Characterization of Structures and Antiviral Effects of Polysaccharides from *Portulaca oleracea* L.

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Three polysaccharides including a neutral polysaccharide (RN), an acidic polysaccharide (RA) and a pectic polysaccharide (RP) were isolated from aerial part of *Portulaca oleracea* L. and evaluated for their anti-herpes simplex virus type 2 (HSV-2) and anti-influenza A virus (IFV-A). RN was found to consist of glucose (Glc), mannose (Man) and arabinose (Ara) with small amounts of galactose (Gal), and identified to be an arabinoglucomannan. RA was mainly composed of Gal and Ara with a small proportion of glucuronic acid (GlcA). It was characterized as a type II arabinogalactan (AGII), which consisted of a 1,3-, 1,6- and 1,3,6-linked galactopyranosyl (Gal*p*) and non-reducing terminal and 1,5-linked arabinofuranosyl (Ara*f*) residues. RP was deduced to be a pectin, which consisted of a predominant amount of galacturonic acid (GalA) with small amounts of Gal, rhamnose (Rha) and Ara. The GalA residues were found to be highly methyl-esterified and partially acetylated. Results of antiviral tests showed that only RP had anti-HSV-2 activity. Furthermore, its anti-HSV-2 target was elucidated to be the step of virus penetration into host cells. No marked virucidal activity of RP was observed.

Key words *Portulaca oleracea* L.; polysaccharide; antiviral effect; herpes simplex virus type 2

Portulaca oleracea L. (Portulacaceae) is an annual plant widely distributed from the temperate to the tropical zones, and has a long history of use as a medicinal and edible plant. As a traditional Chinese medicine, it has been used for treating dysentery with bloody stools, eczema, erysipelas, and used as febrifuge and antiseptic.[1] Recent researches show that it exhibits a wide range of biological effects, including skeletal muscle relaxant effect,[2] analgesic and anti-inflammatory effects,[3] antifungal activity,[4] and antifertility effect.[5] Although *P. oleracea* has been applied in clinical treatment for viral infectious diseases in China, there is no report on the antiviral activity of this plant.[6] In the present paper, we report on the structures and antiviral activities of three polysaccharides isolated from *P. oleracea*.

Results and Discussion

The hot water extract from the defatted aerial part of *Portulaca oleracea* L. was dialyzed against H_2O to obtain a crude polysaccharide. The non-dialyzed fraction was separated by anion-exchange chromatography on Toyopearl DEAE 650M to give two major fractions, RH1 and RH2, eluted with H_2O and 0.5 M NaCl, respectively. Further separation of each fraction was carried out using a combination of ion-exchange column chromatography and gel filtration to yield a neutral polysaccharide (RN), an acidic polysaccharide (RA) and a pectic polysaccharide (RP).

All of the isolated polysaccharides were eluted as a single and symmetrically sharp peak on HPLC chromatogram, and the apparent molecular weight of RN, RA and RP were estimated to be 8.3×10^3 ($M_w/M_n=1.2$), 5.8×10^4 ($M_w/M_n=1.2$) and 8.7×10^4 ($M_w/M_n=1.7$), respectively. In addition, RP was detected as a single band on the cellulose acetate membrane electrophoresis (data not shown). From these results, RN, RA and RP were regarded to be homogeneous polysaccharides. Total carbohydrate contents of RN, RA and RP were determined to be 98.1%, 54.7% and 57.0% by phenol–H_2SO_4 method, respectively. Uronic acid contents of the samples were determined to be 2.8% (RN), 13.2% (RA), 90.7% (RP) using a *m*-hydroxydiphenyl method. In addition, three polysaccharides were estimated to contain trace amounts of protein, RN (0.7%), RA (1.5%) and RP (1.9%) by Bradford assay, respectively.

