, which are located on the surface of intact particles (2,20), may be cleaved in vivo (6) as well as during storage of purified virus preparations (6,15). Such cleavage induces important differences in serological properties (5), because the N-terminus constitutes the major virus-specific epitopes (2,20).

MATERIALS AND METHODS

Virus. The common strain Sv used in this study was propagated on pea cv. Belinda. Seed collected from plants inoculated before flowering transmitted this strain at a 10–20% rate.

Antisera. H is a hyperimmune antiserum to PSbMV provided by R. I. Hamilton (Research Station Agriculture Canada, Vancouver, BC). This serum, prepared with highly purified virus, was obtained by a series of intramuscular injections at 2-wk intervals followed by a booster dose at 10 mo (4).

R_1 is an antiserum obtained by intramuscular injection of PSbMV particles purified according to the method of Alconero et al (1) with only one CsCl gradient centrifugation. One milliliter of incomplete Freund's adjuvant (Sigma Chemical Co., St. Louis) was mixed with 1 ml of virus suspension (500 μg) for the first

intramuscular injection. After a 1-mo delay, intramuscular boosters of freshly prepared virus were administered at 2-wk intervals. R_1 corresponds to the bleeding done 10 days after the second booster.

K is an antiserum to the purified capsid protein of PSbMV. The semipurified virus was prepared according to Alconero et al (1) without any CsCl gradient centrifugation and suspended in 0.2 M Tris, pH 8.2. One volume was mixed with 0.5 volume of a Laemmli denaturation solution of 0.2 M Tris, pH 6.8, containing 6% sodium dodecyl sulphate (SDS), 15% mercaptoethanol, 30% glycerol, and 0.03% bromophenol blue (10); heated in boiling water for 3 min; and loaded onto a 12% polyacrylamide preparative gel. Protein bands were visualized by soaking the gel in cold 0.2 M KCl. The portion of the gel that contained the full-length capsid protein was excised, frozen at −20 C, pressed through a syringe, and stirred in 25 mM Tris and 200 mM glycine, pH 7.4, for 3 h. Two milliliters of incomplete Freund's adjuvant was mixed with 2 ml of capsid protein (200 μg) for the first intramuscular injection. After a 1-mo delay, intramuscular boosters of freshly prepared capsid protein were administered at 2-wk intervals. K_1, K_2, and K_3 correspond to the bleedings done 10 days after the second, third, and fourth intramuscular boosters, respectively.

Mature seed-sample preparation for enzyme-linked immunosorbent assay (ELISA) and/or Western blot assay. Ten mature seeds of pea cv. Belinda collected from infected plants and 10 healthy seeds were soaked overnight in water. After manual separation of embryos and testas, the pooled embryos and pooled testas were ground separately in phosphate buffered saline containing 0.5 ml of Tween 20 per liter (PBST) at 1:20 dilution (w/v) with a mortar and pestle. Each extract was centrifuged for 10 min at 8,000 *g*, and the supernatant was layered onto an ELISA microplate or immediately denatured in the Laemmli solution for Western blot assay.

Immature seed-sample preparation for ELISA and/or Western blot assays. *From infected peas.* Fifty seeds were collected from mechanically inoculated peas and grouped according to their weight into five classes, t1–t5. The average weight of one seed in class t1 was 12 mg; in t2, 42 mg; in t3, 56 mg; in t4, 112 mg; and in t5, 268 mg. Testas of each class were dissected, washed, and ground (1:20 [w/v]) in PBST and centrifuged for 10 min at 8,000 *g* before analysis of the virus in ELISA and Western blots.

From healthy peas. Seeds were collected from healthy peas to select those belonging to classes t2 and t5. The testas were ground and serially diluted in PBST to obtain the 1:10, 1:20, 1:50, and 1:100 (w/v) dilutions. Before loading the ELISA microplate, each of these dilutions was centrifuged (for 10 min at 8,000 *g*) mixed (1:1 [v/v]) with an infected leaf extract (1:50 [w/v] in PBST) prepared with young leaves collected from peas inoculated 4 wk earlier.

