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Alkaline Protease Associated with Virus Particles of a Nuclear Polyhedrosis Virus: Assay, Purification, and Properties

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Proteolytic activity was detected within polyhedra of the nuclear polyhedrosis virus of *Spodoptera littoralis*. The enzyme activity was detected by its ability to degrade the major structural polypeptide of polyhedra (polyhedrin). A quantitative assessment of activity was made by a radioassay technique using ^3H-labeled polyhedrin as the substrate. Of the structural components of polyhedra, virus particles showed the greatest specific proteolytic activity. Preparations of purified nucleocapsids were inactive. The virus particle enzyme displayed a temperature optimum for proteolysis of 30 to 40°C and a pH optimum of 9.6. Its activity was inhibited by H^{2+} and Cu^{2+}, but not by 2-mercaptoethanol. The enzyme was purified from detergent-treated virus particles by affinity column chromatography, using polyhedrin linked to cyanogen bromide-activated Sepharose. Three major envelope polypeptides (L107, L85, and L71) bound to the column at 4°C, but after incubation at 31°C, polypeptide L71 alone was eluted. The fractions containing this protein exhibited a specific enzyme activity more than 80-fold greater than that present in polyhedra. The possible significance of the alkaline protease, and other proteins with affinity for polyhedrin, is discussed.

Several reports have confirmed that alkaline protease enzymes are associated with the inclusion bodies of some baculoviruses (nuclear polyhedrosis virus [NPV] and granulosis virus [2, 3, 6, 10, 12, 18, 20, 21]). When polyhedra are exposed to alkali, the enzyme degrades the major structural polypeptide, polyhedrin (17), into proteins of low molecular weight (10, 12). In the NPV from *Spodoptera littoralis*, the polyhedrin is first cleaved from a polypeptide of 29,300 daltons to one of 24,900 daltons in 0.05 M sodium carbonate (6).

The fact that the protease is involved in the degradation of polyhedrin leads to speculation as to its possible role in the infection process. Summers and Smith (18) state that when enzymatic activity is inhibited, polyhedra show different solubilization properties. This implies that the enzyme may be involved in the breakdown of polyhedra under alkaline conditions. However, as the precise location of the enzyme has not been determined, it is impossible to assess its significance in infection or to exclude the possibility that the activity could be attributed to contaminating protein at the surface of polyhedra.

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Although it is relatively easy to demonstrate the effect of the protease on polyhedrin by electrophoretic analysis of the products, progress in characterizing the enzyme has been hampered by the lack of a rapid assay method and appropriate purification scheme for the presumptive enzyme. An assay involving casein hydrolysis has been used (2), but as casein is not normally a substrate for the protease it is not completely satisfactory for studying the possible biological significance of the enzyme.

The present study was undertaken to develop a suitable assay for the alkaline protease using its natural substrate, to establish the location of the enzyme within polyhedra, and to purify and partially characterize the enzyme activity. The NPV from *S. littoralis* was used as the model for this research.

MATERIALS AND METHODS

Purification of polyhedra, virus particles, nucleocapsids, and polyhedrin. Polyhedra were extracted from moribund infected larvae, as described elsewhere (6). Sodium dodecyl sulfate (SDS; 0.1%) was included in all solutions. This prevented the aggregation of polyhedra and improved the separation from host debris. In addition 0.1% SDS inhibited protease enzymes that might have been present as a result of surface contamination of polyhedra. Before use, the polyhedra were washed twice in distilled water to remove SDS and stored at −20°C. The protein concentration was measured by the method of Lowry et al. (11) and adjusted to 5 mg/ml.

Virus particles and nucleocapsids were prepared as previously described (6, 14). Polyhedrin was recovered from alkali-solubilized polyhedra by precipitation with 0.66 M sodium acetate, pH 5.0.

Assay methods for protease activity. Protease activity in purified samples of polyhedra, virus particles, and subviral components was detected by one of two methods. The first, involving electrophoresis on polyacrylamide gels (PAGE), measured the degradation of polyhedra by gel electrophoresis (6). The second utilized a labeled polyhedrin substrate for the radioassay of proteolytic activity.

(i) SDS-PAGE assay of protease activity. Endogenous protease activity within polyhedra was measured as previously described (6). Protease activity in samples other than polyhedra was measured by using as the substrate polyhedra that had been heat treated at 80°C for 30 min to inactivate the endogenous enzyme.

