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Purification of two potyvirus isolates infecting *Phaseolus vulgaris* L. in Lebanon

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Summary. A purification procedure was developed for the two potyvirus isolates (52-82 and 53-82) recently reported from Lebanon. Virus isolates were propagated in *Phaseolus vulgaris* L. cultivars Sutter Pink and/or Black Turtle Soup and harvested around three weeks after inoculation. Infected tissue was extracted in 0.5M K_2HPO_4 containing 0.005M EDTA, 0.02M Na_2SO_3, 0.01M DIECA, and 3% Triton X-100 pH 8.5, clarified with 25% chloroform +25% carbon tetrachloride, and concentrated by high speed centrifugation. Pellet obtained was suspended in 0.5M sodium citrate buffer pH 7.5, and further purified by centrifugation on sucrose density gradients prepared with the same buffer. Virus concentration at the different steps of the purification procedure was monitored by ELISA, and the final purified material was assessed for purity and recovery of non-aggregated virus particles by sucrose density gradient centrifugation. Virus yield was estimated to range between 20 and 30 mg/kg of tissue. The purified virus (53-82) was injected into a rabbit. The antiserum produced was used for virus detection in infected bean leaves by ELISA. The A_{405} values obtained for infected tissue were high (1.5), whereas those for healthy bean were 0.1 after a 90 min substrate incubation, an indication that the procedure followed produced a fairly purified virus preparation.

Riassunto. Purificazione di due isolati di potyvirus che infettano *Phaseolus vulgaris* in Libano. Viene descritto un metodo di purificazione per due isolati di potyvirus (52-82 e 53-82) trovati recentemente in Libano. Gli isolati virali sono stati propagati in *Phaseolus vulgaris* L. cv. Sutter Pink e/o Black Turtle Soup e raccolti circa tre settimane dopo l'inoculazione quando la concentrazione virale era massima. I tessuti infetti sono stati estratti in K_2HPO_4 0,.5M contenente EDTA 0,005M, Na_2SO_3 0,02M, DIECA 0,01M e 3% di Triton X-100, pH 8,5 chiarificati con 25% di cloroformio e 25% di tetracloruro di carbonio e concentrati con centrifugazione ad alta velocità. I sedimenti sono stati risospesi in tampone citrato sodico 0,5M, pH 7,5 e ulteriormente purificati con centrifugazione in gradienti di densità di saccarosio nello stesso tampone. La concentrazione virale durante le varie fasi della purificazione è stata controllata in ELISA e il grado di purezza e di non aggregazione delle particelle virali dei preparati finali è stato valutato con centrifugazione in gradienti di densità di saccarosio. La resa in virus è risultata di 20-30 mg/kg di tessuto infetto. Un antisiero prodotto in coniglio all'isolato 53-82 è stato adoperato anche per l'accertamento delle infezioni in Fagiolo con ELISA. I valori A_{405} ottenuti con i tessuti infetti erano elevati (1,5) mentre quelli dei tessuti sani erano pari a 0,1 dopo 90 min di incubazione del substrato. Ciò costituisce prova indiretta che, con il metodo di purificazione adottato, sono ottenibili preparati virali soddisfacentemente purificati.

Introduction

Recently, two potyviruses were isolated from French bean (*Phaseolus vulgaris* L.) in Lebanon. These two virus isolates were different from each other and were related to Blackeye cowpea mosaic virus (B1CMV), Azuki bean mosaic virus (AzMV), and Bean common mosaic virus (BCMV-NY 15 strain) (Makkouk, *et al.*, 1985).

To purify these two isolates, namely 52-82 and 53-82, some of the methods reported earlier for the purification of the above mentioned potyviruses were initially tried (Damirdagh and Shepherd, 1970; Uyemoto *et al.*, 1972; Bock, 1973; Huttinga, 1973; Lima *et al.*, , 1979). These procedures resulted in either highly aggregated material or complete loss of virus, but purification was possible when caesium chloride (CsCl) density gradient centrifugation was used (Makkouk *et al.*, 1985).

Caesium chloride is an expensive chemical and using it in equilibrum density gradient centrifugation requires lengthy runs in the centrifuge, which under conditions of some laboratories is not possible. As reported in this paper, it was decided then to develop a procedure where rate zonal sucrose density gradient rather than equilibrum density gradient centrifugation is employed to produce fairly purified preparations.