Serological tests. Double antibody sandwich-ELISA (DAS-ELISA) was conducted as described by Clark and Adams (3). The positive threshold was determined as described previously (13). Protein blots were performed on Bio-Rad Mini-Protean II ready gels (Bio-Rad Laboratories, Richmond, CA) (10% single-percent gels for the experiment reported in Figure 1; 12% for other experiments) and nitrocellulose membranes with the Mini-Protean II electrophoresis and transblot cells. The samples were run along with the Bio-Rad low-range prestained SDS-PAGE (polyacrylamide gel electrophoresis) standards (phosphorylase B, bovine serum albumin [BSA], ovalbumine, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme with respective apparent molecular masses of 106, 80, 49.5, 32.5, 27.5, and 18.5 kDa, respectively; the carbonic anhydrase did not appear clearly) to monitor the duration of electrophoresis, the efficiency of transfer to nitrocellulose membrane, and the integrity of the capsid protein. After saturation with BSA (1%), the membranes were incubated with the (H)IgG-alkaline phosphatase conjugate (1:2,000 [v/v]) or the K_1 conjugate (1:1,000 [v/v]) in 0.05 M Tris, 0.15 M NaCl, and 0.5% Tween 20, pH 7.5; alternatively, they were incubated first with (H)IgG (1 μg/ml) or (K_1)IgG (2 μg/ml) and then with a 1/6,000 (v/v) dilution of a goat–anti-rabbit conjugate (Biosys, 21 quai du clos des roses F-60200 Compiègne). The alkaline phosphatase was visualized with Fast Red TR salt and Naphthol AS-MX phosphate (Sigma).

RESULTS

Heterogeneity of the capsid protein of PSbMV after storage of a prepared semipurified virus. The capsid protein of strain Sv was characterized by sequencing the 3′-terminal region of viral RNA (11). When compared with the sequence of the pathotype P1 (7), the deduced amino acid sequence varied at position 120 with an alanine to arginine substitution. The molecular mass of the intact capsid protein, as deduced from the nucleotide sequence, was 33 kDa.

This 33-kDa protein could be cleaved during storage of leaf extracts or semipurified virus preparations. A range of cleavage products of the capsid protein was observed in Western blots

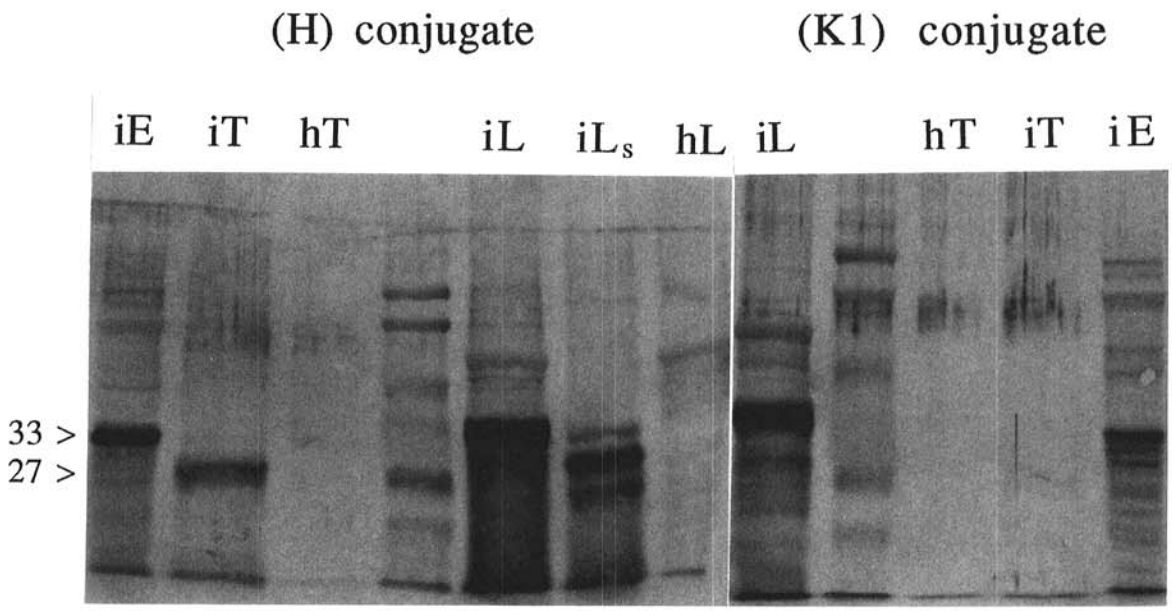

Fig. 1. Western blot analysis of extracts in phosphate buffered saline plus Tween 20 (1:20 [w/v]) of healthy (h) or infected (i) embryos (E), testas (T), and leaves (L). The lane iLs refers to an infected leaf extract stored 4 days at 4 C. The capsid protein was detected, with either a hyperimmune antiserum to pea seed-borne mosaic virus (PSbMV) (H)IgG-alkaline phosphatase conjugate or an antiserum to the purified capsid protein of PSbMV (K_1) conjugate.