(ii) Radioassay of protease activity. For the preparation of ^{3}H-labeled substrate, 5 mg of purified polyhedra was suspended in 1 ml of 0.1 M NaOH for 30 min at room temperature. Undissolved material was removed by low-speed centrifugation, and the supernatant was labeled with [^{3}H]iodoacetate by the method of Parkinson and Kalmakoff (9, 13) in which unreacted [^{3}H]iodoacetate is removed by Sephadex G-50 chromatography. For large-scale preparation of substrate, a batch method was used in which unreacted iodoacetic acid was removed by successive precipitation of the labeled polyhedrin with 0.2 M sodium acetate, pH 5.0. Specific radioactivities in the range of 3,000 to 4,000 dpm/μg of polyhedrin were routinely obtained. Substrate prepared by either the column or batch method gave similar results in the radioassay for protease activity.

Assay conditions were as follows. The standard assay was carried out by using 50 μl of substrate in 0.1 M sodium carbonate buffer, pH 9.6, and 100 μl of protease sample in the same buffer. The mixture was incubated at 31°C for different times, and proteolysis was stopped by the addition of 1.0 ml of 0.2 M sodium acetate, pH 5.0. Samples were left on ice for 30 min, after which time undegraded protein substrate was collected by filtration on Whatman GFA glass-fiber disks. The disks were washed twice with 5-ml volumes of 0.2 M sodium acetate, followed by two 5-ml volumes of absolute ethanol. The disks were dried and counted in a liquid scintillation spectrometer. Protease activity was determined as the difference in acetate-precipitable counts per minute when compared with a control containing the protease fraction that had been heat inactivated at 70 to 80°C for 30 min. All assays were carried out in duplicate. Enzyme activity units were defined as micrograms of polyhedrin hydrolyzed per hour per microgram of test protein.

Affinity column chromatography. An attempt to purify the protease was made by using affinity column chromatography with polyhedrin bound to cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals, Inc.).

(i) Preparation of affinity column. Purified polyhedra of *S. littoralis* NPV were heat treated at 80°C for 30 min and dissolved in 0.1 M NaOH at 5 mg/ml for 30 min at room temperature. Polyhedrin was precipitated by 0.66 M sodium acetate, pH 5.0. The protein was dissolved in 0.1 M sodium carbonate buffer (pH 10.8)–0.5 M NaCl (coupling buffer). A total of 60 mg of protein was then mixed with 3 g of cyanogen bromide-activated Sepharose that had been washed with 600 ml of 10^{-3} M HCl. The mixture was shaken gently for 2 h at room temperature. Excess protein was removed by washing the Sepharose on a sintered-glass filter with 100 ml of coupling buffer. Any remaining active groups were blocked by treatment with 100 ml of 1 M Tris-hydrochloride (pH 8.0) for 2 h. The Sepharose was finally washed with three cycles of 25 ml of coupling buffer, followed by 25 ml of 0.1 M acetate buffer (pH 4.0)–0.5 M NaCl.

(ii) Chromatography. A 3-cm column of polyhedrin coupled to Sepharose was prepared in a 1-cm-diameter water-jacketed column attached to a thermostatically controlled water bath. The column was equilibrated with 0.1 M sodium carbonate buffer (pH 9.6) at 4°C. A total of 5 to 10 mg of purified virus particles pretreated with 1% Nonidet P-40 (NP-40) was run into the column. Material that did not bind to the column was eluted by the successive addition of 5-ml volumes of carbonate buffer. The absorbance of these fractions was monitored at 280 nm. When the absorbance had dropped to a background level, the column temperature was raised to 31°C for 16 h. Fractions were then collected when the column was washed with successive 2-ml volumes of carbonate buffer. All column fractions were assayed for absorbance at 280 nm, total and acid-insoluble (10% trichloroacetic acid) protein, and protease activity.

RESULTS

PAGE assay of protease activity. Preliminary results had shown that *S. littoralis* NPV polyhedrin is degraded in alkali from a polypeptide (P29) of 29,300 daltons to one of 24,900 daltons (P25) (6). Further studies demonstrated that the appearance of P25 is time dependent and shows a product-precursor relationship with P29 (Fig. 1).

The critical temperature of inactivation of this proteolytic event was examined by heating polyhedra at different temperatures for 30 min, followed by dissolution with 0.05 M sodium carbonate. Figure 2 shows that there was a progressive inactivation of the proteolytic activity in the range of 50 to 70°C. At 70°C, there was virtually no residual protease activity since only polypeptide P29 was obtained.