Materials and methods

The plant species *Nicotiana benthamiana* Domin.,

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P. vulgaris cultivars Black Turtle Soup, Monroe and Sutter Pink and *Vigna unguiculata* Welp cv. California Balckeye No. 5 were assayed for their suitability to propagate the virus isolates 52-82 and 53-82. They were maintained in an insect-proof glasshouse where temperatures ranged between 20 and 30 C. The enzyme-linked immunosorbent assay (ELISA) was used to check virus concentration at different times after inoculation. Antisera used in the test were produced against the same isolates purified by a different procedure (Makkouk *et al.*, 1985).

Different extraction buffers and clarifying agents were tested. Furthermore, a number of concentration methods and resuspension buffers were compared. Based on the result obtained, the following procedure was found to be satisfactory: leaves of cv. Sutter Pink of Black Turtle Soup, 3 weeks after inoculation, were homogenised with 0.5M potassium phosphate (K_2HPO_4) containing 0.005M ethylene diaminetetraacetic acid (EDTA), 0.002M sodium sulfite (Na_2SO_3), 0.01M diethyldithiocarbamate (DIECA) and 3% Triton X-100, pH 8.5. The homogenate was filtered through two layers of cheesecloth and subjected to low speed centrifugation at 8000 rpm for 10 min. The supernatant obtained was stirred with 25% chloroform plus 25% carbon tetrachloride for 40 min, and the aqueous phase was separated by low speed centrifugation at 8000 rpm for 10 min. The virus was then concentrated by centrifugation for 90 min at 30000 rpm and the virus pellet was suspended in 0.05M sodium citrate buffer, pH 7.5. The resuspended pellet was centrifuged at 8000 rpm for 5 min to eliminate any non-soluble material and was then layered on top of 10-40% sucrose gradient prepared in 0.05M sodium citrate buffer, pH 7.5 and centrifuged for 2 h at 23000 rpm in a Sorvall AH-627 swing-out rotor. The virus band was collected with an ISCO density gradient fractionator equipped with a UV monitor.

Serology. Four intramuscular injections of 0.5-1 mg of purified virus were given to a rabbit at weekly intervals. Purified material was emulsified (1:1, v/v) with Freund's complete adjuvant for the first injection and incomplete adjuvant for the subsequent injections. The rabbit was bled 8 weeks after the first injection. The gamma globulin fractionation, enzyme conjugation and testing of infected tissue by ELISA was as described by Clark and Adams (1977) with the exception of using 0.2M potassium phosphate buffer pH 6 for tissue extraction.