Sugar composition analysis of RN revealed that it was mainly composed of glucose (Glc) (40.1%), mannose (Man) (38.8%) and arabinose (Ara) (13.7%) with small amounts of galactose (Gal) (5.3%). As shown in Table 1, methylation analysis showed that RN contained 1,4-linked mannopyranosyl (Man*p*) (34.9%), 1,4-linked glucopyranosyl (Glc*p*) (22.0%), and 1,2,4-linked Glc*p* (10.2%) residues with small amounts of 1,4,6-linked Man*p* (1.9%), 1,4,6-linked Glc*p* (0.5%) and terminal-linked galactopyranosyl (Gal*p*) residues (1.8%). In addition, arabinofuranosyl (Ara*f*) residue was

Table 1. Methylation Analysis of RN, RA and RRA

Sugar	Substitution position	RN (mol%)	RA (mol%)	RRA (mol%)
Rha*p*	Terminal		1.2	1.3
	1,2,4-			0.2
Ara*f*	Terminal	11.2	22.3	21.5
	1,3-	0.6	1.7	1.5
	1,5-	3.4	7.8	6.4
Xyl*p*	Terminal	1.0		0.8
Man*p*	Terminal	6.1		
	1,4-	34.9		
	1,3,4-	0.2		
	1,2,4-	0.2		
	1,4,6-	1.9		
Gal*p*	Terminal	1.8	6.5	6.2
	1,3-		14.5	13.9
	1,4-	2.3		0.3
	1,6-	0.2	12.0	11.9
	1,3,4-		0.2	1.3
	1,4,6-		0.2	1.4
	1,3,6-	0.8	28.4	26.5
	1,3,4,6-	0.6	5.2	5.0
Glc*p*	Terminal	0.4		0.6
	1,4-	22.0		1.2
	1,6-	1.6		
	1,2,4-	10.2		
	1,4,6-	0.5		

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suggested to be mainly present as non-reducing terminal linked residues (11.2%). Therefore, RN was suggested to be an arabinoglucomannan type polysaccharide.[7)]

Monosaccharide composition of RA was analyzed using a combination of trifluoroacetic acid (TFA) hydrolysis and methanolysis followed by trimethylsililation. RA was revealed to be mainly composed of Ara (23.2%), Gal (67.0%) with small proportions of rhamnose (Rha) (2.8%), xylose (Xyl) (2.9%) and glucuronic acid (GlcA) (4.1%). The results of methylation analysis of RA and its reduced product (RRA) were summarized in Table 1. RA was found to mainly consist of 1,3-linked Gal*p* (14.5%), 1,3,6-linked Gal*p* (28.4%), 1,6-linked Gal*p* (12.0%) and 1,5-linked Ara*f* (7.8%), 1,3-linked Ara*f* (1.7%) and non-reducing end-residues of Ara*f* (22.3%) and Gal*p* (6.5%), indicating that RA is a typical type II arabinogalactan (AGII) which consists of a 1,3-linked β-D-Gal*p* main chain, partially substituted at C-6 by 1,6-linked β-D-Gal*p* side chains.[7)] On the other hand, the appearance of terminal-linked Glc*p* (0.6%) and 1,4-linked Glc*p* (1.2%) residues in the case of carboxyl reduced polysaccharide (RRA) indicated that GlcA was present in RA at the forms of terminal-linked and 1,4-linked residues.

Monosaccharide composition of RP was determined in the same manner used for RA. RP was mainly composed of galacturonic acid (GalA) (67.8%), Gal (11.3%) and GlcA (10.6%) with small amounts of Ara (5.8%) and Rha (4.3%). The predominant amount of GalA with small amount of Rha indicated that RP might be a pectic polysaccharide. In addition, other small proportions of sugars Xyl, Glc and GlcA were also detected, which have been reported sometimes present in the side chains.[8)]