Radioassay of protease activity. Although protease activity can be detected by PAGE, this method cannot be easily quantified and is inconvenient for large numbers of samples. Since undegraded polyhedrin could be obtained by 0.1 M NaOH treatment (6) and/or heat inactivation at 70 to 80°C, polyhedrin was labeled under these conditions with the alkylating agent iodoacetic acid. Protein prepared in this way had the same molecular weight as did undegraded polyhedrin, with only a small amount of lower-molecular-

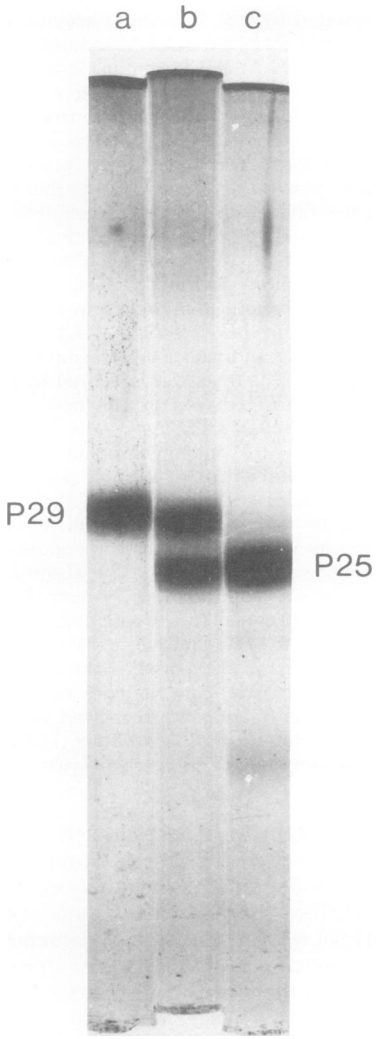

FIG. 1. *Time-dependent degradation of polyhedrin by an endogenous protease. Electrophoretic separation of the polypeptides of polyhedra on 10% SDS-polyacrylamide gels. (a) Polyhedra untreated with alkali; (b) polyhedra dissolved in 0.05 M sodium carbonate for 15 min; (c) polyhedra dissolved in carbonate for 30 min.*

weight material (Fig. 3a). The labeled polyhedrin was also shown to be antigenically "native" in configuration as it gave a line of serological identity with the major antigen of dissolved polyhedra (Fig. 3b).

When proteolytically active fractions of *S. littoralis* NPV were mixed with this substrate, the labeled protein was degraded to acetate-soluble products. Although the assay could also be stopped with 10% trichloroacetic acid, the precipitation of undegraded polyhedrin by acetate buffer gave more sensitive results. The degradation of substrate was initially linear (Fig. 4). However, when approximately 70 to 75% had been degraded, further breakdown proceeded very slowly (Fig. 4). Provided such substrate-limiting conditions were not reached, it was possible to use the radioassay to measure the specific activities of proteolytically active samples.

Location of the enzyme within polyhedra. With the exception of some early studies of Yamafuji et al. (20), previous studies of baculovirus alkaline proteases have made little attempt to determine the location of the enzymes within polyhedra. In the present study, polyhedra (both undissolved and dissolved in 0.05 M sodium carbonate), polyhedrin, virus particles, and nucleocapsids of *S. littoralis* NPV were assayed for the presence of protease activity by the radioassay method. Undissolved polyhedra showed no activity (Table 1), indicating that the enzyme is not a surface contaminant. Polyhedra dissolved in 0.05 M carbonate before use had an activity of 0.016 enzyme unit, but the highest activity was measured in virus particles. Treatment of virus particles with 1% NP-40 did not increase or reduce activity, but purified nucleocapsids were inactive. This implies that the enzyme is located primarily in the envelope fraction of virus particles.

Properties of the virus-associated enzyme. The enzyme associated with virus particles demonstrated a linear time and concentration dependence characteristic of an enzymatic reaction (Fig. 4). It also had a high pH optimum (pH 9.6) and a broad temperature optimum of 35 to 50°C (Fig. 5). In accordance with the heat inactivation studies (Fig. 2), protease activity was markedly reduced at 60°C. The enzyme activity was inhibited by high concentrations of several divalent cations (Hg^{2+}, Cu^{2+}, Mg^{2+}) and by the detergent SDS (Table 2). However, activity was not significantly affected by the addition of 2-mercaptoethanol (2-ME), EDTA, or Na^{+}. We did not find any conditions that significantly increased the relative activity of the enzyme (Table 2).

