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Short communication

Sensitivity of *Invertebrate iridescent virus 6* to organic solvents, detergents, enzymes and temperature treatment

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Abstract

The sensitivity of *Invertebrate iridescent virus 6* (IIV-6) to a selection of organic solvents, detergents, enzymes and heat treatment was assayed in *Spodoptera frugiperda* (Sf9) cells and by injection of inoculum into larvae of *Galleria mellonella*. In several cases, the degree of sensitivity of the virus depended on the method of assay; cell culture assays indicated greater losses of activity than insect bioassay. IIV-6 was sensitive to chloroform but sensitivity to ether was only detected by cell culture assay. Sensitivity (defined as a reduction of at least 1 log activity) was detected following treatment by 1 and 0.1% SDS, 1% Triton-X100, 70% ethanol, 70% methanol, 1% sodium deoxycholate, pH 11.1 and 3.0. No sensitivity was detected to 1% Tween 80, 1 M $MgCl_2$, 100 mM EDTA, lipase, phospholipase A2, proteinase K, or trypsin at the concentrations tested. Viral activity was reduced by approximately 4 logs following heating to 70 °C for 60 min or 80 °C for 30 min. The above observations highlight the need for studies on the role of the virus lipid component in the process of particle entry into cells, and may explain why vertebrate and invertebrate iridoviruses have been reported to differ in their sensitivity to organic solvents and enzymes.

Keywords: *Invertebrate iridescent virus 6*; *Spodoptera frugiperda*; *Galleria mellonella*; Lipid membrane; Iridovirus; Internal

Invertebrate iridescent viruses (IIVs) (family *Iridoviridae*) are icosahedral particles containing a linear, circularly permuted, dsDNA genome (Williams et al., 2000). IIVs typically infect arthropods, especially insects, in damp and aquatic habitats (Williams, 1998; Tonka and Weiser, 2000). IIVs appear to be highly stable in water and this probably is due to their structure (Marina et al., 2000). The icosahedral capsid is composed of a single major peptide of around 50 kDa which exists as a weakly linked trimer on the outer surface and a covalent disulphide-bonded trimer on the interior of the capsid (Cerutti and Devauchelle, 1990); together these proteins from capsomer structures of ~8 nm diameter. Small fibres of approximately 3.5 nm protrude from the surface of the capsid (Yan et al., 2000). Polypeptide complexes pass from the outer capsid, through an intermediate lipid bilayer approximately 4 nm thick, to a hydrated DNA and nucleoprotein core (Cuillel et al., 1979).

The composition of the lipid layer differs from that of the host cell and de novo synthesis of this component has been assumed (Williams and Thompson, 1995). However, due to their close structural similarity to iridoviruses, recent observations of the wrapping of poxviruses and African swine fever virus by membrane cisternae derived from specific cellular compartments such as the trans Golgi network or endoplasmic reticulum (Gershon et al., 1994; Schmelz et al., 1994; Roullier et al., 1998) have placed in doubt the exact origin of the lipid bilayer of iridoviruses.

Iridoviruses from vertebrates are sensitive to treatment by ether, chloroform, sodium deoxycholate and phospholipase A (Granoff, 1969; Willis and Granoff,

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1974; Langdon et al., 1986; Ahne et al., 1989). In contrast, iridoviruses from invertebrates have been reported to be unaffected by treatment with ether (Day and Mercer, 1964; Matta and Lowe, 1970) but have recently been shown to be sensitive to chloroform (Marina et al., 2000). The sensitivity of IIVs to other lipid active compounds has not been reported.

Such characters are fundamental in defining the importance of the lipid component in the infection process and may also be of use as fundamental characteristics in systems of virus classification. We have undertaken a study to determine the sensitivity of *Invertebrate iridescent virus 6* (IIV-6), the type species of the *Iridovirus* genus, to different organic solvents, detergents and enzymes. Estimates of virus inactivation were made using two different assay systems based upon either whole insects or insect cell cultures (Constantino et al., 2001). The effect of heat-treatment on IIV-6 activity was also quantified as these viruses are known to be thermolabile (Day and Mercer, 1964).