Results

Propagative host and extraction buffer. To determine optimal harvest

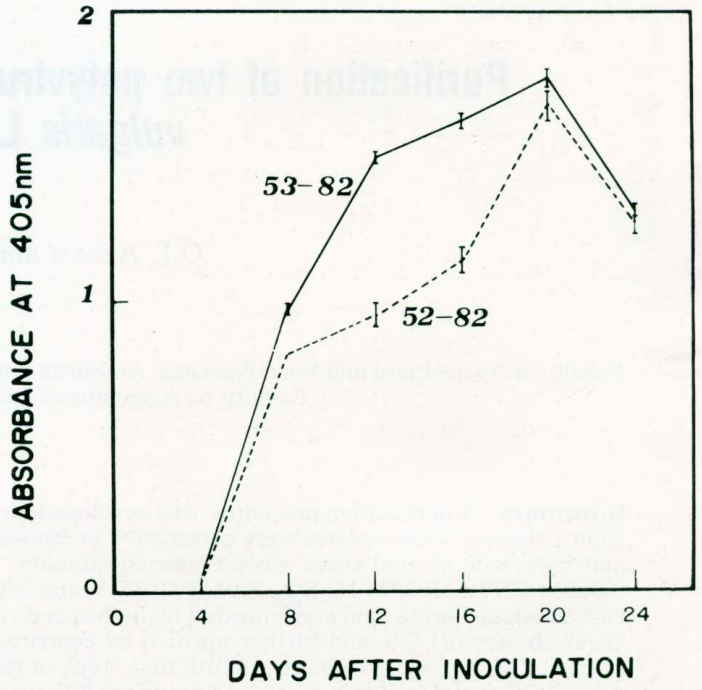

Fig. 1 - Effect of time after inoculation on virus concentration of the potyvirus isolates (52-82 and 53-82) in *Phaseolus vulgaris* cv. Sutter Pink leaves as detected by ELISA.
Fig. 1 - Effetto del tempo dopo l'inoculazione sulla concentrazione dei due isolati virali (52-82 e 53-82) in foglie di Phaseolus vulgaris *cv. Sutter Pink, determinate con ELISA.*

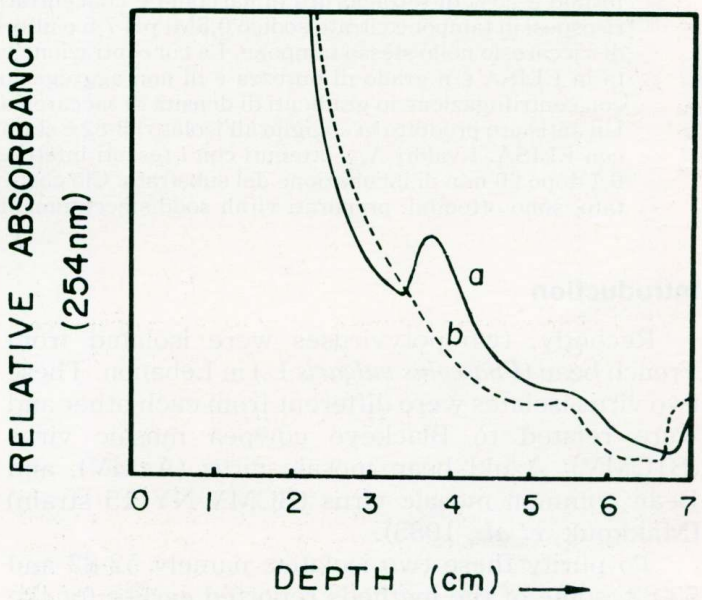

Fig. 2 - Ultraviolet-absorbance profile of a partially purified virus preparation from 52-82 infected *Phaseolus vulgaris* cultivars a) Black Turtle Soup; b) Monroe centrifuged on a 10-40% sucrose density gradient for 2 hours at 23,000 rpm.
Fig. 2 - Profilo di assorbimento UV di un preparato dell'isolato 52-82 parzialmente purificato da Phaseolus vulgaris *cultivars Black Turtle Soup (a) e Monroe (b) dopo centrifugazione in gradienti di densità di saccarosio 10-40% per 2 h a 23.000 giri/min.*

time after inoculation, when virus concentration is highest, infected cv. Sutter Pink plants were checked by ELISA 4, 8, 12, 16, 20 and 24 days after inoculation. The highest concentration was reached 20 days after inoculation (Fig. 1). Therefore in all subsequent experiments, infected bean leaves were harvested around 3 weeks after inoculation. *P. vulgaris* cv. Monroe proved to be an inadequate propagative host for 52-82 isolate purification (Fig. 2) whereas cv. Sutter Pink and cv. Black Turtle Soup were found satisfactory for the purification of both 52-82 and 53-82 isolates. Efficacy of many extraction buffers was evaluated directly by ELISA and by UV analysis of partially purified preparations after density gradient centrifugation. The buffers 0.5M K_2HPO_4 + 0.005M EDTA + 0.02M Na_2SO_3 + 0.01M DIECA, pH 8.5 (PE buffer) and 0.2M K_2HPO_4 + 0.005M EDTA, 0.02M Na_2SO_3 + 0.01M DIECA, pH 8.5 were both superior to 0.1M Tris buffer, pH 9 (Table I). In later experiments in which other extraction buffers were tested, best separation of healthy material from virus particles was obtained

