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Measurement of Surface Charge of Baculovirus Polyhedra

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The isoelectric points of three baculoviruses, *Trichoplusia ni* nuclear polyhedrosis virus (NPV), *T. ni* granulosis virus, and *Spodoptera littoralis* NPV were identified by cell electrophoresis. At neutral pH polyhedra were negatively charged. *T. ni* NPV polyhedra were reacted with a number of reagents which could potentially attach to or degrade their surface structure. This gave information on the components that contribute to the charge profile of *T. ni* NPV. This is discussed in relation to the use of polyhedra as biological control agents against insect pests.

Baculoviruses are double-stranded DNA viruses that infect insects (8, 9). The virus particles are occluded within a proteinaceous polyhedral inclusion body, and this is surrounded by a densely staining outer envelope (9).

Baculoviruses have been used successfully on many occasions as biological control agents against insect pests (5–7, 12, 13, 16). This successful use depends on the ability of the virus to persist within and between generations of the pest. Hence, the mechanism by which polyhedra attach to leaf surfaces, resulting in their maintenance in the environment, is an area which merits more study. This is of particular interest with respect to developing optimum formulations in which to apply the virus.

Despite the extensive use of baculoviruses as field control agents, there has been relatively little effort directed towards studying the surface characteristics of the polyhedra and hence identifying the factors important to strong attachment. In this work, cell electrophoresis was used to determine the electrokinetic behavior of three baculovirus polyhedra. Cell electrophoresis was the technique selected as it permitted the charge on the intact polyhedra to be measured without any form of denaturation. The three baculoviruses studied were *Trichoplusia ni* nuclear polyhedrosis virus (NPV), *T. ni* granulosis virus (GV), and *Spodoptera littoralis* NPV. These viruses were selected because of their frequent use in controlling pests of cabbage and cotton (for example, *T. ni*, the cabbage looper, and *S. littoralis*, the cotton bollworm).

The surface charge was measured at various pH values to determine the isoelectric points. The effect of various treatments that had site-directed actions were then used to give information on the components that contribute to the surface charge on *T. ni* NPV polyhedra. Polyhedra were digested with enzymes to identify the involvement of surface protein carbohydrate and lipid on the charge profile. Also, the effects of nonionic cationic and anionic detergents were tested to observe the effect of changes in the ionogenic composition of the suspending medium, especially as many of these reagents are used in virus spray formulations. In this way, it was possible to determine the charge characteristics of polyhedra and also to increase our understanding of the surface structure.

T. ni NPV and *S. littoralis* NPV polyhedra were produced in third-instar larvae of *T. ni* and *S. littoralis* infected with 10^5 polyhedra and maintained at 28°C with defined diet (11). After 5 to 7 days, when larvae were terminally infected, they were harvested, mascerated in water, and filtered through muslin. Polyhedra were purified by differential sucrose gradient centrifugation (10) and counted by the dry counting method of Wigley (PhD. thesis, University of Oxford, Oxford, England, 1976). Suspensions were then stored at −20°C until required. Tn GV was purified essentially as described by Russell and Consigli (15).

A rank-electrophoresis apparatus, Mark II with binocular microscope head (Rank Brothers, Cambridge, England), was used for cell electrophoresis (1). This contained a flat cell which enabled relatively large particles to be observed. The binocular head was fitted to a Philips video camera linked via a video cassette recorder to a television monitor. The cell was firmly clamped to the electrophoresis stage and closed at both ends to eliminate all external movements.

Convection currents were minimized by using a heat filter system and by immersing the cell in a temperature-controlled water bath. Convection was also minimized by using low currents and a low salt concentration in all cases. Phosphate or glycyl glycine buffer, 0.01 M, was used, and consecutive timings were made for polyhedra to travel a distance of 10 cm on the monitor screen. This corresponded to a distance of 200 μm in the viewing chamber. Eight individual polyhedra were timed to move this distance at each stationary phase, with the current first flowing from the positive to the negative electrode and then in the opposite direction. The mean time for each treatment was calculated, and from this the mobility was expressed as follows: mobility = velocity (μm/s)/field strength (V/cm), where field strength = v/l, and velocity = d/t, in which v = potential difference across the electrodes (60 V), l = interelectrode distance (6.01 cm), d = distance of polyhedra migration (200 μm), and t = time (seconds). Therefore, mobility = $t^{-1}(d \times l)/v$. Since $(d \times l)/v$ was constant, mobility varied only with the time and could be expressed as: mobility = $t^{-1}K$. Under the experimental conditions K was 20.036, so mobility was $(1/t) \times 20.04$.