A sample of IIV-6 was obtained from CSIRO Entomology, Canberra, Australia, and passaged by injection into third and fourth instar *Galleria mellonella* larvae that were subsequently maintained on semi-synthetic diet at 25 °C. When larvae developed the characteristic signs of infection they were individually triturated in 1ml sterile distilled water and the virus was purified by differential centrifugation as described previously (Marina et al., 2000). Purified suspensions of IIV-6 were quantified by mixing the virus with known concentrations of polystyrene beads (460 nm diameter, Aldrich Chem. Co. USA) in a solution of 68% (v/v) isopropanol (Constantino et al., 2001). The concentration of each virus stock suspension was then determined by scanning electron microscope (SEM) observation of virus preparations dried onto polylysine coated glass coverslips. A minimum of eight fields of vision from each of five replicate virus-polystyrene mixtures were counted for each virus preparation, representing >1000 virus particles in all cases. For assays carried out in insects, virus suspensions were stored at 4 °C and used within 3–5 days of purification.

The first series of experiments was carried out in insects. To determine IIV-6 sensitivity to lipophilic substances virus suspensions were divided into two aliquots of 500 μl in 1.5 ml microcentrifuge tubes. One of the aliquots was subjected to the experimental treatment and the other, control sample, was left untreated. Treatment details are given in Table 1. Samples were placed on an orbital shaker and briskly agitated for 1 h, or 2 h in the case of the lipase treatment. All samples were treated at 25 °C, except lipase and proteinase K which were incubated at 37 °C (Table 1). When virus was treated with solvent, the solvent was removed and the sample was held at 4 °C for 24 h to permit complete evaporation of the solvent prior to dilution and bioassay. The volume of the virus suspension (500 μl) was not affected by this procedure. In the case of detergents and enzymes, the dilution and bioassay procedure was performed immediately. In all cases, control suspensions were treated identically, but were not exposed to the substance being tested. Dilutions of treated and control virus suspensions were made using sterile distilled water in siliconized microcentrifuge tubes. Groups of 30 third instar *G. mellonella* larvae from a laboratory culture were injected with doses of 1, 2.5, 5, 10, 50 or 100 IIV-6 particles in a mean volume of 8.4 μl using a manual microinjector fitted with a 1 ml syringe (Burkard Co., UK), as described by Constantino et al. (2001). Following injection, *G. mellonella* larvae were individually placed in 30 ml plastic cups half filled with semi-synthetic diet (Hunter and Boraston, 1979) and held at 25 ± 1 °C. The number of patently infected larvae and pupae was recorded at 8 and 14 days post-injection. Each reagent was tested on three separate occasions. Infectious dose (ID_{50}) values were determined by logit analysis using a binomial error structure in GLIM and the Fieller macro present in the program (Numerical Algorithms Group, 1993). For each individual test, the ratio of the activity of the treated sample relative to the untreated sample was calculated. In many cases, injection of a single particle of IIV-6 caused more than 50% infection in *G. mellonella* larvae reflecting the very high sensitivity of the bioassay technique (Constantino et al., 2001). Therefore, where the ID_{50} for a given treatment was less than one particle a value of 0.69 was assumed i.e. the Poisson distribution predicted average number of virus particles need to produce 50% infection.

