JOURNAL OF VIROLOGY, June 1978, p. 702-711
0022-538X/78/0026-0702$02.00/0
Copyright © 1978 American Society for Microbiology
Vol. 26, No. 3
Printed in U.S.A.

Characterization of an Alkaline Protease Associated with a Granulosis Virus of *Plodia interpunctella*†

KATHLEEN A. TWEETEN,[1] LEE A. BULLA, JR.,[2] AND RICHARD A. CONSIGLI[1]*

Division of Biology, Kansas State University, Manhattan, Kansas 66506,[1] and U.S. Grain Marketing Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Manhattan, Kansas 66502[2]

Received for publication 21 October 1977

An alkaline protease was found to be associated with the granulosis virus of the Indian meal moth, *Plodia interpunctella*. The protease was located within the protein matrix of the occluded virus and hydrolyzed the major constituent of this matrix, a 28,000-dalton protein (granulin), to a mixture of polypeptides ranging in molecular weight from 10,000 to 27,000. A rapid, sensitive assay for the protease was developed using radioactively labeled granulosis virus as substrate. With this assay, the proteolytic activity could be detected by measuring the release of acid-soluble peptides from the labeled virus. The protease had a pH optimum of 10.5 and a temperature optimum of 40°C and was inhibited by diisopropyl phosphorofluoridate, phenylmethylsulfonyl fluoride, and L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone. Purification of the protease from matrix protein was achieved by anion-exchange and gel permeation chromatography. The molecular weight of the isolated protease, determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and gel filtration, was approximately 14,000.

The occluded insect viruses of the *Baculoviridae* consist of enveloped nucleocapsids enclosed within a crystalline matrix of protein. A number of studies indicate that the baculoviruses (nuclear polyhedrosis virus [NPV] and granulosis virus [GV]) have alkaline proteases associated with them. Yamafugi et al. (22) first reported the occurrence of such proteases in NPV preparations from the silkworm, *Bombyx mori*. Kozlov et al. (8) provided evidence that proteolytic activity was directed against the protein that comprises the crystalline matrix. These latter investigators found that the protein matrix isolated from *B. mori* or *Galleria melonella* NPVs by dissolution in alkaline carbonate (pH 11) consisted of several protein components. These polypeptides ranged in molecular weight from 11,000 to 23,000, whereas the protein matrix extracted with 67% acetic acid consisted of a single 28,000-dalton component. Similar proteolytic activity was observed during alkaline solubilization of the protein matrix from the NPVs of the cabbage looper, *Trichoplusia ni* (2, 3) and *Porthetria dispar* (12), and from GVs of *T. ni* and the fall armyworm, *Spodoptera frugiperda* (16). Nondegraded matrix protein was obtained only if inhibitors of proteolytic activity such as mercury (2), diisopropyl phosphorofluoridate (DFP) (19), or phenylmethylsulfonyl fluoride (PMSF) (12) were present during alkaline dissolution.

Our laboratory is investigating the insecticidal activity and molecular biology of the GV of the Indian meal moth, *Plodia interpunctella*. Proteolytic activity was observed when highly purified preparations of this virus were incubated at alkaline pH. As a first step in the characterization of this activity, a sensitive assay using radiolabeled GV as substrate was developed for measuring proteolysis. This assay was used to localize the protease in the occluded virus and to determine the effect of temperature, pH, and various inhibitors on its activity. Furthermore, isolation of the protease was accomplished, and properties of the purified enzyme are presented in this report.

MATERIALS AND METHODS

Rearing of insects and production of GV. A laboratory colony of *P. interpunctella* was reared as previously described (20). Early fourth-instar larvae were infected with GV, and the virus was purified from larvae as described (20). Briefly, infected larvae were disrupted in a Sorvall Omni-mixer and the resulting homogenate was centrifuged at 1,000 × *g* for 30 min. The supernatant was further centrifuged at 16,000 × *g* for 30 min. The pellet was resuspended in distilled water and treated with sodium deoxycholate at a final concentration of 1% for 15 min at room temperature. The GV was then pelleted through a 4-ml shelf of 50% (wt/wt in distilled water) sucrose and centrifuged in a 45 to 60% sucrose (wt/wt in distilled water) gradient.

† Contribution no. 78-108-J, Kansas Agricultural Experiment Station, Kansas State University, Manhattan, KS 66506.