Stationary phase. As the electrophoretic cell was closed, there was an electro-osmotic shearing of fluid near the glass boundary which caused a return of fluid in the reverse direction, through the center of the tube. The stationary phase was the position in the electrophoretic cell where there was no net movement of fluid. All readings were made at this position to ensure that the true electrophoretic mobility was determined.

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TABLE 1. Charge characteristics for three baculoviruses: mobility over a range of pH values[a]

pH	*T. ni* GV		*T. ni* NPV		*S. littoralis* NPV	
	Time (s)	Mobility ($\mu m\ s^{-1}/V\ cm^{-1}$)	Time (s)	Mobility ($\mu m\ s^{-1}/V\ cm^{-1}$)	Time (s)	Mobility ($\mu m\ s^{-1}/V\ cm^{-1}$)
2	36.49 ± 10.71	+0.55	27.75 ± 5.26	+0.72	25.24 ± 4.94	+0.79
3	48.35 ± 17.09	+0.41	31.96 ± 9.57	+0.69	28.94 ± 5.09	+0.69
4	38.67 ± 5.42	−0.52	9.38 ± 3.15	−2.13	53.98 ± 9.75	+0.37
5	5.42 ± 0.76	−3.69	5.98 ± 0.97	−3.34	10.20 ± 1.25	−1.96
6	5.23 ± 1.16	−3.83	6.60 ± 1.32	−3.03	5.09 ± 0.68	−3.63
7	5.54 ± 0.29	−3.65	6.04 ± 1.81	−3.17	5.01 ± 0.64	−3.99
8	5.83 ± 0.85	−3.44	6.89 ± 3.34	−2.90	4.93 ± 0.73	−4.06
9	6.05 ± 0.70	−3.31	5.64 ± 1.21	−3.55	5.61 ± 0.56	−3.52
10	5.99 ± 0.98	−3.35	5.34 ± 0.88	−3.75	4.72 ± 0.59	−4.25

[a] The experiment was repeated on three separate occasions and the values obtained did not differ greatly from those presented above. These values were obtained in the experiment shown in Fig. 1. In all cases, the mean time for polyhedra to travel 200 μm was taken from 32 observations. (This was the time for eight polyhedra to move in each direction at each stationary phase.) From the mean time the electrophoretic mobility was calculated. Means ± standard deviations are given for time measurements.

The stationary phase was found by moving a constant distance from the point of focus of the electrophoretic cell wall. The point of focus was recognized by polyhedra remaining fixed even when current was applied. The distance moved to reach the stationary position was determined from the ratio l/d, where l = length of cell and d = width of cell. For the cell used, l/d was ±0.17. This value was subtracted from the focus reading for the outer cell wall and added to the focus reading for the inner cell wall.

Determination of isoelectric point. Glycyl glycine at 0.01 M was prepared over a pH range of 1.5 to 10. The pH was adjusted with either 0.01 M HCl or 0.01 M NaOH. This buffer was selected for use because of its low ionic strength and its buffering capacity over a wide pH range. A 9-ml portion of buffer was added to 1 ml of buffer containing 10^7 polyhedra. After 30 min of incubation at 25°C, the solutions were placed into the illuminated viewing chamber of the microelectrophoresis apparatus, also at 25°C. An electrical potential of 60 V (1.15 mA) was applied across the viewing chamber via two platinum electrodes inserted into the electrode parts of the chamber. The mobility was determined and graphs were plotted of mobility against pH. The point on the graph at which the line joining the individual mobility points crossed the horizontal axis was taken to be the point of neutral charge (pI).