performed with the H antiserum (Figs. 1 and 2). The fastest migrating band had a molecular mass of 27 kDa. The value calculated for the trypsin-treated core protein, which migrated similarly, was 26.4 kDa, as deduced from the observation that trypsin cleaves the N-terminus of the capsid protein of different potyviruses at the same site: DR/DVDAG (19). These findings support the hypothesis that the N-terminal domain of the capsid protein of PSbMV was cleaved serially. These cleavages of the capsid protein might involve a proteolytic activity from leaves, present in leaf extracts or in partially purified virus preparations.

Interestingly, the K_1 polyclonal antiserum did not detect the capsid protein cleavage products as shown in lanes corresponding to the infected leaf extract (Fig. 3); thus, K_1 is specific to the intact form of the capsid protein.

Capsid protein stability in embryos and testas of mature seeds. Embryo and testa extracts from infected pea seed were analyzed by Western blots in comparison with extract of infected leaves. The capsid protein subunit in embryos had a molecular mass of 33 kDa. In testas, the antigen consisted essentially of 29- to 27-kDa degradation products (Figs. 1 and 3).

Unlike the H antiserum, the K_1 antiserum did not detect the capsid protein in testas, using either the alkaline phosphatase conjugate (Fig. 1) or, in a different experiment, the immunoglobulins visualized by an indirect method (Fig. 3). It recognized only the intact capsid protein present in infected embryos.

The failure of the K_1 antiserum to detect the truncated protein in testas of mature seeds also was found in DAS-ELISA with several antisera resulting from early bleedings (K_1, K_2, K_3, and R_1). Whatever immunogen was used for preparing the antiserum, the capsid protein (K_1, K_2, or K_3) or the intact virus particle (R_1), these antisera detected the virus in infected embryos but did not detect it in infected testas (Table 1).

Thus, in mature seeds, the capsid protein was present only in its degraded form in testas, and the K_1 antiserum, either in Western blots or ELISA, detected only the virus present in embryos (i.e., the seed-transmitted virus). It was interesting, therefore, to investigate at what earlier stage of seed development the K_1 antiserum detected the virus in embryos only.

Cleavage of the capsid protein in testas according to seed-development stage. To determine at what stage of seed development the capsid protein is degraded, immature seeds were collected from mechanically inoculated pea plants. Because the weight of a green seed constitutes a reliable criterion of its physiological evolution, these immature seeds were distributed into five classes of weight, t1–t5. PSbMV was analyzed in the corresponding testas by comparing first the reaction of the H and K_1 antisera in DAS-ELISA.

The results with the H antiserum indicated a constant level of virus in each of the classes of testas. The K_1 antiserum similarly detected the virus in the first three classes, however, a drastic reduction and a complete loss of the ability to detect the virus occurred in the fourth and fifth classes (Fig. 4).

However, in Western blots, the intact form of the capsid protein was observed in each class of testas, as reported for t5 in Figure 5 (lane 2). This result was not consistent with the ELISA results. To test whether degradation was due to incubation, new extracts of testas were obtained corresponding to the t5 class. Two aliquots of the t5 testa extract were mixed with the Laemmli denaturing solution, one immediately after obtaining the extract, the second after an overnight incubation at 6 C to reproduce the conditions of the ELISA procedure: The capsid protein was indeed degraded during the overnight incubation (Fig. 5).

These results showed that although the cleavages of the capsid protein did not occur to a great degree in vivo in t5 immature testas a proteolytic activity capable of degrading the capsid protein to 27 kDa was induced in testas at the t4 and t5 stages.