The virus was classified as sensitive to solvent, detergent and enzyme treatments when a difference of at least 1 logarithm was observed in the activity of treated and untreated virus (Burleson et al., 1992). This magnitude of loss of activity is considered to be biologically meaningful and takes into account normal variation in bioassay systems due to differences in the infectivity of different batches of virus, and the susceptibility of insects or cells that may vary over time or with passage history, even though virus/cell production and insect rearing conditions have been standardized (Revozzo and Burke, 1973; Jones, 2000). In the case of IIV-6, the degree of variability of the bioassay systems used varies approximately 3-fold for the insect bioassay (ID_{50} typically between one and three particles of IIV-6 per larva of *G. mellonella*), and approximately 5-fold for end point dilution assays in *Spodoptera frugiperda* (Sf9) cells, described below (Marina et al., 2000; Constantino et al., 2001).

In the initial experiments, in which the assays were carried out in insects, only three treatments appeared to produce a substantial reduction in virus titre namely, chloroform, 1% SDS and 1% sodium deoxycholate

Table 1
Organic solvent, detergent and enzyme treatments applied to IIV-6

Treatment	Assay system	Concentration[b]	Buffer	Exposure time and temperature
pH 11.1	Cell culture	–	–	1 h, 25 °C
pH 3.0	Cell culture	–	–	1 h, 25 °C
Chloroform	Insects	50%	–	1 h, 25 °C
	Cell culture	10%	–	1 h, 25 °C
Ether	Insects	50%	–	1 h, 25 °C
	Cell culture	10%	–	1 h, 25 °C
SDS	Insects and cell culture	0.1%		
1.0%	–	1 h, 25 °C		
Tween 80	Insects	1.0%	–	1 h, 25 °C
Sodium deoxycholate	Insects and cell culture	1.0%	–	1 h, 25 °C
Triton X-100	Cell culture	1.0%	–	–
Phospholipase A[a]	Insects and cell culture	25 units/ml	20 mM Tris pH 7.4, 2 mM $CaCl_2$, 0.1% BSA	1 h, 25 °C
Lipase[a] (*Candida rugosa*)	Insects and cell culture	0.5 mg/ml	PBS	2 h, 37 °C
Trypsin	Cell culture	0.025 mg/ml	PBS	1 h, 25 °C
Proteinase K[a]	Insects and cell culture	0.04 mg/ml	–	1 h, 37 °C
EDTA	Cell culture	100 mM	–	1 h, 25 °C
$MgCl_2$	Cell culture	1 M	–	1 h, 25 °C
Ethanol	Cell culture	70% v/v	–	1 h, 25 °C
Methanol	Cell culture	70% v/v	–	1 h, 25 °C

Viral activity was subsequently assayed by insect bioassay in *G. mellonella* larvae or by cell culture assay in Sf9 cells.
[a] Reaction stopped by addition of EDTA.
[b] For solvents, percentage figures indicate percent volume used (e.g. 50% represents equal volumes of solvent and virus suspension). Ratio of solvent:virus suspension was lower in cell culture assays, compared with insect bioassays, to avoid adverse effects of residual solvent on cells.

Table 2
Relative proportion of activity remaining after treatment of IIV-6 with a range of organic solvents, detergents and enzymes

	Assay system	Proportion of activity remaining[a]			Sensitivity[b]
		Replicate 1	Replicate 2	Replicate 3	
pH 11.1	Cell culture	0.159	0.100	0.100	+
pH 3.0	Cell culture	0.019	0.045	0.010	++
SDS 1%	Cell culture	< 0.00001	< 0.00001	< 0.00001	+++
	Insects	0.0180	< 0.0069	< 0.0069	++
SDS 0.1%	Cell culture	0.063	0.0077	0.0043	++
	Insects	0.244	0.554	0.244	NS
1% Triton-X100	Cell culture	0.3174	0.1193	0.0100	+
1% Tween 80	Insects	1.000	1.000	1.000	NS
Trypsin	Cell culture	0.501	0.549	10.000	NS
Proteinase K	Insects	0.500	1.000	1.000	NS
Ether	Cell culture	< 0.00001	< 0.00001	< 0.00001	+++
	Insects	1.000	1.000	1.000	NS
Phospholipase A2	Cell culture	0.292	1.000	0.245	NS
	Insects	1.000	1.000	1.000	NS
Lipase	Cell culture	0.235	0.616	0.246	NS
	Insects	1.0137	1.0814	1.011	NS
Chloroform	Cell culture	< 0.00001	< 0.00001	< 0.00001	+++
	Insects	< 0.078	< 0.0061	< 0.0061	++
100 mM EDTA	Cell culture	0.6826	1.000	0.1193	NS
Sodium deoxycholate 1%	Cell culture	0.0069	0.0050	0.0041	++
	Insects	0.0431	0.1882	0.1022	+
70% Ethanol	Cell culture	< 0.00001	< 0.00001	< 0.00001	+++
70% Methanol	Cell culture	< 0.00001	< 0.00001	< 0.00001	+++
1 M $MgCl_2$	Cell culture	0.5023	0.8126	–	NS