For enzyme digestion, all enzymes were tested at a concentration of 5 mg/ml: pepsin (porcine stomach mucosa), trypsin (type III from bovine pancreas), hyaluronidase (type 1-S from bovine testes), α-amylase (type IIA from *Bacillus* bacteria), protease (type I crude pancreatic), protease (type VIII from *Bacillus subtilis*; also known as subtilisin), phospholipase D (type III from peanuts), and lipase (type VII from *Candida cylindracea*). All incubations were initially for 1 h at 37°C, pH 7. The temperature was adjusted to 25°C before the mobility was read. Pepsin digestion was performed at pH 3, but the pH was adjusted to 7 before the mobility was determined. If one treatment demonstrated a decrease in mobility, then several digestion times were used together with observations on the effect of temperature and enzyme concentration. For example, hyaluronidase was tested at 0.05, 0.5, and 1 mg/ml at 27 and 37°C. The reaction mixture in all cases was 9 ml of buffer, 1 ml of buffer containing 10^7 polyhedra, and 200 μl (5 mg/ml) of enzyme solution. The controls were buffer and polyhedra without enzyme.

Treatments with detergents and chelating agents were in 0.01 M phosphate buffer and included the following: sodium dodecyl sulfate (SDS)—anionic detergent, 1 mg/ml, pH 5; Tween 80—nonionic detergent, 0.03 mg/ml, pH 8; cetyl trimethyl ammonium bromide (CTAB)—catonic detergent, 1 mg/ml, pH 8; EDTA—chelating agent, 1 mg/ml, pH 5. A 9-ml amount of detergent or chelating agent was incubated with 1 ml of buffer containing 10^7 polyhedra at room temperature for 30 min.

The use of a video cassette recorder during cell electrophoresis had the advantage of allowing short readings to be made at each stationary point and polarity. Subsequent replay then enabled all replicate measurements to be taken at the same contact time. It also presented a much clearer picture of the initial changes in surface charge following a specific treatment. This was a valuable improvement first used by Broxton and Gilbert (2) to overcome the difficulty of only observing the effect of treatments when equilibrium had been reached. In some cases it was found that polyhedral mobility was low and the time taken to move the fixed distance was very long. In these cases the video cassette recorder enabled a "freeze frame" facility to be used to measure the distance moved by individual polyhedra in a fixed time. This considerably reduced the time required to make recordings.

All baculoviruses tested were negatively charged at most pH values (Table 1). *S. littoralis* NPV had the greatest charge, followed by *T. ni* NPV. The isoelectric points, determined from the graphs of mobility against pH, also varied slightly for the various viruses (Fig. 1). *S. littoralis* NPV was electrically neutral at pH 4.2; *T. ni* NPV, at pH 3.2; and *T. ni* GV, at pH 3.45. The greater charge and higher isoelectric point of *S. littoralis* NPV seemed to correlate with the larger size of this baculovirus polyhedra and therefore the greater number of ionizable groups.

Multiple analysis of variance showed that, of the enzymes tested, only hyaluronidase and pancreatic protease significantly reduced the electrophoretic mobility of *T. ni* NPV ($P < 0.001$; Table 2). Time course experiments were therefore performed to investigate the effect of these enzymes further. It was found that the binding of pancreatic protease was instantaneous and no time-dependent activity could be identified (data not shown). Further analysis was therefore not pursued. Treatment with hyaluronidase demonstrated a progressive decrease in mobility (Table 3), and this was sensitive to higher concentrations of enzyme and elevated temperatures (Fig. 2). However, the mobility curves under these different conditions were not related, and as no typical enzyme kinetics could be demonstrated, it was not possible

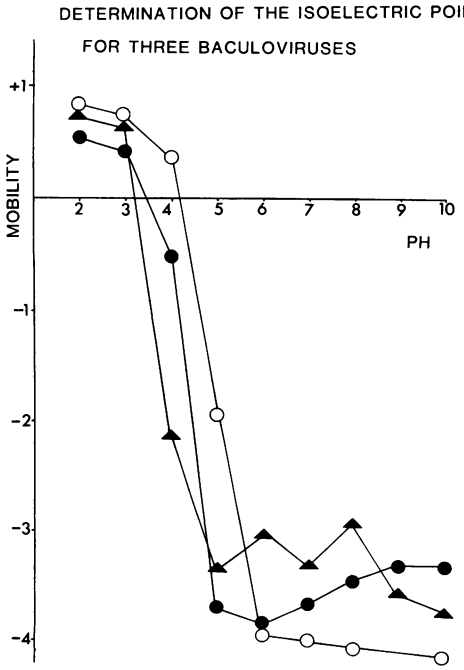

FIG. 1. Determination of isoelectric point for three baculoviruses by cell electrophoresis. The pH at which the line joining the individual mobility values crossed the horizontal axis was taken as the point of neutral charge (pI). Symbols: (●) *T. ni* GV, pI = 3.45; (▲) *T. ni* NPV, pI = 3.2; (○) *S. littoralis* NPV, pI = 4.2.

to conclude that the hyaluronidase activity observed was specific. That very few of the enzymes tested showed any effect on mobility demonstrated the extraordinary resistance of polyhedra to enzyme attack. This agrees with the observations of Jaques and Huston (13) on the resistance of polyhedra to microbial degradation.