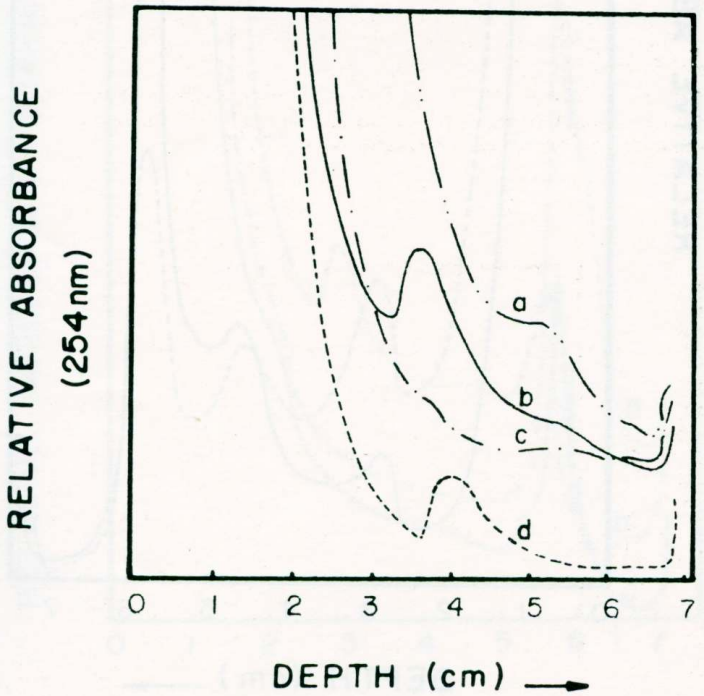

Fig. 3 - Effect of different extraction buffers on the ultraviolet absorbance profile of a partially purified virus preparation of the potyvirus (isolate 53-82) centrifuged for 2 hours in 10-40% sucrose density gradient when the following extraction buffers were used: (a) 0.5M borate + 0.005M EDTA + 0.1% thioglycolic acid, pH 8.0, (b) 0.5M K_2HPO_4 + 0.005M EDTA + 0.02M Na_2SO_3 + 0.01M DIECA, pH 8.5, (c) 0.1M K_2HPO_4, 0.01M EDTA + 0.02M Na_2SO_3 + 0.1% Thioglycolic acid + 0.5M urea, pH 8.5, and (d) 0.5M K_2HPO_4 + 0.005M EDTA + 0.02M Na_2SO_3 + 0.01M DIECA + 3% Triton X - 100, pH 8.5.
Fig. 3 - Effetto di diversi tamponi di estrazione sull'assorbimento UV di un preparato dell'isolato 53-82 centrifugato per 2 h in gradienti di saccarosio 10-40%. I tamponi usati sono indicati sopra da a) a d).

TABLE I. - Virus levels of isolates 52-82 and 53-82 after treatment of plant homogenates with different extraction buffers and clarifying agents as monitored by ELISA using the homologous antisera.
TABELLA I. - *Valori ELISA delle concentrazioni virali degli isolati 52-82 e 53-82 dopo trattamento degli estratti di piante con differenti tamponi di estrazione e agenti chiarificanti.*

	Antiserum	
Steps	52-82	53-82
Extraction buffers		
0.1M Tris-thioglycolic acid, pH 9.0	0.184	0.175
0.2M K_2HPO_4 + 0.02Na_2SO_3 + 0.005M EDTA + 0.01M DIECA, pH 8.5	0.882	0.497
0.5M K_2HPO_4 + 0.02Na_2SO_3 + 0.005M EDTA + 0.01M DIECA, pH 8.5	0.919	0.447
Clarifying agents		
Butanol 8% (V/V)	0.000	0.000
25% Butanol + 25% chloroform (V/V)	0.000	0.000
50% Chloroform (V/V)	0.945	0.387
25% Chloroform + 25% carbon tetrachloride (V/V)	0.995	0.425
Healthy bean	0.019	0.008

when 3% Triton X-100 was added to 0.5M PE buffer (Fig. 3).

Clarification and virus concentration. A series of experiments were carried out to determine the proper combination of organic solvents that leads to high virus yield and highest possible freedom from host contaminants. Infected tissue was ground in 0.5M PE buffer, centrifuged at 8000 rpm for five min and the supernatant was then treated with the solvent, stirred for 30-40 min at 4°C and centrifuged at 8000 rpm for 10 min. Virus concentration in the aqueous layer was measured by ELISA. The virus was completely lost when n-butanol or n-butanol + chloroform were used (Table I). Chloroform alone or mixed with carbon tetrachloride provided adequate clarification without significant virus loss.

