

Stabilization of Purified Potato Virus X by Dextran T-10 during Lyophilization

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Received October 23, 1968

Abstract. Purification and preservation of potato virus X from leaf sap of tobacco plants before lyophilization was carried out by two methods: 1) precipitation by polyethylene glycol and ultracentrifugation, and 2) precipitation by ammonium sulphate, chromatography on Sephadex G-50 and ultracentrifugation. The first method is preferable to the second because the final preparation contains more virus antigen. Both preparations were strongly infectious and maintained antigenic properties after lyophilization.

To achieve a more gentle course of lyophilization of virus preparations, addition of urotropine and dextran T-10 to the virus suspension, purified by the precipitation by polyethylene glycol-6000, was examined. Addition of urotropine was proved unsatisfactory, because only antigenic properties were maintained after lyophilization while the infectivity disappeared. But we can recommend addition of dextran T-10 up to a concentration of 6% to the preparation of virus antigen before lyophilization. The course of lyophilization is much rapider, the lyophilized product can be very easily dissolved in water and is not hygroscopic. The product is strongly infectious and gives the serological precipitation reaction in a dilution four times that of X virus antigen lyophilized without addition of dextran T-10.

Preservation of plant viruses with full biological activity is very important for virological research, because it permits work in all seasons without requiring field or greenhouse growing of host and indicator plants.

The best method of preservation of purified and concentrated virus preparations is lyophilization. The quality of the final product of lyophilization is affected not only by the method of purification and concentration of virus but also by the way lyophilization is carried out.

Preservation by lyophilization suspensions of potato virus X and Y which were obtained from the leaf sap of tobacco plants infected with these viruses has been previously described by JERMOLJEV and PRŮŠA (1957) and by BOJARSKY (1961). Antigenic properties were preserved after lyophilization, but the titres of these preparations were low. A lyophilized preparation of potato virus X of higher titre was later prepared by ALBRECHTOVÁ et al. (1968). But reduction of the titre of lyophilized virus preparation occurs

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during lyophilization. We have observed that one reason is the considerable hygroscopicity of the lyophilized preparation; another reason is the residual humidity in preparations. For this reason we wanted to test substances which would eliminate those undesirable phenomena and would stabilize the virus suspension during lyophilization. We used urotropine (Schering) and dextran T-10 (Pharmacia, Uppsala). In addition we compared two methods for preparation of purified and concentrated potato virus X. In the first method precipitation by polyethylene glycol is used (LEBERMANN 1966, ALBRECHTOVÁ and KLÍR 1968), and in the second method ammonium sulphate, column chromatography on Sephadex G-50 and ultracentrifugation (ALBRECHTOVÁ et al. 1968).

Material and Methods

Chemical and Plant Material

We used polyethylene glycol-6000 (Koch and Light), original solution of 20% urotropine (Hexamethylentetramin) (Schering) and original solution of 20% dextran T-10 (Pharmacia Uppsala). Plants of tobacco hybrid (*Nicotiana tabacum* L.) S 390-1 from Velký Báb, infected with potato virus X, were the virus source.

Crude Extract

One hundred grams of frozen leaves of tobacco plants (freezing time one hour) infected with potato virus X were homogenized in a mortar with 80 ml of 0.007 M phosphate buffer, pH 7.8, which contained 0.01 M veronal, 0.01 M cysteine and 0.007 M EDTA (MUNDY and RÖHMER 1958). The sap was expressed through silon cloth and centrifuged at $9\,000 \times g$ for 10 minutes. We then proceeded according to two different methods.

Virus Purification — Method I.

We added to a 50 ml portion of supernatant 0.73 g NaCl (solution 0.25 M NaCl) and 6.25 g polyethylene glycol-6000 (to a concentration of 12.5%) slowly and under stirring, and the mixture was then centrifuged at $10\,000 \times g$ for 15 minutes. The sediment was dissolved in 12 ml of 0.01 M phosphate buffer, pH 7.0, and centrifuged in a Spinco L centrifuge for one hour at 40000 rev./min, the sediment was dissolved in 5 ml of 0.01 M phosphate buffer, pH 7.5, and insoluble material was removed by centrifugation at $5000 \times g$ for 10 minutes. The supernatant containing the purified X virus in the concentration 1.27 mg of virus protein in 1 ml, was then lyophilized.