Purification of the enzyme. Affinity chromatography was selected as a method for the purification of the protease on the basis that the enzyme should have considerable affinity for its natural substrate, polyhedrin. Earlier results (Table 1) had suggested that the enzyme associated with virus particles was located in the envelope fraction, but was not affected by NP-40 treatment. For this reason, virus particles were treated with 1% NP-40 to solubilize the envelope proteins before they were applied to the affinity column. The sample application was also carried out at a low temperature (4°C) to

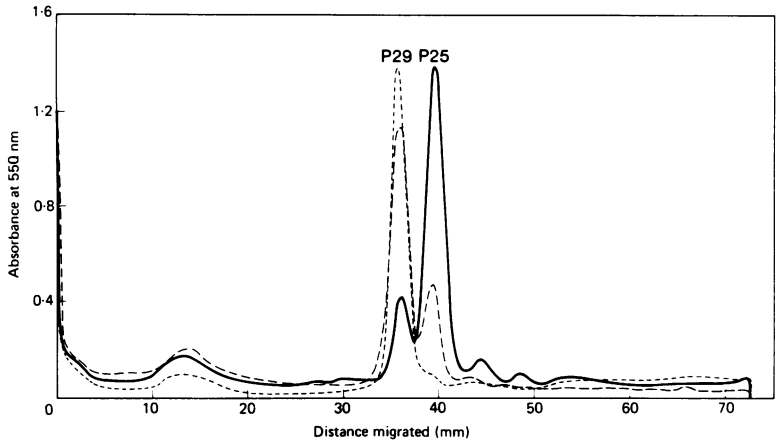

FIG. 2. *Thermal inactivation of alkaline protease. Composite densitometer traces at 550 nm of three 7% polyacrylamide gels. Samples of polyhedra were treated at three different temperatures before dissolving for 30 min in 0.05 M sodium carbonate. The direction of electrophoresis is from left to right.* (——) *Polyhedra pretreated at 50°C;* (- - -) *polyhedra pretreated at 60°C;* (·····) *polyhedra pretreated at 70°C.*

minimize enzyme activity. Successive washes at this temperature with 0.1 M carbonate buffer eluted several fractions with a high optical density at 280 nm. The fractions contained some protease activity, but of lower specific activity than that normally present in intact virus particles (Fig. 6 and Table 1). When the proteins of these fractions were subjected to SDS-PAGE, it could be seen that at least three major polypeptides (L107, L85, and L71 [6]) were not eluted and must have bound to the column (Fig. 7). These three polypeptides had previously been determined to be envelope components of *S. littoralis* NPV particles (6).

The column temperature was then raised to 31°C overnight to activate the protease. Subsequent washing should then elute any displaced enzyme and degraded polyhedrin from the column. As expected, the most proteolytically active samples were eluted after the overnight incubation (Fig. 6). These samples contained a large proportion of acid-soluble products (degraded polyhedrin). When their specific activity was plotted on the basis of acid-insoluble protein instead of total amino acids, the enzyme activity was almost 84 times greater than the original activity of protease in polyhedra (Table 3).

When the protein in these fractions (fractions 5 and 6 of Fig. 6) was subjected to electrophoresis on a 7% gel, of the three major polypeptides that had bound to the column, only polypeptide L71 was detected (Fig. 8a). When this protease sample was added to heat-inactivated polyhedra and incubated in 0.05 M sodium carbonate for 16 h, the polyhedrin was almost completely degraded to the 24,900-dalton component (P25, Fig. 8b). It is therefore likely that polypeptide L71 is the alkaline protease of *S. littoralis* NPV particles.

DISCUSSION

Although the protease assay methods used in this work were different in experimental design, they complemented each other in the measurement of enzyme activity. The PAGE assay measured the appearance of P25 from undegraded polyhedrin (P29). In this case the product was precipitated by the addition of sodium acetate (pH 5.0), whereas the radioassay measured the appearance of small-molecular-weight hydrolysis products that were not acetate precipitable. The fact that the purified protease cleaved polyhedrin in the same way (Fig. 8b) as did the unpurified enzyme in polyhedra (Fig. 1) indicates that the two assays were measuring the same enzymatic activity.

The development of the radioassay made it possible to measure the specific activity of the enzyme in viral and subviral components by using the natural substrate. The results showed that virus particles had a protease activity almost six times greater than that in polyhedrin and that in virus particles the enzyme was located in the envelope fraction.