The virus was washed twice with distilled water and stored at −20°C. This procedure was confirmed by mixing experiments (in which radioactively labeled, uninfected larvae or host proteins were mixed with unlabeled, infected larvae before purification) to free the GV of all detectable host contamination and to preserve its structural integrity and biological infectivity (20).

Radiolabeling of GV. Radioactively labeled ([^{3}H]valine, 23 Ci/mmol; Schwarz/Mann) GV was produced in vivo by injection of 1 μl (0.5 μCi) of the isotope into larvae at 96, 120, and 144 h after infection. The GV was purified from injected larvae at 8 days after infection. The proteins of purified GV were labeled in vitro with [^{14}C]formaldehyde by a modification of the method of McMillen and Consigli (13). Purified GV was pelleted by centrifugation at 10,000 rpm (HB-4 rotor) for 30 min and was resuspended in 2 ml of 0.1 M sodium borate, pH 9.0. [^{14}C]formaldehyde (0.25 mCi; New England Nuclear) was added, and, after incubation for 10 min at 37°C, sodium borohydride (160 μg/mg of viral protein) was added. The preparation was incubated for 10 min at room temperature. Unreacted [^{14}C]formaldehyde and sodium borohydride were removed by pelleting the GV two times through a 4-ml shelf of 50% sucrose (25,000 rpm [SW 27 rotor] for 30 min at 10°C). The labeled GV was resuspended in distilled water and stored at −20°C until used.

Protease assay. A modification of the method of Kunitz (9) with casein as substrate was initially used for determination of protease activity. The assay was conducted by adding 1 ml of 1% (wt/vol) heat-denatured casein in 0.1 M sodium carbonate, pH 10.6, to 0.1 ml of purified GV (5 to 150 μg of protein). After incubation for 24 h at 37°C, cold trichloroacetic acid was added to a final concentration of 10% (vol/vol). The mixture was incubated for 20 min at 4°C and centrifuged at 10,000 rpm (HB-4 rotor) for 20 min, and the absorbance of the supernatant at 280 nm was determined. Three controls consisting of casein only, GV only, and complete reaction mixture to which the trichloroacetic acid was added at zero time were included in the assay.

A more sensitive assay for GV protease was developed using in vitro ^{14}C-labeled GV as substrate. The radioactive GV was heated for 30 min at 70°C to inactivate endogenous protease. The reaction mixture consisted of 0.1 ml of protease preparation (containing 5 to 150 μg of protein), 50 μg of labeled substrate (8×10^6 cpm/μg of protein), and 0.3 ml of 0.1 M sodium carbonate (pH 10.6) and was incubated for 2 h at 37°C. Cold trichloroacetic acid was added to a final concentration of 10% (vol/vol), and the tubes were incubated for 20 min at 4°C and then centrifuged at 10,000 rpm (HB-4 rotor) for 20 min. Radioactivity of acid-soluble material was measured in Triton scintillation mixture in a LS-233 Beckman scintillation counter. A control consisting of labeled substrate only produced background levels of acid-soluble, radioactive material.

Inhibition of protease. GV (100 μg of protein) was preincubated for 1 h at room temperature with the following inhibitors: 1 to 50 mM DFP, 1 to 25 mM PMSF, 10 mM L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone (TPCK), 1 to 50 mM mercuric chloride ($HgCl_2$), 1 to 50 mM iodoacetate, and 25 mM EDTA. Unreacted inhibitor was removed by pelleting the virus at 10,000 rpm (HB-4 rotor) for 30 min and washing it twice. The virus was resuspended in 0.1 ml of water, and proteolytic activity was assayed by the ^{14}C-labeled substrate method described above. The effect of the inhibitors was also determined by adding them directly to the assay mixture. Inhibitors were tested on the isolated protease by conducting the ^{14}C-labeled substrate assay after 1 h of preincubation of the protease with the various inhibitors.

Effect of pH and temperature on protease activity. The influence of pH was determined over the range of pH 3 to 12 in 0.1 M sodium glycinate, using [^{14}C]GV and heat-denatured casein as substrates in the assays described above. The effect of temperature on protease was determined by preincubating purified GV in a water bath at temperatures ranging from 40 to 70°C for 30 min and then assaying for protease by the [^{14}C]GV method. The optimum temperature for protease activity was determined by conducting the [^{14}C]GV assay over a temperature range of 25 to 60°C.