For virus concentration, homogenised tissue in PE buffer was centrifuged for 10 min at 8000 rpm, clarified with 25% chloroform + 25% carbon tetrachloride and recentrifuged for 10 min at 8000 rpm. The aqueous layer obtained was divided into four equal volumes. The virus in each aliquot was concentrated differently. For the first two, polyethylene glycol (PEG) was added, while stirring, to a final concentration of 4% and 8% respectively, in the presence of 0.15M NaCl. After one h incubation at 4°C the mix-

ture was centrifuged at 8000 rpm for 10 min and the pellet obtained was suspended in 0.05M sodium citrate, pH 7.5. The third aliquot was concentrated by high speed centrifugation at 30000 rpm for 90 min and the fourth by low speed at 15,000 rpm for 90 min. Pellets obtained were dissolved in 0.05M sodium citrate buffer, pH 7.5. When virus concentration in the final preparation was measured by ELISA, it was found that the high speed, low speed and 8% PEG concentration procedures were far better than the 4% PEG procedure (Table II). However, when the 8% PEG pellets were resuspended in 0.05M sodium citrate buffer, pH 7.5 and subjected to centrifugation in 10-40% sucrose density gradients, no virus peak was observed, an indication that most virus particles were lost due to aggregation. Consequently, the 8% PEG concentration procedure was not adopted. Subsquent experiments indicated that high speed centrifugation proved to be the most reliable method to concentrate both isolates (Fig. 4).

To reduce virus aggregation further, the following resuspension buffers which have been reported previously to reduce aggregation of potyviruses were tested: 0.02M K_2HPO_4 + 0.01M EDTA + 0.02M Na_2SO_3, pH 7.4; 0.2M K_2HPO_4 + 1M Urea + 0.5% mercaptoethanol, pH 7.4; and 0.05M sodium citrate, pH 7.5. Virus recovery was evaluated by ELISA and UV analysis of sucrose density gradient tubes. Results

TABLE II. - Virus levels of isolates 52-82 and 53-82 after treatment of plant homogenates with different concentration methods as monitored by ELISA using the homologous antisera.
TABELLA II. - *Valori ELISA delle concentrazioni degli isolati 52-82 e 53-82 dopo trattamento degli estratti di piante con diversi metodi di concentrazione.*

Steps	Antiserum	
	52-82	53-82
Extraction (a)	2.125	1.840
Clarification (b)	2.112	1.761
High speed at 30000 rpm for 90 min (c)	2.095	1.735
Low speed concentration at 15000 rpm for 90 min	2.025	1.574
8% Polyethylene glycol (PEG) + 0.15M NaCl (c)	2.093	1.801
4% PEG + 0.15M NaCl (c)	0.414	0.499
Healthy bean	0.026	0.054

(a) Extraction buffer used was 0.5M K_2HPO_4 + 0.02 Na_2SO_3 + 0.005M EDTA + 0.01M DIECA, pH 8.5
(b) Clarifying agent was 25% chloroform + 25% carbon tetrachloride.
(c) Preparations were brought up to the original volume before testing by ELISA.