Virus activity was assayed in larvae of *G. mellonella* and/or in Sf9 cells, NS, not sensitive.
[a] Figures indicate proportional reduction in virus activity compared with untreated virus suspensions.
[b] Sensitivity classified as +1 log reduction, ++ 2–3 logs reduction in activity, +++ very sensitive > 3 logs reduction in activity.

(Table 2). Treatment with chloroform caused a decreased in viral activity of more than two orders of magnitude, whereas, ether treatment caused no detectable change in viral activity. The sensitivity to SDS depended on concentration, with little change at 0.05 or 0.1%, compared with a change of more than 2 logarithms at 1%. No sensitivity to 1% Tween 80 was observed. Sodium deoxycholate (1%) caused approximately a one logarithm reduction in IIV-6 activity, whereas, none of the enzymes tested caused a significant effect (Table 2).

As ether, Tween 80 and 0.1% SDS did not immediately reduce the infectivity of IIV-6, we decided to determine the effect of these substances on the stability of IIV-6 in storage. Viral suspensions that had been treated with ether, Tween 80 and 0.1% SDS and their respective controls were stored in a refrigerator at 4 °C for 8 months and were then serially diluted. Each dilution was injected into *G. mellonella* larvae as described above and the ID_{50} value was calculated by logit analysis. During this period control virus suspensions lost 2.5–4.2 logarithms of activity but ether and Tween-treated virus ID_{50} values were not significantly different from their respective controls (ether, $\chi^2_1 = 0.45$, $P = 0.50$; Tween, $\chi^2_1 = 0.90$, $P = 0.33$). The SDS-treated virus had lost all activity, however, and had become a clear viscous liquid, presumably due to the breakdown of virus particles and the liberation of viral DNA. Scanning electron microscopy of the SDS-treated suspensions confirmed that virus particles had completely broken down.

To determine the sensitivity of IIV-6 to heat treatment, virus was purified and quantified as described above. Aliquots of virus suspension were adjusted to a concentration of 2.5×10^{11} particles per ml and heated to 50–80 °C for between 15 and 60 min using a laboratory heating block with a precision of ±0.5 °C. The virus suspension was then diluted by a factor of 10^5 and volumes of 8.4 μl representing a dose of 2.1×10^4 particles, were injected into groups of 30 *G. mellonella* larvae. These larvae were maintained in plastic pots with semi-synthetic diet at 25 °C and checked for signs of patent infection at 5–12 days post-inoculation. Control virus suspension was held at 25 °C prior to dilution and injection in *G. mellonella* larvae. Importantly, dilutions for insect bioassay were carried out after heating such that all heat treatments were performed at the same virus concentration (2.5×10^{11} particles per ml). The procedure was performed three times.