The pH optimum for enzyme activity was 9.6, very similar to that reported for the alkaline protease of *Trichoplusia ni* NPV (pH 9.5 [2]). This is also remarkably close to the pH of the larval gut of *S. littoralis* larvae (pH 9.5) and may indicate some adaptation for activity in the alkaline gut juice of lepidopterous larvae (8). In addition, enzymes from both *T. ni* and *S. littoralis* NPVs are inhibited by Hg^{2+} and Cu^{2+}, but not by 2-ME (2).

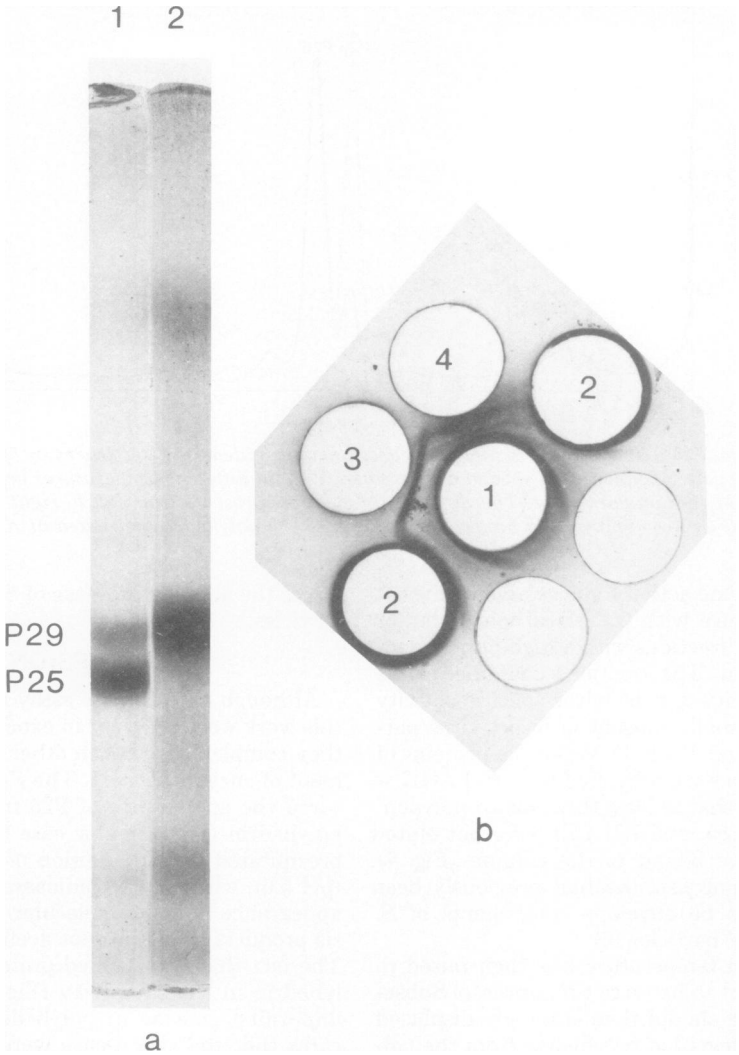

FIG. 3. *Properties of the ^{3}H-labeled polyhedrin. (a) Electrophoretic mobility on 7% gels of the labeled substrate (2) compared with polyhedrin polypeptides P29 and P25 (1). (b) Comparison of the antigenic properties of labeled polyhedrin and NPV polyhedra by gel diffusion in 1% agarose. (1) Antiserum to S. littoralis NPV polyhedra (prepared as previously described [6]); (2) "mock-labeled" polyhedrin prepared in the same way as the labeled substrate but without the addition of [^{3}H]iodoacetate; (3) ^{3}H-labeled polyhedrin; (4) S. littoralis NPV polyhedra. All antigens were dissolved in 0.05 M sodium carbonate before use.*

Our results agree with the earlier studies of Yamafuji et al. (20) in that virus particles show a greater enzymatic activity than does polyhedrin. In contrast, Eppstein and Thoma (2) imply that, in *T. ni* NPV, an alkaline protease is closely associated with the polyhedrin fraction. It is possible that the virus particle enzyme described in the present study may not account for all of the protease activity in polyhedra and that two or even more distinct enzymes may be present. However, many of the properties of the virus particle enzyme are consistent with properties of other alkaline protease enzymes described in NPV (2, 3, 12). It is also known that a proportion of NPV particles lose the viral envelope when they are exposed to alkali during the dissolution of polyhedra (5).