In the ^{1}H-NMR spectrum of RP, the large signal at δ 3.81 ppm was considered to be derived from esterified methyl groups and two characteristic signals at δ 2.13 and 2.04 ppm was assigned to acetyl groups substituted at C-2 and C-3 of GalA, as compared with the reported values.[9,10)] In the ^{13}C-NMR spectrum of RP, the signal at δ 55.5 ppm was assigned to esterified methyl groups of GalA and signals at δ 175.5 and 173.3 ppm were attributed to carboxyl groups of GalA and esterified GalA, respectively.[9)] In addition, the presence of two anomeric carbon signals at δ 102.2 and 102.7 ppm indicated that two sugar repeating units were present in RP. The ^{1}H-NMR spectrum of RP showed a complexity to assign each signal of sugar residues due to esterification of carboxyl groups. Thus, de-esterified RP (DRP) was applied to further NMR analysis. The signal at δ 3.81 ppm in the ^{1}H-NMR spectrum and two signals at δ 55.5 and 173.3 ppm in the ^{13}C-NMR spectrum of RP disappeared. These findings supported the notion that GalA residues were highly methyl-esterified and partially acetylated. In the ^{1}H-NMR spectrum of DRP, five major signals were observed and assigned to H-1 (δ 5.08 ppm), H-2 (3.76), H-3 (3.98), H-4 (4.44) and H-5 (4.83) of α-D-GalA. Six signals were clearly observed and assigned as C-1 (δ 102.0 ppm), C-2 (70.9), C-3 (71.4), C-4 (80.8), C-5 (73.6) and C-6 (176.2) of α-D-GalA in the ^{13}C-NMR spectrum of DRP. These spectral data indicate that RP was suggested to be a galacturonan mainly composed of α-1,4-linked GalA residues which were highly methyl-esterified and partially acetylated. The degree of methyl-esterification (DM) was estimated to be 73.2% according to the reported method,[11)] indicating that RP was a highly methyl esterified pectic polysaccharide.

The three polysaccharides were evaluated for their inhibitory effects on replication of herpes simplex virus type 2 (HSV-2) and influenza virus type A (IFV-A) (Table 2). No anti-IFV-A activity was observed in these polysaccharides. Among the three polysaccharides, only RP showed potential anti-HSV-2 activity with selectivity index (SI) of more than 20. The SI for HSV-2 was higher when RP was added during viral infection (Experiment A) as compared with that when it was added immediately after infection (Experiment B). Since the GalA residues in RP were found to be highly methyl-esterified and partially acetylated as mentioned above, the de-esterified RP (DRP) was prepared and subjected to the same assay to elucidate the role of these groups in the antiviral action of RP. The result revealed that the anti-HSV-2 activity of RP disappeared after de-esterification on the basis of low SI values of 1.8 and 1.9 in the experiments A and B, respectively.

In order to determine the most sensitive phase of HSV-2 replication to RP, time-of-addition experiments were performed. In these experiments, Vero cells were infected with HSV-2 at a high multiplicity of infection of 10. As shown in Fig. 1, RP suppressed viral replication occurred most efficiently when added at the same time as virus infection and throughout the incubation thereafter. A relatively high anti-HSV-2 effect was also observed when the sample was added only during viral infection. However, RP showed less anti-HSV-2 effect when the sample was added to the medium at various times after infection. These results suggested that RP

Table 2. Anti-HSV-2 and IFV-A Activities of RN, RA and RP

Virus	Samples	Cytotoxicity (CC_{50}, μg/ml)	Antiviral activity (IC_{50}, μg/ml)		Selectivity index (CC_{50}/IC_{50})	
			A	B	A	B
HSV-2	RN	>10000	>1000	>1000	>><10	>><10
	RA	>10000	>1000	>1000	>><10	>><10
	RP	7000	210	320	33	22
IFV-A	RN	>10000	>1000	>1000	>><10	>><10
	RA	>10000	>1000	>1000	>><10	>><10
	RP	5100	>1000	>1000	<5	<5

A: Sample was added during infection and throughout the incubation thereafter.
B: Sample was added immediately after viral infection.

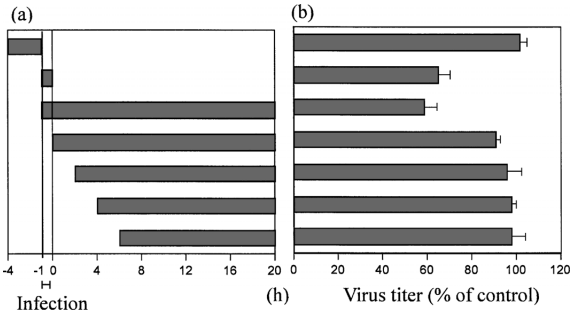

Fig. 1. Effect of Time of Addition of RP on HSV-2 Replication

Vero cells were infected with HSV-2 at 10 PFU/cell. RP was added to the culture medium at the times shown in the panel (a). Briefly, RP (500 μg/ml) was added at various time intervals: 3 h before infection, during infection for 1 h, during viral infection and throughout the incubation, and post-infection (0, 2, 4, 6 h). No test compound was added to the control. At 20 h post-infection, virus yields were determined by plaque assay (b). The plaque number of control was taken as 100%. Each value is the mean±S.D. from triplicate assays.