Method II.

We added to another 50 ml portion of supernatant a cold saturated solution of ammonium sulphate to a concentration of 38% and the mixture was kept for about 30 minutes in an icebox. The precipitate was then separated by centrifugation for 10 minutes at $9\,000 \times g$. The sediment was dissolved in 10 ml of 0.01 M phosphate buffer and briefly centrifuged to remove coarse insoluble particles. The supernatant was chromatographed on a Sephadex G-50 column, equilibrated with 0.01 M phosphate buffer, pH 7.8, and eluted

with the same buffer. Fractions which reacted positively with homologous antiserum, were combined and centrifuged on the Spinco L. ultracentrifuge for 1 hour at 40 000 rev./min. The sediment was dissolved in 5 ml of 0.01 M phosphate buffer, pH 7.5 and again centrifuged for 5 minutes at 3 000 x g. The virus purified in this way was lyophilized on a special apparatus for lyophilization "Laboratorní potřeby".

Experiments with Dextran

In experiments with dextran we proceeded as follows: Purified virus was obtained from 50 g of leaves of tobacco plants infected with potato virus X using precipitation by polyethylene glycol. The sediment after ultracentrifugation was suspended in 5 ml of phosphate buffer and divided into 4 portions of 1.25 ml for lyophilization. Dextran T-10 was added in these variants: 1) without addition of dextran, 2) addition of dextran to 3%, 3) to 4.5%, and 4) to 6%. Lyophilization was carried out on a laboratory glass apparatus.

Results

Both virus preparations obtained from the same clarified sap were tested for their relative amount of virus antigen. The relative amount of virus antigen was found by the determination of virus end-point dilution of sap with positive reaction. The results of serological precipitation reactions are given in Table 1.

TABLE 1
PRECIPITATION reaction with homologous antiserum

Preparation of potato virus X	Dilution of virus antigen								Dilution of leaf sap from healthy plants
	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/4
Before lyo- philization									
Method I.	+++3	+++3	+++3	+++3	+++3	+++2	+++2	+++2	—
Method II.	+++3	+++3	+++3	+++3	+++2	+1	—	—	—
After lyo- philization									
Method I.	+++3	+++2	+++2	—	—	—	—	—	—
Method II.	+++2	+++2	—	—	—	—	—	—	—

Amount of virus antigen in the preparation obtained with the first method, and that obtained by precipitation with polyethylene glycol, was before lyophilization about fourfold that of the preparation obtained with the second method (using ammonium sulphate, Sephadex G-50 and ultracentrifugation). Precipitation with polyethylene glycol results in a more concentrated virus

preparation in comparison with that obtained by precipitation with ammonium sulphate, when the starting material is the same. One possible reason could be the fact that the non-ionogenic polymer, polyethylene glycol, acts more gently on the virus protein than ammonium sulphate. Losses in virus protein take place when the virus is precipitated with cold saturated solution of ammonium sulphate to 38% saturation, because of its partial denaturation.

However during lyophilization there were losses of X virus antigen prepared by both methods. The X virus preparation obtained using precipitation by polyethylene glycol resulted in a better lyophilized product only because of the higher content of virus antigen before lyophilization.

Infectivity of both lyophilized preparations was determined, after dissolving, on plants of *Nicotiana tabacum* and *Datura stramonium*. Both preparations were strongly infectious.

Because of the high hygroscopicity of the end product which is the main reason for losses during lyophilization, we tried, as we had done with antisera (JERMOLJEV and ALBRECHTOVÁ 1968), to lyophilize purified and concentrated X-virus preparations with addition of stabilizing substances such as urotropine and dextran T-10.