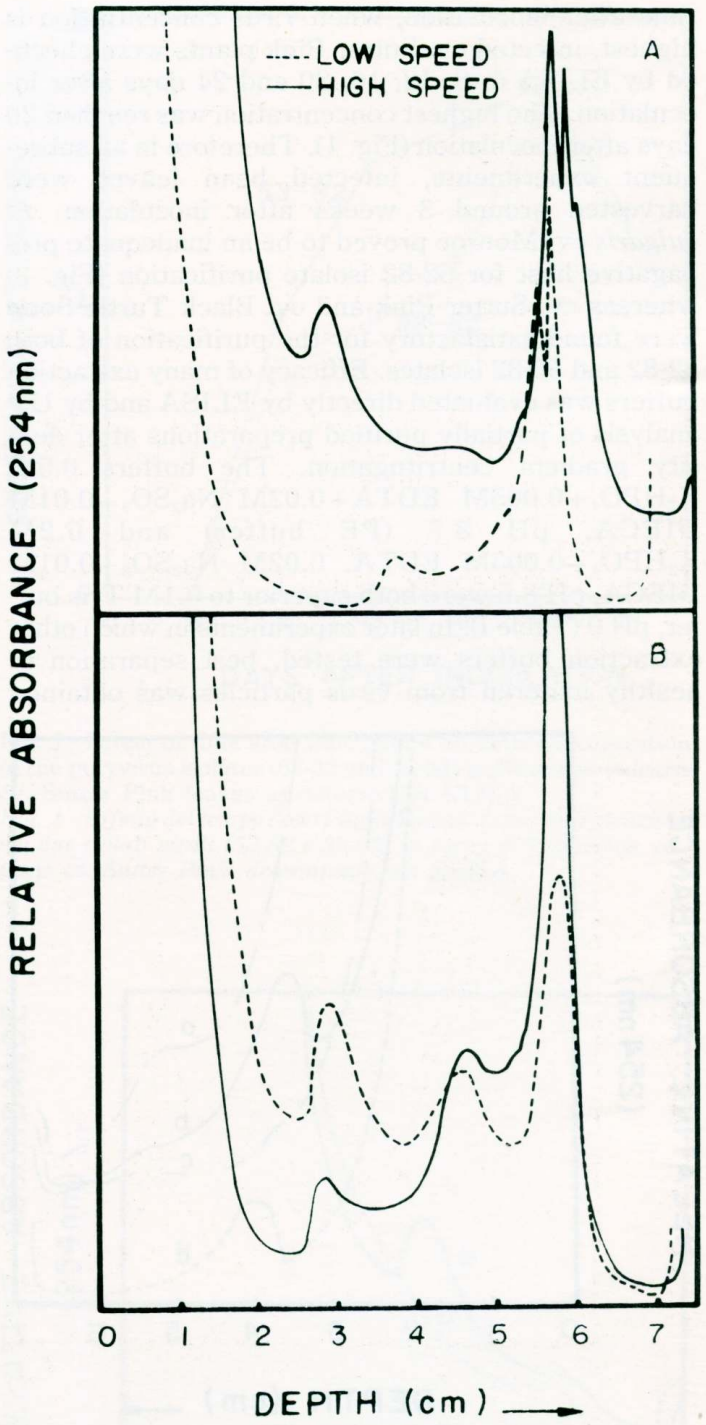

Fig. 4 - Effect of low and high speed concentration on the sedimentation patterns of the potyvirus isolates 52-82 (A) and 53-82 (B) purified from *Phaseolus vulgaris* cv. Black Turtle Soup. Ultraviolet absorbance profiles after 2 hours centrifugation in 10-75% sucrose gradient prepared 0.05M Sodium citrate, pH 7.5
Fig. 4 - Effetto della centrifugazione a bassa ed alta velocità sulla sedimentazione degli isolati virali 52-82 (A) e 53-82 (B) purificati da Phaseolus vulgaris *cv. Black Turtle Soup. Curve di assorbimento UV dopo 2 h di centrifugazione in gradienti di saccarosio 10-75% in citrato sodico 0,05M, pH 7.5.*

indicated that the addition of either 0.01M EDTA or 1M urea to the resuspension buffer did not reduce virus aggregation of either isolate during purification (Fig. 5). The 0.05M sodium citrate buffer proved to be the most adequate for the resuspension of the high speed pellets. When the resuspended high speed pellets were layered on 10-40% and 10-75% sucrose gradients and centrifuged for 2 h, three virus peaks were noticed in the 10-75% gradient as compared to only one peak in the 10-40% gradient (Fig. 6). The virus purification procedure followed yielded 20-30 mg/kg of fresh bean tissue.

Serology. Using the antiserum produced against the virus preparation purified by the procedure reported in this study in direct ELISA, the A_{405} value obtained for healthy bean tissue was 0.10 as compared to 1.78 for the homologus antigen, after 90 min of substrate incubation. Gamma globulin concentration used for coating the plate was 1 μg/ml and conjugate dilution used was 1/1000. When the same test was done using an antiserum produced by injecting a virus preparation purified by the CsCl equilibrum density gradient centrifugation procedure, the A_{405} values obtained for healthy and infected bean tissues were 0.10 and 2.11, respectively. Such results are an indication that the purification procedure followed in this study yielded a fairly purified virus preparation.