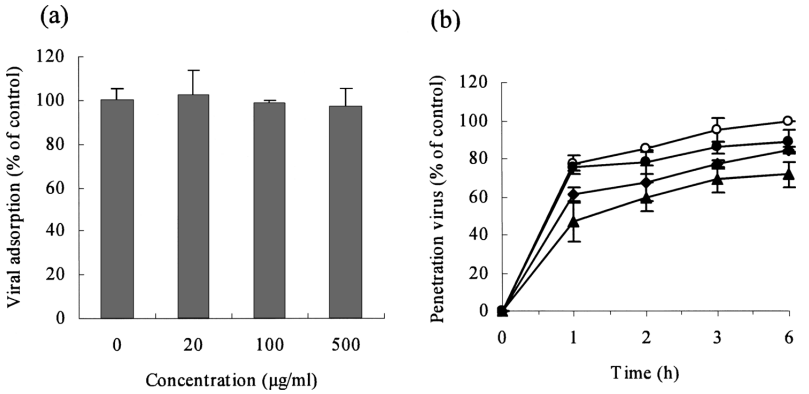

Fig. 2. Effect of RP on HSV-2 Adsorption and Penetration

Vero cell suspensions were infected with HSV-2 (1 PFU/cell) in the absence or presence of RP (20, 100 or 500 μg/ml) at 4 °C (a). Virus-adsorbing cells were counted by an infection center assay. In penetration assay, cell monolayers were infected at 4 °C with HSV-2 in the absence of RP, and then shifted to 37 °C to penetrate the adsorbed virus in the presence of RP (b). The penetrated viruses were titrated by plaque assay. ○, no drug control; ●, 20 μg/ml; ◆, 100 μg/ml; and ▲, 500 μg/ml of RP. The plaque number of control at 6 h after temperature shift was taken as 100%. Each value is the mean±S.D. from triplicate assays.

might interfere with early events including virus binding to the receptor on the host cell membrane surface and/or virus penetration into host cells by fusion between the virus envelope and cell membrane. Thus, the effect of RP on virus adsorption was evaluated using an infectious center assay, which determined the number of cells binding the virus particles at low temperature (4 °C) in the presence of different sample concentrations. As shown in Fig. 2a, no dose-dependent interference with virus adsorption was observed, indicating that the antiviral target of RP was not the virus-cell interaction. Then, the effect of RP on HSV-2 penetration step was examined. As shown in Fig. 2b, RP exerted a moderate inhibitory effect on virus penetration in dose- and time-dependent manners. Therefore, it was confirmed that virus penetration step was at least one of antiviral targets of RP.

When the virucidal effect of RP on HSV-2 infectivity was examined, no marked inactivation of the virus was observed at 100 μg/ml even after 6 h of incubation at 37 °C (data not shown). These results do not contradict the data from the time-of-addition experiments, because more reduction of virus titers would be seen even after addition of RP at 6 h post-infection (p.i.) if the released viruses were inactivated by RP.

So far, numerous sulfated polysaccharides were reported to be endowed with the capacity to inhibit HSV infection by blocking the adsorption, entry and cell-to-cell spread of viruses. The molecular target of sulfate polysaccharides was found to be binding to HSV envelope glycoproteins *via* negative charged sulfate/carboxylate groups, therefore inhibiting virions from binding to and penetrating target cells.[12,13] In the present study, a pectic polysaccharide RP, with highly methyl-esterified and partially acetylated GalA residues, was demonstrated to exert a potential anti-HSV-2 activity by inhibiting virus penetration but not virus adsorption step. After de-esterification of RP, its anti-HSV-2 activity ceased, indicating that methyl-esterification and/or acetylation of GalA residues might be responsible for exerting the antiviral action. This is the first report of an antiviral pectic polysaccharide that was isolated from *P. oleracea*.

Experimental

Plant Material *P. oleracea* L. was collected in August, 2008 at Toyama, Japan.