Proteinase activity in healthy t5 testa extracts. To determine if the proteolytic activity observed in extracts of immature infected t5 testas was induced by PSbMV infection, similar extracts were prepared from healthy testas and mixed with an infected leaf extract from peas inoculated 4 wk earlier. Two stages of immature testas, t2 and t5, were compared in this experiment. The proteolytic activity from t5 testa extract appeared to be significantly efficient on the virus capsid protein to a dilution of 2.10^{-2}, whereas the t2 extracts were not able to degrade the capsid protein at any

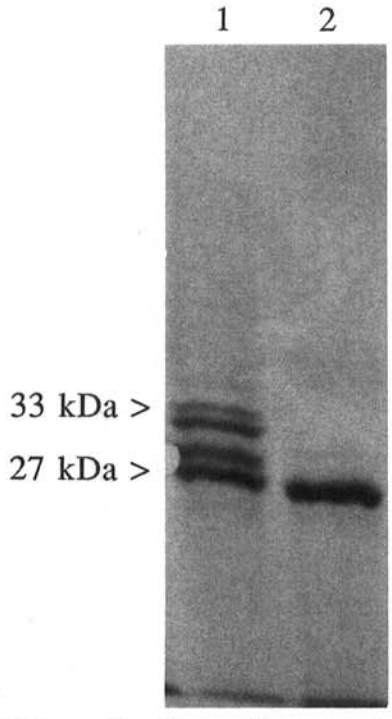

Fig. 2. Length heterogeneity of the capsid protein analyzed by Western blot with the hyperimmune antiserum to pea seed-borne mosaic virus (PSbMV) (H) conjugate. Lane 1: semipurified virus preparation after storage for 1 wk at 6 C. Lane 2: 10 μg of freshly purified PSbMV treated with 1 μg of trypsin for 1 h at 37 C.

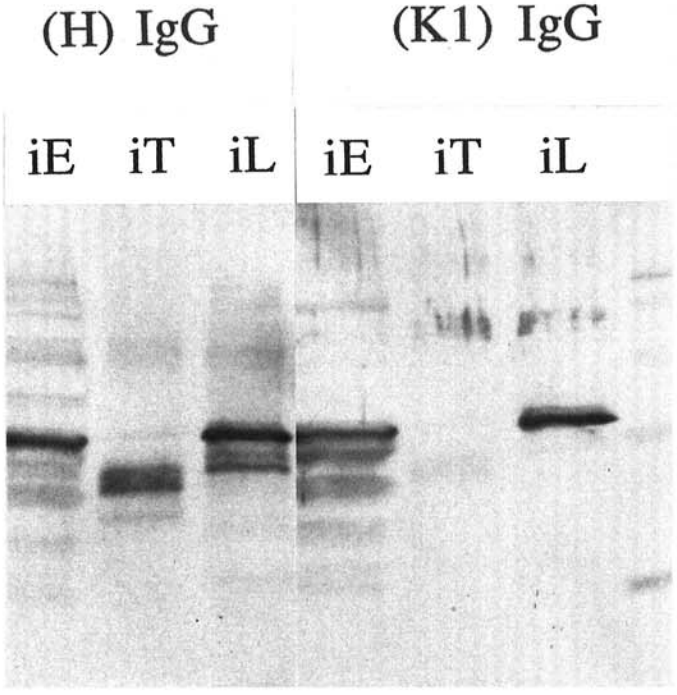

Fig. 3. Western blot analysis of extracts in phosphate buffered saline plus Tween 20 (1:20 [w/v]) of infected embryos (iE), testas (iT), and leaves (iL), with hyperimmune antiserum to pea seed-borne mosaic virus (PSbMV) (H)IgG-alkaline phosphatase conjugate or an antiserum to the purified capsid protein of PSbMV (K_1) conjugate and a goat-anti-rabbit immunoglobulin-alkaline phosphatase conjugate.

dilution (Fig. 6). This experiment showed that the factors able to cleave the capsid protein in the t5 testas exist in healthy seeds.

DISCUSSION

The capsid protein of PSbMV does not have the same structure in both components of mature pea seed. It is intact in embryos (33 kDa) and partially cleaved in seed testas (29–27 kDa), as might result from a mild trypsin treatment. This cleavage in testas results from a proteolytic activity that is inconstant but develops with time. Thus, the proteinase involved has some similarities with a proteinase induced in *Ipomoea nil*, which partially cleaved the capsid protein of sweet potato feathery mottle potyvirus (19). In that system, the induction of a proteinase was associated with a phenomenon of recovery in symptomless and virus-free upper leaves of infected plants (19). In contrast, the proteinase activity in pea seed testas was independent of PSbMV infection. Its induction also was observed in healthy testas. The physiological role of this enzyme in vivo is unknown, but its induction in testas when the embryo is in a very active growth phase might suggest