Proteases have been found associated with a number of enveloped viruses, including influenza, vesicular stomatitis, and sowthistle yellow vein viruses (7, 22). With influenza virus and sowthistle yellow vein virus, the autodigestion of viral proteins was activated by treatment with NP-40. With *S. littoralis* NPV, there is no evidence of the degradation of virus particle polypeptides after NP-40 treatment (6). However,

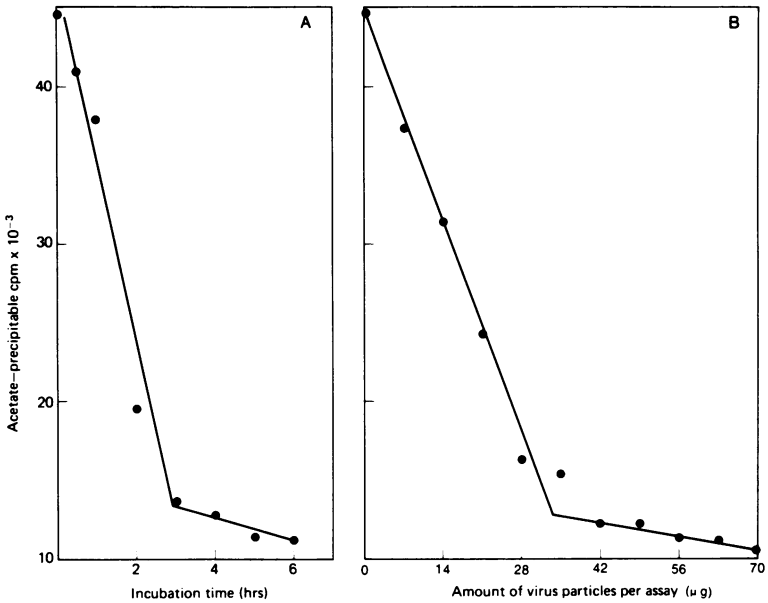

FIG. 4. *Assay of the protease activity in NPV particles, demonstrating time dependence (A) and concentration dependence (B) of the radioassay.*

TABLE 1. *Specific activities of protease in components of S. littoralis NPV*

Sample	Protease activity (enzyme units)	Relative activity
Intact polyhedra	NA[a]	
Dissolved polyhedra	0.016 ± 0.005[b]	1.2
Polyhedrin	0.013 ± 0.005	1.0
Virus particles	0.075 ± 0.018	5.8
Virus particles plus 1% NP-40	0.076 ± 0.013	5.8
Nucleocapsids	NA	

[a] NA, No activity detected.

[b] Standard deviation calculated from a minimum of four measurements.

modifications that would be attributed to proteolytic degradation were observed when particles of another baculovirus were treated with the detergent (1).

It has been suggested that membrane-associated enzymes could be of some significance for infection, possibly during the fusion of the viral envelope with the plasma membrane before the introduction of the viral nucleocapsid into the cell cytoplasm (7). Recent studies of an enzyme associated with a granulosis virus of *Pseudaletia unipuncta* have shown that the enzyme will increase the uptake of NPV nucleocapsids by

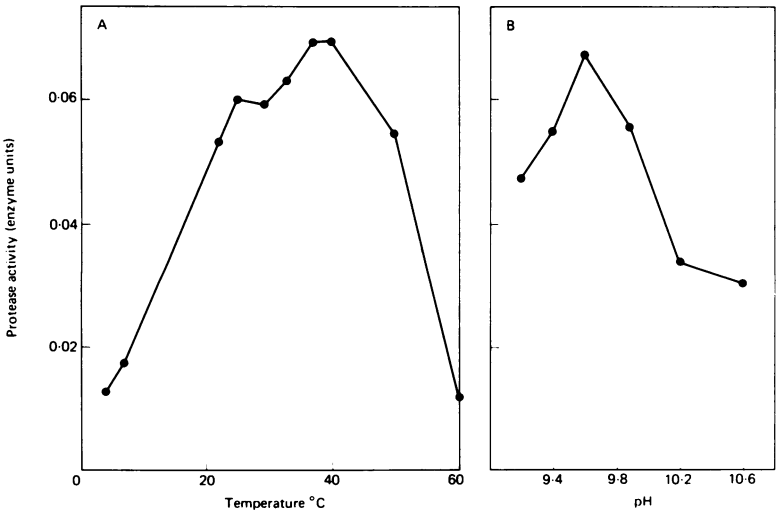

FIG. 5. *Temperature (A) and pH (B) optima of the protease activity in virus particles.*