In the virus suspension diluted after heating, viral activity was completely eliminated by treatment at 70 °C for 60 min or 80 °C for 30 min, and activity was almost zero at 80 °C for 15 min (Fig. 1). The ID_{50} value for IIV-6 in *G. mellonella* larvae is usually one to three particles per larvae and as the dose injected was 2.1×10^4 particles per larva, this represents approxi-

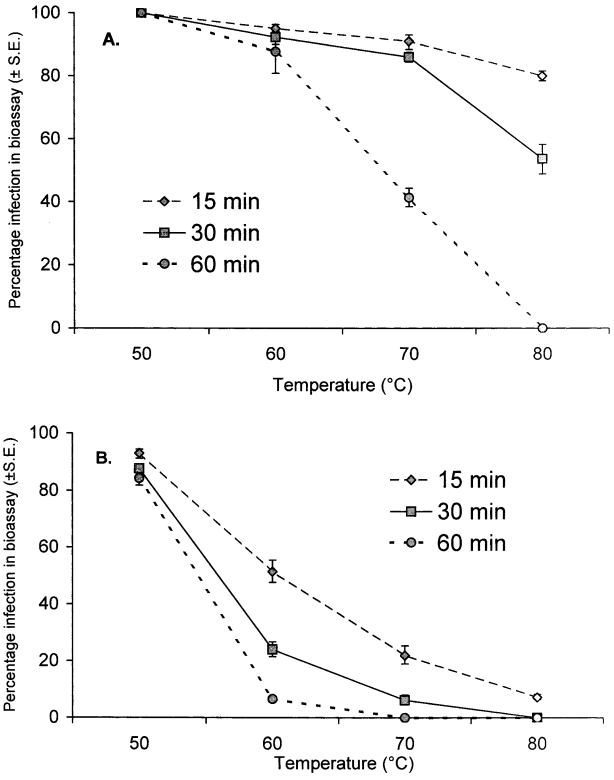

Fig. 1. Effect of heat treatment at 50–80 °C for periods of 15–60 min on the activity of IIV-6 assayed by injection into *G. mellonella* larvae at doses of (A) 2.1×10^9 particles per larva (stock suspension) and (B) 2.1×10^4 particles per larva (after being heated, the stock suspension was diluted by a factor of 10^5). Each point represents the mean of three independent trials.

mately four orders of magnitude inactivation. Virus suspension subjected to bioassay without prior dilution (representing a dose of 2.1×10^9 particles per *G. mellonella* larva) was completely inactivated at 80 °C for 60 min, representing approximately nine orders of magnitude inactivation. In contrast, the virus suspension that had been treated at 80 °C for 15 or 30 min still caused between 80 and 54% infection of *G. mellonella* larvae, respectively, when injected in undiluted form.

During the period in which the studies using the *Galleria* bioassay system were being been completed, we also developed and calibrated a cell culture assay for IIV-6 (Constantino et al., 2001). The apparent inability of ether to inactivate IIV-6 when chloroform was clearly capable of inactivating the virus seemed anomalous. We, therefore, decided to assess whether changing the assay system had any apparent effect on the results obtained with several of the compounds used previously.

For the assays carried out in cultured cells, three separate preparations of IIV-6 grown in *G. mellonella* were diluted to approximately 1×10^{11} virus particles per ml in phosphate buffered saline (PBS), divided into aliquots and stored frozen at −20 °C until use. A single aliquot was thawed for each experiment and discarded

after use. Sf9 cells (Summers and Smith, 1987) cells were maintained in Grace's medium (Sigma Cell Culture, St Louis, Missouri, USA) supplemented with 10% Foetal Calf Serum (FCS) (Commonwealth Serum Laboratories, Melbourne), 2 mM glutamine, penicillin (50 units/ml) and streptomycin (50 μg/ml). The cells were maintained at 26 ± 1 °C and were sub-cultured at weekly intervals.