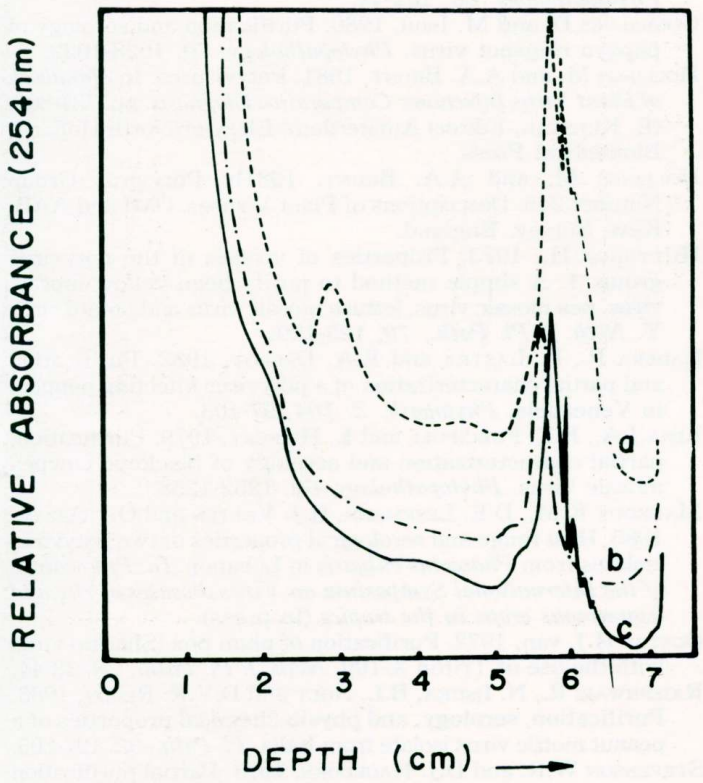

Fig. 5 - Effect of three resuspension buffers on the ultraviolet-absorbance profile of the potyvirus (isolate 52-82) purified from *Phaseolus vulgaris* cv. Black Turtle Soup and centrifuged two hours in 10-75% sucrose density gradient. Virus in high speed pellet was resuspended in a) 0.05M sodium citrate, pH 7.5; b) 0.02M K_2HPO_4 + 0.01M EDTA + 0.02M Na_2SO_3, pH 7.4; c) 0.02M K_2HPO_4 + 1.0M Urea + 0.1% 2-Mercaptoethanol.
Fig. 5 - Effetto di tre tamponi di risospensione sull'assorbimento UV dell'isolato 52-82 purificato da Phaseolus vulgaris *cv. Black Turtle Soup e centrifugato per 2 h nei gradienti di densità di saccarosio 10-75%. I sedimenti delle alte centrifugazioni sono stati risospesi nel modo indicato sopra da a) a c).*