General Experimental Procedure NMR spectra were recorded using a Unity plus 500 spectrophotometer (Varian, Palo Alto, CA, U.S.A.). Each sample was dissolved in D_2O (99.9%), and all spectra were recorded at 70 °C. All chemical shifts were relative to external tetramethylsilane. GC was carried out on a GL-Science GC-353 gas chromatograph equipped with a FID detector, and nitrogen was used as carrier gas. GC-MS analysis was performed on a Shimadzu GC-MS QP, and helium was used as carrier gas. Cellulose acetate membrane electrophoresis and HPLC analysis were performed as described elsewhere.[14] Toyopearl DEAE 650 M and $GMPW_{XL}$ columns were purchased from Tosoh (Tokyo, Japan). Sepharose 6B, Sephacryl S-100 HR and S-300 HR were from GE Healthcare (Piscataway, NJ, U.S.A.). Eagle's minimal essential medium (MEM) was obtained from Nissui Pharmaceutical (Tokyo, Japan). Other chemicals were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Extraction and Isolation Fresh aerial parts of *P. oleracea* (6.9 kg) were cut into pieces and extracted with EtOH (8.1 l) at room temperature for 15 h three times. After filtration, the residue was extracted with H_2O (12.2 l) under reflux for 1 h three times. Then, the combined extracts were filtered through defatted cotton and concentrated *in vacuo* and lyophilized. The yielded residue (45.4 g) was dissolved in H_2O and centrifuged at 3000 rpm for 15 min to remove the insoluble portion. The supernatant was subjected to dialysis to yield dialysate (PHs-L, 35.2 g) and nondialysate (PHs-H, 3.7 g).

PHs-H (3.7 g) dissolved in H_2O was applied to a Toyopearl DEAE 650M anion exchange column (5.0×20 cm), which was successively eluted with H_2O, 0.5 M NaCl, 1.0 M NaCl, 2.0 M NaCl, and 0.2 N NaOH to give RH1 (7.4%), RH2 (38.0%), RH3 (3.5%), RH4 (2.2%) and RH5 (5.7%). RH1 (260 mg) was subjected to a Sepharose 6B gel filtration (4.4×90 cm) and eluted with 0.01 M citrate buffer containing 0.1 M NaCl. The fractions were collected and monitored using the phenol–H_2SO_4 method [15] and UV absorbance at 480 nm to give three sub-fractions, RH1A (5.6%), RH1B (15%) and RH1C (1.8%). Then RH1B (39 mg) was applied to a Sephacryl S-100 HR gel filtration (3.0×90 cm) and eluted with distilled H_2O to yield a neutral polysaccharide RN (30 mg).

RH2 (1.4 g) was separated by anion exchange chromatography on a Toyopearl DEAE 650M column (5.0×20 cm) by using a linear gradient system prepared by H_2O and 2 M NaCl. Fractions were collected and monitored by phenol–H_2SO_4 method and UV absorbance at 480 nm to give four fractions (RH2A—RH2D). Yield: RH2A (2.7%), RH2B (16.7%), RH2C (42.8%), RH2D (9.4%). RH2B (215 mg) was applied to a gel filtration (4.4×90 cm) on Sepharose 6B and eluted with 0.01 M citrate buffer containing 0.1 M NaCl. Fractions were collected and monitored using the phenol–sulfuric acid method and UV absorbance at 480 nm to give two fractions (RH2B-1, 78 mg, RH2B-2, 84 mg). Then RH2B-1 (78 mg) was applied to a gel filtration (3.0 i.d.×90 cm) on Sephacryl S-300 HR and eluted with 0.1 M NaCl to give an acidic polysaccharide RA (70 mg).

RH2C (570 mg) was applied to a gel filtration (4.4×90 cm) on Sepharose 6B and eluted with 0.01 M citrate buffer containing 0.1 M NaCl. Fractions were collected and monitored by the phenol–H_2SO_4 method and UV absorbance at 480 nm to give 4 sub-fractions, RH2C-1 (4.8%), RH2C-2 (9.8%), RH2C-3 (59.6%), RH2C-4 (9.1%). RH2C-3 (340 mg) was subjected twice to a Sephacryl S-300 HR gel filtration (3.0 i.d.×90 cm) and eluted

with 0.1 M NaCl to give a pectic polysaccharide RP (234 mg).