For the test treatments of the virus the stored aliquots were diluted 100-fold to a concentration of approximately 1×10^9 IU/ml in the treatment buffer and incubated at the appropriate conditions (Table 1). After incubation the test sample was immediately diluted 100 fold in Grace's medium before titration. For titrations 80 μl of Sf9 cell suspension containing 2×10^5 cells were seeded into 96 well plates (Falcon, Becton Dickinson Co, Franklin Lakes, NJ). Ten-fold dilutions of the samples to be titrated were made in Grace's medium supplemented with FCS, glutamine and penicillin and streptomycin as described above. For each dilution, 50 μl of diluted virus suspension was added to each of eight wells and then scored for cytopathic effect after 7 days. Titres were calculated using the method of Reed and Muench (1938). In each series of experiments an untreated control and a solvent, detergent or enzyme treated control were included along with the test samples. For each individual test, the ratio of the activity of the treated sample relative to the untreated sample was calculated.

Cell culture assays indicated that IIV-6 was sensitive to ethanol, methanol and high and low pH (pH 3; pH 11; Table 2). IIV-6 was also sensitive to treatment with sodium deoxycholate, and Triton-X100, but not to Tween 80, trypsin, proteinase K, phospholipase A_2, lipase, EDTA or $MgCl_2$ solution. No adverse effects were observed in control cells. The sensitivity of IIVs to alcohols has not been previously reported whereas the sensitivity to low and high pH had previously been detected but only qualitatively (Marina et al., 2000). The fact that such basic tests have not been reported previously in IIVs is due to the minor attention paid to this group of viruses (Williams, 1998). Although believed to have low potential as agents for the biological control of insect pests, they can lethally infect important agricultural pest species (Henderson et al., 2001; Just and Essbauer, 2001; Funk et al., 2001) and insect vectors of human diseases (Hernández et al., 2000).

Interestingly, several of the treatments tested produced different results depending on the assay system employed. The most striking contrast was with ether treatment. In this case, no inactivation was observed when assays where conducted in *G. mellonella* whereas when assays were conducted in cell culture over 5 logarithms reduction in activity was recorded. Anomalous results were also obtained after 1 and 0.1% SDS treatment. In both cases, there are clear indications that a greater loss of activity is observed when samples are assayed in the cell culture system than in whole insects. As before, no adverse effects were observed in control cells.

These observations tend to suggest that disruption of the lipid bilayer in IIVs has a profound effect on their ability to infect cultured cells but not to infect whole insects when the virus is injected into the haemocoel. In the latter instance it is possible that the lipid bilayer is disrupted but the virus is still able to enter and subsequently replicate in cells of the haemolymph i.e. phagocytotic cells such as macrophages (Lea, 1985; Charpentier et al., 1986). Presumably when infecting cultured cells the lipid plays an important role in virus binding and/or entry into the cell. In contrast, the integrity of the external layer of the capsid may be relatively unimportant as proteinase and trypsin treatments did not alter viral activity although they have been shown to degrade capsid structure (Heppell and Berthiaume, 1992). The above is obviously speculative, but does lead to some clearly testable hypotheses about the ability and pathways by which IIVs are able to enter certain cell types. In this respect, comparative studies on the process of IIV particle entry into different insect cell lines and in primary cell culture derived from insect haemolymph would be of great interest.

Our observations may explain why vertebrate and invertebrate iridoviruses apparently differ in their sensitivity to organic solvents and enzymes. The activity of vertebrate iridoviruses has habitually been assayed in cell culture systems (Willis and Granoff, 1974) whereas the sensitivity of invertebrate iridoviruses has been assayed in living insects (Day and Mercer, 1964; Matta and Lowe, 1970; Marina et al., 2000). Our results also have also have implications for the study of these and other non-occluded insect viruses. Treatment by ether or chloroform could be used as a method of eliminating IIV activity permitting the isolation of viruses that are not sensitive to organic solvents, e.g. picornaviruses. This technique may be especially applicable to environmental samples or insect samples with mixed viral infections. Our results also indicate that when biological characteristics such as solvent sensitivity are employed as part of a virus taxonomic system, it is important that all the relevant parameters, in this case the assay system, are adequately prescribed.

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