TABLE 2. *Effect of metal ions, etc., on the radioassay of virus particle protease activity*

Treatment[a]	Relative activity (%)[b]
None	100
10^{-2} M Hg^{2+}	2
10^{-3} M Hg^{2+}	17
10^{-4} M Hg^{2+}	20
10^{-2} M Cu^{2+}	9
10^{-3} M Cu^{2+}	5
10^{-4} M Cu^{2+}	40
10^{-1} M Mg^{2+}	0
10^{-2} M Mg^{2+}	46
10^{-3} M Mg^{2+}	102
10^{-2} M Na^{+}	95
10^{-3} M Na^{+}	102
0.1% 2-ME	118
0.01% 2-ME	85
10^{-2} M EDTA	87
10^{-3} M EDTA	80
10^{-4} M EDTA	84
1% SDS	0
0.1% SDS	0
0.01% SDS	66

[a] The radioassays were carried out in a final volume of 150 μl for 30 min at 31°C.

[b] Averages of two measurements.

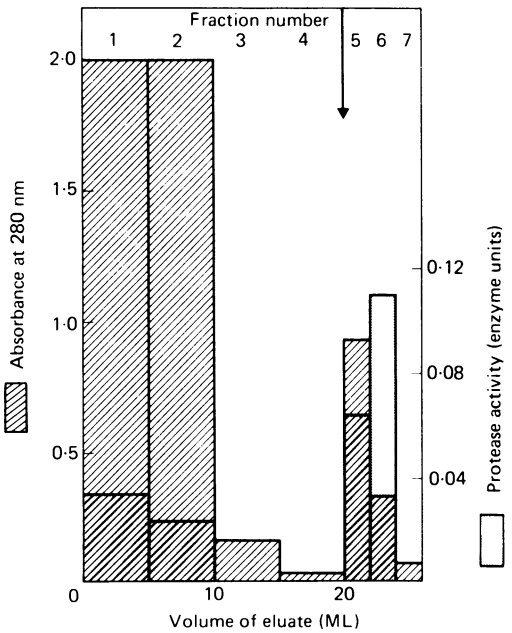

FIG. 6. *Elution profile of virus particles (treated with 1% NP-40) from a 3-cm column of polyhedrin attached to cyanogen bromide-activated Sepharose. Fractions 1 to 4 were eluted at 4°C, and fractions 5 to 7 were eluted at 31°C.*

the gut epithelial cells of this insect (19). It is interesting to speculate that this "synergistic" enzyme could be an alkaline protease associated

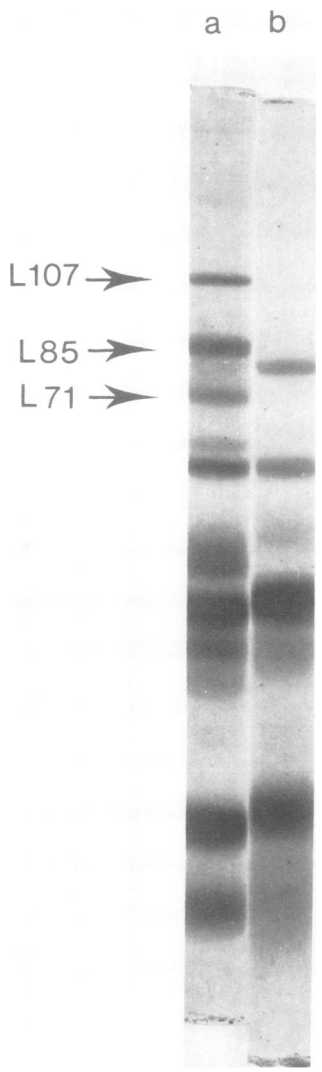

FIG. 7. *SDS-PAGE of the proteins of (a) S. littoralis NPV particles and (b) viral proteins that did not bind to the polyhedrin affinity column (fraction 2, Fig. 6).*

TABLE 3. *Comparative specific activities of the alkaline protease of S. littoralis NPV at three stages of purification*

Sample	Protease activity (enzyme units)[a]	Relative activity
Dissolved polyhedra	0.016	1.0
Virus particles	0.123	7.7
Purified enzyme from affinity column	1.340	83.5

[a] Enzyme activity was determined per microgram of 10% trichloroacetic acid-precipitable protein in each sample. Values are an average of two measurements.