The mobility of polyhedra was most influenced by charged detergents. SDS (anionic) very rapidly reduced the mobility, but over a 15-min period this steadily increased back to the values recorded for untreated control preparations (Table 4). CTAB (catonic) also reduced the mobility, but this occurred progressively and remained stable at a value approaching neutrality. The nonionic detergents Triton X and Tween 80 had no effect on mobility. Clearly, the effect of detergents was complicated and involved an association with the electrostatic double layer surrounding the polyhedra.

The initial decrease in electrophoretic mobility following

TABLE 2. Effect of various enzyme treatments on electrophoretic mobility of *T. ni* NPV polyhedra

Enzyme	Mean time (s) ± SD	Mobility ($\mu m\ s^{-1}/V\ cm^{-1}$)
Hyaluronidase[a]	13.68 ± 3.0	−1.46
Pancreatic protease[a]	17.38 ± 3.0	−1.15
Pepsin	5.40 ± 0.9	−3.71
Protease (*B. subtilis*)	7.50 ± 0.4	−2.67
Phospholipase D	9.57 ± 1.7	−2.09
Lipase	5.95 ± 0.8	−3.37
Trypsin	6.24 ± 1.0	−3.21
α-Amylase	7.09 ± 1.7	−2.82
Control	5.48 ± 2.2	−3.65

[a] Enzymes that significantly reduced the mobility of polyhedra ($P < 0.0001$).

TABLE 3. Effect of hyaluronidase on electrophoretic mobility of *T. ni* NPV polyhedra over 60 min[a]

Time (min)	Mean time (s) ± SD	Mobility ($\mu m\ s^{-1}/V\ cm^{-1}$)
2.5	13.43 ± 1.20	−1.49
7.5	14.27 ± 1.80	−1.41
30	15.12 ± 4.29	−1.33
60	17.07 ± 3.50	−1.17

[a] A progressive decrease in mobility was observed, so the enzyme was tested at different concentrations and at different temperatures.

SDS treatment was attributed to the detergent stripping off surface material from the polyhedra and exposing positively charged sites. The return to the original mobility values after 10 min can be explained in a number of ways. SDS adsorption to the polyhedra surface will itself enhance the negative charge. It may also cause release of negatively charged surface components or induce reorientation of surface complexes that change the charge composition. A further possibility is that the material released by the initial action of SDS may adsorb back to the surface and hence induce the mobility to return to its original value. Anionic detergents in conjunction with cell electrophoresis have been shown to increase the mobility of *Micrococcus aureus* (3). They have also been used by Dyar and Ordal (4) to quantitatively estimate the amount of surface lipid present in certain mycobacterium species.

The gradual decrease in electrophoretic mobility by the cationic detergent CTAB was attributed to surface adsorption of detergent, which progressively negated the inherent negative charge on the polyhedra. It is suggested that attachment of a certain amount of detergent to polyhedra may facilitate further uptake. This is envisaged to occur in a manner similar to that described by McQuillen (14) in which strong Van der Waals forces between adjacent nonpolar parts of detergent molecules (16 carbon atoms in the case of