TABLE 1. Detection of pea seed-borne mosaic virus in testas and embryos with antisera (H, K_1, K_2, K_3, and R_1) able or unable to detect the cleaved forms of the capsid protein[a]

Sample[b]	Antisera				
	H	K_1	K_2	K_3	R_1
Testas	0.618[c]	0.060	0.012	0.030	0.049
Testas (h)	0.016	0.029	0.015	0.015	0.065
Embryos	0.255	0.885	0.248	0.334	0.247
Embryos (h)	0.015	0.039	0.012	0.011	0.045

[a] Underlined values indicate positive reactions.
[b] $n = 10$ for each sample. h = healthy.
[c] Double antibody sandwich enzyme-linked immunosorbent assay value A_{405}.

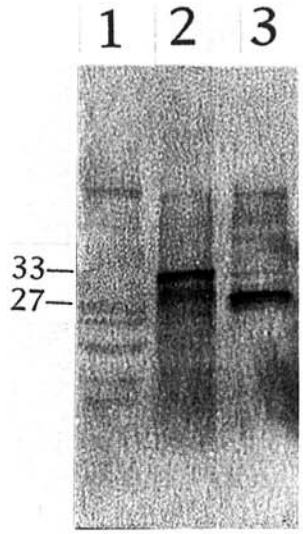

Fig. 5. Western blot analysis of the capsid protein of pea seed-borne mosaic virus (PSbMV) in t5 (seed weight class 5, 268 mg) testa extracts. Lane 1: healthy extract; lane 2: infected extract immediately denatured in the Laemmli solution; lane 3: infected extract denatured after overnight incubation at 6 C. The capsid protein bands were visualized with the hyperimmune antiserum to PSbMV (H) conjugate.

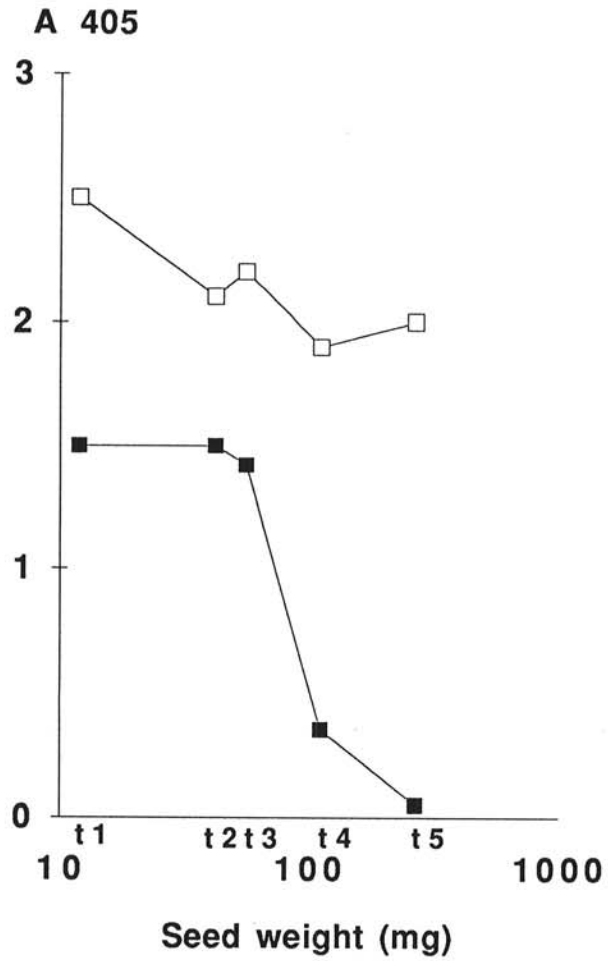

Fig. 4. Comparative detection of pea seed-borne mosaic virus (PSbMV) capsid protein, with hyperimmune antiserum to PSbMV (H) (□) and an antiserum to the purified capsid protein of PSbMV (K_1) (■), in infected immature testas at different stages of seed development.