Chemical Analysis of Polysaccharides Total carbohydrate, uronic acid and protein content analyses were performed using the methods reported previously.[14] Sugar composition analysis of RN was carried out by hydrolysis with 2 M trifluoroacetic acid at 120 °C for 2 h, followed by derivatization into alditol acetates as described previously.[16] The obtained alditol acetates were analyzed by GC using a fused silica capillary column (SP 2330, 30 m×0.32 mm i.d.; Supelco, Bellefonte, PA, U.S.A). The oven temperature was set at 220 °C and the detector temperature was maintained at 240 °C.

Monosaccharide composition of polysaccharides (RA and RP) was determined by a combination of hydrolysis with methanolysis.[17] The methyl-glycosides were trimethylsilylated and extracted with *n*-hexane, and then the partially methylated trimethylsilyated derivatives were analyzed on GC using a SPB-1 fused silica capillary column (30 m×0.32 mm i.d., Supelco, Bellefonte, PA, U.S.A). The temperature program was set from 150—250 °C at 2 °C/min, then to 300 °C at 5 °C/min, and the injector temperature was kept at 250 °C.

De-esterification of RP RP (30 mg) was dissolved in 15 ml of 0.05 M NaOH solution at 4 °C. The mixture was kept for 1 h under mild stirring. After adjusting pH to 6 with 1 M AcOH, it was dialyzed and lyophilized.

Methylation Analysis Prior to methylation, RA was reduced with 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluene sulfonate (CMC) and $NaBD_4$.[18] Methylation of RN and reduced product of RA (RRA) was performed according to the method described by Ciucanu and Kerek.[19] The resulting methylated polysaccharides were hydrolyzed with 2 M TFA at 120 °C for 2 h, followed by reduction with $NaBD_4$, acetylation with acetic anhydride to yield partially methylated alditol acetates. Then the partially methylated alditol acetates were quantified by GC with a SP-2330 fused silica capillary column at an oven temperature from 160—210 °C at 2 °C/min, then to 240 °C at 5 °C/min, finally kept in 240 °C for 4 min. Peak areas were corrected using published molar response factors.[20] And the derivatives were analyzed by GC-MS using a DB-5 MS fused silica capillary column (30 m×0.32 mm i.d., J&W Scientific Inc., CA, U.S.A.). The column temperature was 100 °C when injected, then increased with 4 °C/min to 300 °C. Helium was used as carrier gas. The compound at each peak was identified by an interpretation of the characteristic mass spectra and relative retention to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol.[21]

Cells and Viruses Vero and Madin–Darby canine kidney (MDCK) cells were grown in minimal essential medium (MEM) containing 5% fetal bovine serum (FBS). HSV-2 (UW268 strain) and influenza A virus (A/NWS/33, H1N1) (IFV-A) were propagated on Vero and MDCK cells, respectively.

Evaluation of Cytotoxicities and *in Vitro* Antiviral Activities Evaluation of cytotoxicities and antiviral tests of the polysaccharides were performed according to the previous report.[14] Briefly, for cytotoxicities, cells were incubated for 72 h in the presence of increasing concentration of test compound. The 50% cytotoxic concentration (CC_{50}) was calculated from concentration–response curves after viable cells were counted using the trypan blue exclusion test. In the antiviral assays, cells were infected with virus at 0.1 plaque-forming units (PFU) per cell, and then incubated at 37 °C in the presence of test compound. Virus yields were determined by plaque assay at 1-d incubation. The 50% inhibitory concentration (IC_{50}) was obtained from a concentration–response curve. Time-of-addition experiment was carried out as follows: Vero cell monolayers were infected with HSV-2 at 10 PFU per cell. RP was added at a concentration of 500 μg/ml before viral infection for 3 h, during viral infection for 1 h, during viral infection and throughout the incubation thereafter, immediately after infection, at 2 h p.i., at 4 h p.i., and at 6 h p.i.. At 20 h p.i., the cell cultures were harvested and subjected to plaque assay. Inhibitory effect of RP on virus adsorption to and penetration into Vero cells were estimated as described previously.[14] The direct inactivation of HSV-2 by RP was evaluated as follows: Virus (2×10^4 PFU/100 μl) was treated with an equal volume of RP at 37 °C. At 0, 1, 2, 3 and 6 h of incubation, 100-fold dilutions of the mixtures were overlaid with media containing 0.8% methylcellulose to be plaque-assayed.

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