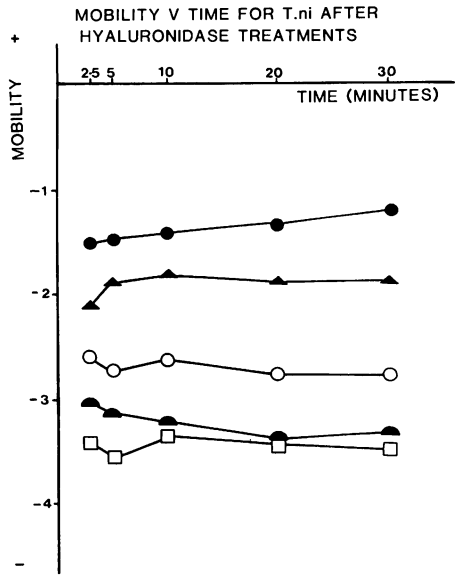

FIG. 2. Electrophoretic mobility versus time for *T. ni* NPV after hyaluronidase treatments. The enzyme was tested at various concentrations and temperatures to observe the kinetics of binding. Symbols: (●) 1 mg/ml, 27°C; (○) 0.5 mg/ml, 27°C; (□) no treatment, 37°C; (▲) 0.5 mg/ml, 37°C; (◠) 0.05 mg/ml, 27°C. The mobility curves under these different conditions were not related, so the binding of hyaluronidase did not appear to be specific.

CTAB) will tend to anchor further molecules in a cooperative way. It is therefore conceivable that a series of concentric shells of detergent are built up around the polyhedra, with polar heads facing alternatively inwards and outwards. However, this is purely speculative and the electrophoresis data do not conform that this is occurring.

The chelating agent EDTA also had a marked effect on polyhedra mobility. There was a rapid decrease in mobility which remained stable over a 15-min period. This permanent change in charge profile was attributed to a change in the charge composition of the surface.

The cell electrophoresis experiments described have enabled the surface charge on various baculovirus polyhedra to be characterized under fixed conditions. As the polyhedra were not processed in any way before the readings were made, it is assumed that the measurements of surface charge accurately reflect their behavior with changes in their environment. This favors the use of this technique over other forms of electrophoresis that involve measuring the surface charge and isoelectric points of denatured components. The use of treatments with a site-directed action prior to cell electrophoresis also yielded useful information on the polyhedra surface composition. The resistance of polyhedra to enzymatic attack illustrates that they are not readily dissociated in the environment. Also, their sensitivity to detergents demonstrated that small changes in the spray formulation of polyhedra can have quite a marked effect on their surface characteristics.

The experiments described have shown that under most conditions polyhedra are negatively charged, but the extent of this charge is considerably influenced by changes in the environment.

TABLE 4. Effect of detergents and a chelating agent on polyhedra mobility over 60 min[a]

Time course (min)	SDS, pH 5		Triton X-100, pH 5		Tween 80, pH 8		CTAB, pH 8		EDTA, pH 5		Control (0.01 M phosphate buffer)			
											pH 5		pH 8	
	Time (s)	Mobility ($\mu m\ s^{-1}\ V^{-1}$)	Time (s)	Mobility ($\mu m\ s^{-1}\ V^{-1}$)	Time (s)	Mobility ($\mu m\ s^{-1}\ V^{-1}$)	Time (s)	Mobility ($\mu m\ s^{-1}\ V^{-1}$)	Time (s)	Mobility ($\mu m\ s^{-1}\ V^{-1}$)	Time (s)	Mobility ($\mu m\ s^{-1}\ V^{-1}$)	Time (s)	Mobility ($\mu m\ s^{-1}\ V^{-1}$)
0	10.07 ± 5.40	−1.99	5.78 ± 0.47	−3.47	6.57 ± 0.92	−3.08	7.73 ± 1.10	−2.59	11.59 ± 1.69	−1.73	5.79 ± 0.48	−3.46	6.56 ± 0.89	−3.06
5	6.70 ± 1.13	−2.98	5.87 ± 0.56	−3.42	5.80 ± 1.08	−3.45	7.67 ± 0.68	−2.61	14.92 ± 5.44	−1.34	5.87 ± 0.57	−3.41	6.74 ± 1.17	−3.49
10	6.45 ± 1.14	−3.10	5.49 ± 0.65	−3.69	6.10 ± 0.59	−3.28	9.08 ± 1.00	−2.20	12.60 ± 5.22	−1.59	5.42 ± 0.66	−3.69	6.10 ± 0.59	−3.28
15	6.49 ± 1.09	−3.08	5.59 ± 0.94	−3.58	6.09 ± 1.18	−3.29	10.16 ± 1.88	−1.97	19.46 ± 10.70	−1.03	5.59 ± 0.94	−3.58	6.09 ± 1.18	−3.28

[a] For times measurements, means ± standard deviations are given.

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