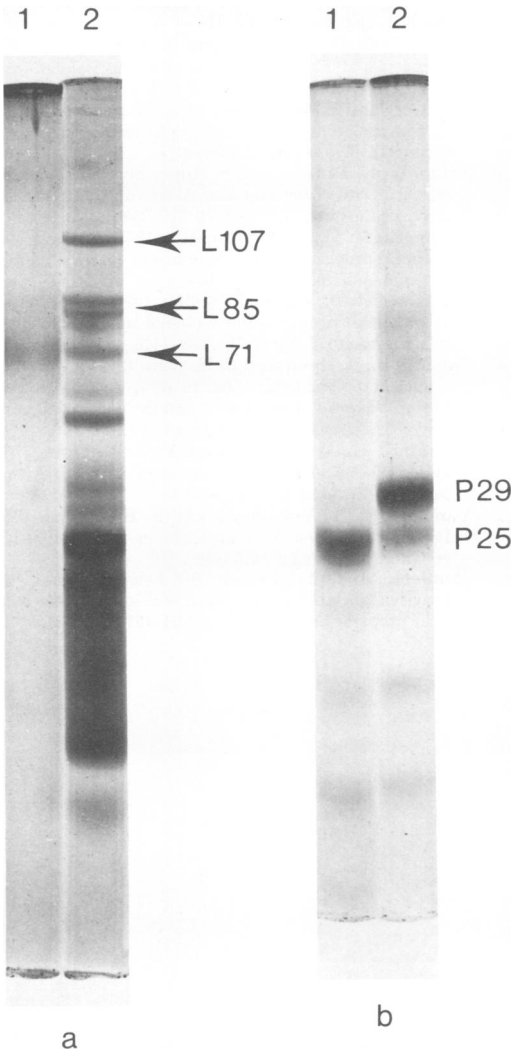

FIG. 8. *Properties of the protease-active fractions eluted from polyhedrin-cyanogen bromide-activated Sepharose at 31°C. (a) Electrophoretic separation on 7% gels of (1) trichloroacetic acid-precipitable protein in column fractions 5 and 6 and (2) trichloroacetic acid-precipitable proteins in a preparation of S. littoralis NPV particles pretreated with 1% NP-40 (polypeptide L85 has resolved into two components on this gel). (b) Proteolytic cleavage of polyhedrin by the purified protease. Seven percent SDS gels. (1) Polyhedra (heat denatured at 70°C for 30 min) incubated with protease for 16 h at 31°C. (2) Heat-denatured polyhedra incubated without protease for the same time. A small amount of endogenous activity is evident (accounting for the presence of polypeptide P25), but this is insufficient to account for the disappearance of P29 from the protease-treated sample.*

with the granulosis virus. Like the alkaline protease of *T. ni* NPV (2), the enzyme shows esterase activity (4).

It seems unlikely that the alkaline protease of occluded baculoviruses is responsible for the solubilization of polyhedra. Although Summers and Smith (18) state that the solubilization of *T. ni* granulosis virus is affected when enzyme activity is inhibited, we failed to detect any significant difference in the time taken for *S. littoralis* NPV polyhedra to dissolve in alkali, regardless of whether the enzyme was active or denatured. However, when the enzyme is inhibited, a small amount of polyhedrin is still found associated with purified virus particles (6). Thus, the protease may be important in releasing virions from the layer of polyhedrin that immediately surrounds them.

It is, therefore, impossible at present to draw firm conclusions as to the role of this virus-associated enzyme. No evidence is yet available to determine whether it is a host- or virus-directed protein. In this context, some interesting studies should be possible with virus particles grown in vivo and in vitro. Recent research has shown that polyhedra produced in cell culture appear to lack the protease activity (W. McCarthy, personal communication; C. C. Payne and D. A. Brown, unpublished data).

For some time, the mechanism of virus occlusion within polyhedra has puzzled insect virologists. In this study we have reported that three virus particle envelope polypeptides have a strong affinity for binding polyhedrin. It is possible that these polypeptides could act as initial points of attachment of virus to developing polyhedra. In this way, the occlusion of NPV virions within polyhedra could resemble the occlusion of vaccinia virions within type A inclusions, where incorporation is dependent upon the presence of a virus-occluding factor located at the surface of the virion (16). This factor is also proteolytically active (15).

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