Urotropine, added to the purified X virus before lyophilization up to the concentration 4.5% was found unsuccessful. Losses of X virus antigen were greater during lyophilization with urotropine than without it. Though the preparation maintained antigenic properties, the infectivity disappeared completely; the preparation without urotropine was infectious.

Good results were obtained with dextran T-10 as stabilizing material. Lyophilization of X virus antigen occurred with dextran much quicker than without it, and the lyophilized product could be very easily dissolved in water. The course of lyophilization and the characteristics of the product are given in Table 2.

TABLE 2

OBSERVATION of the course of lyophilization

Lyophilized antigen Variant	Time necessary for lyophilization Hours	Character of the lyophilized preparation	Character after dissolving	Infectivity on <i>Datura</i> <i>stramonium</i> plants
Original	3	compact	faintly troubled	strong
+ 3% of dextran T-10	3	very light, soft	very faintly troubled	medium
+ 4.5% of dextran T-10	2	light, soft	very faintly troubled	strong
+ 6% of dextran T-10	1.5	white, light, soft	very faintly troubled	strong

Infectivity of the lyophilized X virus antigen with addition of dextran T-10 was tested on *Datura stramonium* plants and it was found that the lyophilized

virus antigen maintained strong infectivity. It gave a serological precipitation reaction with homologous antiserum even at the dilution 1/512—1/1024 in comparison with the dilution 1/256 of the original lyophilized antigen. The presence of dextran T-10 did not disturb the serological precipitation reaction.

Sensitivity of serological precipitation reaction of different variants with homologous antiserum is given in Table 3.

TABLE 3

SEROLOGICAL PRECIPITATION reaction of potato virus X antigen with homologous antiserum. — Sensitivity of different variants.

Lyophilized antigen Variant	Dilution of virus antigen						Dilution of leaf sap from healthy plants
	1/32	1/64	1/128	1/256	1/512	1/1024	1/32
Original	+++3	+++3	+++3	++2	—	—	—
+ 3% of dextran T-10	+++3	+++3	+++3	+++3	++2	—	—
+ 4,5% of dextran T-10	+++3	+++3	+++3	+++3	+1	—	—
+ 6% of dextran T-10	+++3	+++3	+++3	+++3	++2	+1	—

Lyophilization of potato virus X antigen, purified and concentrated using precipitation by polyethylene glycol 6000 and ultracentrifugation, occurred most favourably when 6% of dextran T-10 was added. The lyophilized X virus antigen, light, soft and easily soluble in water, is best sealed in small vials in vacuum and kept in an icebox. We suppose that under these conditions the lyophilized X virus antigen will maintain all biological properties and will store better than lyophilized X virus antigen without addition of dextran T-10.

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Čištění a konzervace X-viru bramboru ze šťávy z listů rostlin tabáku před lyofilizací byla provedena dvěma způsoby: 1. vysrážením polyethylenglykolem a ultracentrifugací a 2. vysrážením síranem amonným, chromatografií na Sephadexu G-50 a ultracentrifugací. První metoda je výhodnější, poněvadž v konečném preparátu zůstává více antigenu viru než u druhé metody. Oba preparáty byly po lyofilizaci silně infekční a zachovaly si antigenní vlastnosti.

Abychom docílili šetrnějšího průběhu lyofilizace virových preparátů byl vyzkoušen přídavek urotropinu a dextranu T-10 k suspensi viru, čištěné srážením polyethylenglykolem-6000. Přídavek urotropinu se neosvědčil, poněvadž po lyofilizaci se zachovali jen antigenní vlastnosti, kdežto infektivita se úplně ztratila. Můžeme však doporučit přídavek dextranu T-10 do koncentrace 6 % k preparátu antigenu viru před lyofilizací. Lyofilizace probíhá mnohem rychleji, lyofilizovaný produkt se velmi snadno rozpouští ve vodě a není tak hygroskopický. Je silně infekční a poskytuje s homologním antisérem sérologickou reakci precipitace kapkovou metodou ve čtyřikrát větším zředění než antigen X-viru lyofilizovaný bez přídavku dextranu T-10.