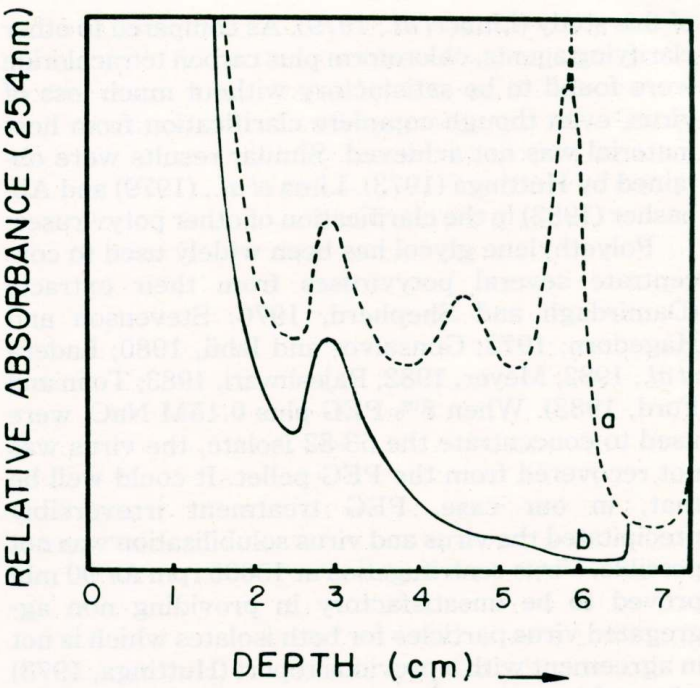

Fig. 6 - Ultraviolet-absorbance profile of the potyvirus (isolate 53-82) centrifuged on (a) 10-75% and (b) 10-40% sucrose density gradient prepared in 0.05M Sodium citrate, pH 7.5
Fig. 6 - Assorbimento UV dell'isolato 53-82 centrifugato in gradienti di densità di saccarosio 10-75% (a) e 10-40% (b), in citrato sodico 0,05M pH 7,5.

Discussion

Purification of the 52-82 isolate was not successful when cv. Monroe was used as propagative host, even though high concentration of the virus was detected by ELISA. Such cultivar might have some components which during tissue homogenisation lead to instability of virus particles or enhance aggregation and subsequent loss of virus during purification.

The addition of Triton X-100 to PE buffer improved the purification procedure, which is in agreement with previous findings (Oosten, 1972; Ladera *et al.*, 1982). Clarification with n-butanol caused significant virus loss. This finding was not in agreement with previous work where n-butanol was found to be useful for the purification of a number of potyviruses (Bock, 1973 a; Hollings and Brunt, 1981 and 1981a; Rajeshwari, 1983) and particularly to BlCMV which is very closely related to the two virus isolates subject

of this study (Lima *et al.*, 1979). As compared to other clarifying agents, chloroform plus carbon tetrachloride were found to be satisfactory without much loss of virus, even though complete clarification from host material was not achieved. Similar results were obtained by Huttinga (1973), Lima *et al.*, (1979) and Abbasher (1983) in the clarification of other potyviruses.

Polyethylene glycol has been widely used to concentrate several potyviruses from their extracts (Damirdagh and Shepherd, 1970; Stevenson and Hagedorn, 1973; Gonsalves and Ishii, 1980; Ladera *et al.*, 1982; Meyer, 1982; Rajeshwari, 1983; Tolin and Ford, 1983). When 8% PEG plus 0.15M NaCl were used to concentrate the 53-82 isolate, the virus was not recovered from the PEG pellet. It could well be that, in our case, PEG treatment irreversibly precipitated the virus and virus solubilisation was not possible. Virus centrifugation at 15000 rpm for 90 min proved to be unsatisfactory in providing non aggregated virus particles for both isolates which is not in agreement with a previous report (Huttinga, 1973) where this treatment was found satisfactory with a number of potyviruses. Damirdagh and Shepherd (1970) obtained highly purified preparation of Tobacco etch virus when they included urea in their resuspension buffer. Similarly, Tolin and Ford (1983) succeeded in purifying large amount of unaggregated and pure Peanut mottle virus when they included EDTA and urea in the resuspension buffer. When these two additives were incorporated to the phosphate buffer and used to resuspend the 52-82 and 53-82 pellets, a complete loss of virus in sucrose gradients resulted. Our findings are in agreement with previous work which indicated that EDTA and urea may possess a detrimental effect on virus stability during purification of some viruses (Lauffer and Stanley, 1943; Price 1963; Shepherd and Shalla, 1969). As noted by Damirdagh and Shepherd (1970) and as concluded by Tolin and Ford (1983), effectiveness of reagents like EDTA and urea in minimising aggregation of several viruses may be variable and experimental determination of the right concentration is recommended for each individual virus. It is probable that EDTA and/or urea concentrations used in this study were at levels detrimental to the viral capsid.

The procedure adopted in this study did not lead to complete virus recovery in an unaggregated manner since more than one virus peak was observed when 10-75% sucrose gradients were used. The bottom two bands were, most likely, aggregated virus since they both gave strong reaction against 53-82 antiserum in ELISA. Such aggregated material which is lost in the 10-40% sucrose gradient are collected when caesium chloride gradients are used. However, when laboratory conditions available do not permit the use of equilibrium density gradient centrifugation, reduced virus yield by using sucrose density gradient centrifugation would be the second best.

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