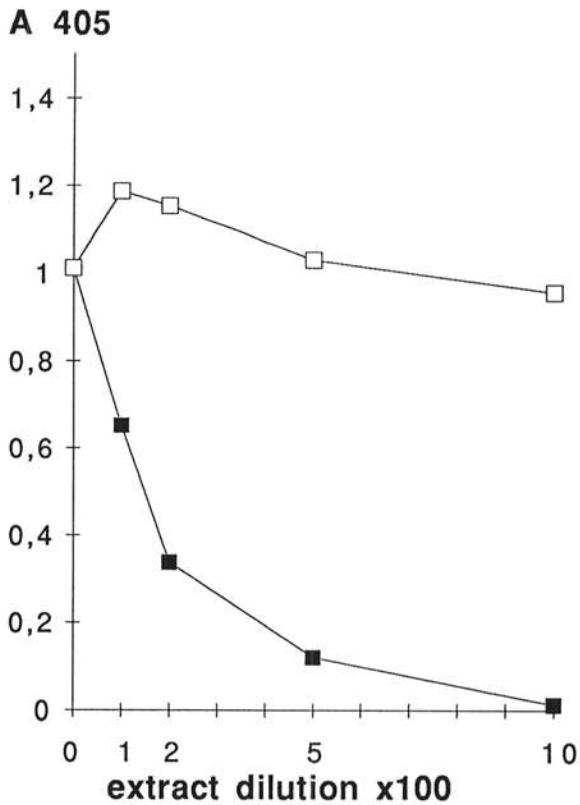

Fig. 6. Detection of proteolytic activity in healthy t5 (seed weight class 5, 268 mg) (■) and t2 (seed weight class 2, 42 mg) (□) testas. Four dilutions of testa extracts in phosphate buffered saline plus Tween 20 were mixed (v/v) with an infected leaf extract and incubated overnight at 6 C in a microplate. The enzyme-linked immunosorbent assay was performed with the antiserum to the purified capsid protein of pea seed-borne mosaic virus (K_1).

some involvement in the remobilization of seed-testa proteins for supplying nitrogen to the growing embryo (16,18).

The conversion of the capsid protein to a 27-kDa form occurs in vivo between the mature stage and the immature t5 stage (i.e., during seed maturation). Indeed, at the t5 stage of immature green seeds, the predominant form was still a 33-kDa form. However, at that stage, the ELISA test indicated that the amount of proteinase synthesized was high enough for cleaving all the molecules of capsid protein present in infected testas during overnight incubation of the antigen at 6 C. Such a cleavage did not occur at t2 stage in the conditions of the ELISA test.

Therefore, The DAS-ELISA test performed with both antisera—an antiserum (H) specific to both 33- and 27-kDa forms and an antiserum (K_1) that detects only the 33-kDa capsid protein—appears to be a sensitive assay for detecting this proteinase at early stages of development. In further experiments, we observed that the detection of an extremely low proteolytic activity at the t2 stage needs a much longer incubation period (1 wk at 6 C).

The antiserum (K_1) specific to the 33-kDa capsid protein resulted from an early bleeding. Presumably, the antibodies are, as demonstrated in similar cases, directed to the degraded N-terminus, which constitutes the main immunogenic part of the capsid protein of potyviruses (21).

From a practical point of view, it has been shown that the cleavage of a part of the capsid protein can cause a failure of potyvirus detection in the context of the production of virus-free propagation material (22). Concerning potyvirus detection in pea seed, this phenomenon should also be taken into account, and the antiserum selected according to the objectives of the programs. In the context of detection experiments in which seed testas are used as representative of the mother plant, a hyperimmune antiserum should be selected: This was the case while screening pea germ plasm for resistance to PSbMV, where most of the susceptible lines were eliminated on the basis of an ELISA test on a few testas from seed obtained directly from the germ plasm collection (8). In the context of quality control programs, the simultaneous presence of a complete capsid protein in the embryo and of a deleted form in the testas enables a serological differentiation to be made between the seed-transmitted and seedborne virus, using for seedborne a definition adopted for most pathogens carried in, on, or with the seed but not necessarily transmitted (17). The specific detection, by appropriate antisera, of the virus in embryos avoids the prior decortication of thousands of seeds for screening infected seed lots on the basis of their seed transmission (12).

LITERATURE CITED

1. Alconero, R., Provvidenti, R., and Gonsalves, D. 1986. Three pea seedborne mosaic virus pathotypes from pea and lentil germ plasm. Plant Dis. 70:783-786.
2. Allison, R. F., Dougherty, W. G., Parks, T. D., Willis, L., Johnston, R. E., Kelly, M., and Armstrong, F. B. 1985. Biochemical analysis of the capsid protein gene and capsid protein of tobacco etch virus: N-terminal amino acids are located on the virion's surface. Virology 147:309-316.
3. Clark, M. G., and Adams, A. N. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. Gen. Virol. 34:475-483.
4. Hamilton, R. I., and Nichols, C. 1978. Serological methods for detection of pea seed-borne mosaic virus in leaves and seeds of *Pisum sativum*. Phytopathology 68:539-543.
5. Hiebert, E., and McDonald, J. G. 1976. Capsid protein heterogeneity in turnip mosaic virus. Virology 70:144-150.
6. Hiebert, E., Tremaine, J. H., and Ronald, W. P. 1984. The effect of limited proteolysis on the amino acid composition of five potyviruses and on the serological reaction and peptide map of the tobacco etch virus capsid protein. Phytopathology 74:411-416.
7. Johansen, E., Rasmussen, O. F., Heide, M., and Borkhardt, B. 1991. The complete nucleotide sequence of pea seed-borne mosaic virus RNA. J. Gen. Virol. 72:2625-2632.
8. Khetarpal, R. K., Maury, Y., Cousin, R., Burghofer, A., and Varma, A. 1990. Studies on resistance of pea to pea seed borne mosaic virus. Ann. Appl. Biol. 116:297-304.
9. Kohnen, P. D., Dougherty, W. G., and Hampton, R. O. 1992. Detection of pea seed borne mosaic potyvirus by sequence specific enzymatic amplification. J. Virol. Methods 37:253-258.
10. Laemmli, V. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond.) 227:680-685.
11. Masmoudi, K. 1993. Etude du pea seed borne mosaic virus dans la graine de pois. Ph.D. thèse. Université Pierre et Marie Curie, Paris VI, Paris.
12. Masmoudi, K., Duby, C., Suhas, M., Guo, J. Q., Guyot, L., Olivier, V., Taylor, J., and Maury, Y. Quality control of pea seed for pea seed borne mosaic virus. Seed Sci. Technol. In press.
13. Maury, Y., Bossennec, J. M., Boudazin, G., Hampton, R. O., Pietersen, G., and Maguire, J. D. 1987. Factors influencing ELISA evaluation of transmission of pea seed borne mosaic virus in infected pea seed: Seed group size and seed decortication. Agronomie 7:225-230.
14. Maury, Y., and Khetarpal, R. K. 1992. Pea seed borne mosaic virus. Pages 74-92 in: Plant Diseases of International Importance. Vol 2. H. S. Chaube, J. Kumar, A. N. Mukhopadyay, and U. S. Singh, eds. Prentice Hall, New York.
15. Michelin-Lansarot, P., and Papa, G. 1975. The coat protein of the Alliaria strain of turnip mosaic virus: Molecular weight and degradation products formed during purification and upon storage. J. Gen. Virol. 29:121-126.
16. Murray, D. R., and Kennedy, I. R. 1980. Changes in activities of enzymes of nitrogen metabolism in seedcoats and cotyledons during embryo development in pea seeds. Plant Physiol. 66:782-786.
17. Neergaard, P. 1979. Seed Pathology. Vols. 1 and 2. Macmillan Press Ltd., London.
18. Rochat, C., and Boutin, J. P. 1991. Metabolism of phloem-borne amino acids in maternal tissues of fruit of nodulated or nitrate-fed pea plants (*Pisum sativum* L.). J. Exp. Bot. 42:207-214.
19. Salomon, R. 1989. Partial cleavage of sweet potato feathery mottle virus coat protein subunit by an enzyme in extracts of infected symptomless leaves. J. Gen. Virol. 70:1943-1949.
20. Shukla, D. D., Strike, P. M., Tracy, S. L., Gough, K. H., and Ward, C. W. 1988. The N and C termini of the coat proteins of potyviruses are surface-located and the N terminus contains the major virus-specific epitopes. J. Gen. Virol. 69:1497-1508.
21. Shukla, D. D., and Ward, C. W. 1989. Identification and classification of potyviruses on the basis of coat protein sequence data and serology. Arch. Virol. 106:171-200.
22. Stein, A., Salomon, R., Cohen, J., and Loebenstein, G. 1986. Detection and characterisation of bean yellow mosaic virus in corms of *Gladiolus grandiflorus*. Ann. Appl. Biol. 109:147-154.
23. Wang, D., and Maule, A. J. 1992. Early embryo invasion as a determinant in pea of the seed transmission of pea seed borne mosaic virus. J. Gen. Virol. 73: 1615-1620.