Isolation of granulin and enveloped nucleocapsids. The protein matrix or "granulin" (17) was solubilized by incubating purified GV for 10 min at 37°C in 0.05 M Na_2CO_3–0.05 M NaCl, pH 10.6. The dissociated virus was layered on a 30 to 70% (vol/vol in water) glycerol gradient, which was centrifuged at 25,000 rpm (SW41 rotor) for 30 min at 10°C. The granulin remained on top of the gradient, and the enveloped nucleocapsids banded half-way down the gradient (6, 7, 12, 18). Both viral components were recovered from the gradient by use of 1-ml pipettes inserted into a Pipet-aid (Drummond Scientific Co.) and were diluted severalfold with water. Glycerol was removed in a model 12 Amicon ultrafiltration cell, using a UM-10 membrane for the granulin fraction and an XM-300 membrane for the enveloped nucleocapsids. Protein was determined by the method of Lowry et al. (11), using bovine serum albumin as a standard, and the protease activity in each component was determined by the [^{14}C]GV assay.

Protease isolation. The alkaline protease was isolated from the granulin fraction that was separated from the enveloped nucleocapsids as described above. Approximately 300 mg of granulin preparation was applied to a DEAE-Sepharose (CL-6B, Pharmacia Fine Chemicals) column (2 by 10 cm) equilibrated with 0.01 M Tris-hydrochloride (pH 8.5)–0.01 M NaCl. The protein was eluted with a linear salt gradient of 125 ml of 0.01 M Tris–0.01 M NaCl (pH 8.5) and 125 ml of 0.01 M Tris–0.5 M NaCl (pH 8.5). Fractions of 3 ml each were collected, protein was measured by absorbance at 280 nm using a type 8303A LKB UV absorptiometer, and the protease activity was determined by the hydrolysis of [^{14}C]GV. The active fractions were pooled and concentrated in a model 52 Amicon ultrafiltration cell (UM-10 membrane). The protease preparation was then applied to a Sephadex G-100 column (1.7 by 80 cm) equilibrated with 0.01 M Tris–0.1 M NaCl (pH 8.0). The column was washed with this buffer, and 1-ml fractions were collected. Protease activity was determined using the [^{14}C]GV assay, and active fractions were pooled and stored at −20°C.

Gel electrophoresis. Proteins were subjected to electrophoresis on 15% sodium dodecyl sulfate (SDS)-polyacrylamide slab gels (1.5 by 14 by 18 cm; model SE-500, Hoefer Scientific Instruments), using the discontinuous buffer system of Laemmli (10). Samples were prepared for electrophoresis by boiling for 2 min in 2% SDS, 5% 2-mercaptoethanol, and 10% glycerol. Electrophoresis was carried out at 30 mA/slab until the tracking dye (bromophenol blue) reached the bottom of the gel. Slabs were stained overnight in 0.1% Coomassie brilliant blue R (Sigma Chemical Co.), 50% methanol, and 7.5% acetic acid. Destaining was in 50% methanol–7.5% acetic acid for 1 h and in 25% methanol–1.5% acetic acid for 48 h. Gels were dried using a SE-540 Hoefer Scientific Instruments slab gel dryer. Molecular weights were determined by the method of Weber and Osborn (21), using cytochrome *c*, chymotrypsinogen, ovalbumin, and bovine serum albumin (Schwarz/Mann) as standards.

Gel filtration. The molecular weight of purified alkaline protease was determined on Sephadex G-100 according to Andrews (1). The gel column (1.7 by 80 cm) was calibrated with cytochrome *c*, chymotrypsinogen, ovalbumin, bovine serum albumin, and blue dextran (Pharmacia). These reference proteins (except for blue dextran) were labeled in vitro with [^{14}C]formaldehyde (13), and their elution volumes were measured by monitoring radioactivity of column fractions. Protease was detected using the [^{14}C]GV assay.

RESULTS

Protease activity associated with GV of *P. interpunctella*. Proteolytic activity was initially observed in the GV of *P. interpunctella* by SDS-polyacrylamide gel electrophoresis of virus that had been incubated in 0.1 M Na_2CO_3 at pH 10.6. The alkaline carbonate was being used to solubilize the protein matrix of the GV, and, with prolonged exposure to it, extensive protein degradation was observed (Fig. 1). The most evident cleavage was that of the 28,000-dalton matrix protein or granulin. After exposure to carbonate for 30 min at room temperature, three degradation products of molecular weights of 27,000, 25,000, and 23,000 were observed in addition to the granulin (Fig. 1A). When the exposure was increased to 1 to 2 h, increased amounts of the 23,000- to 27,000-dalton polypeptides were generated (Fig. 1B and C). Incubation

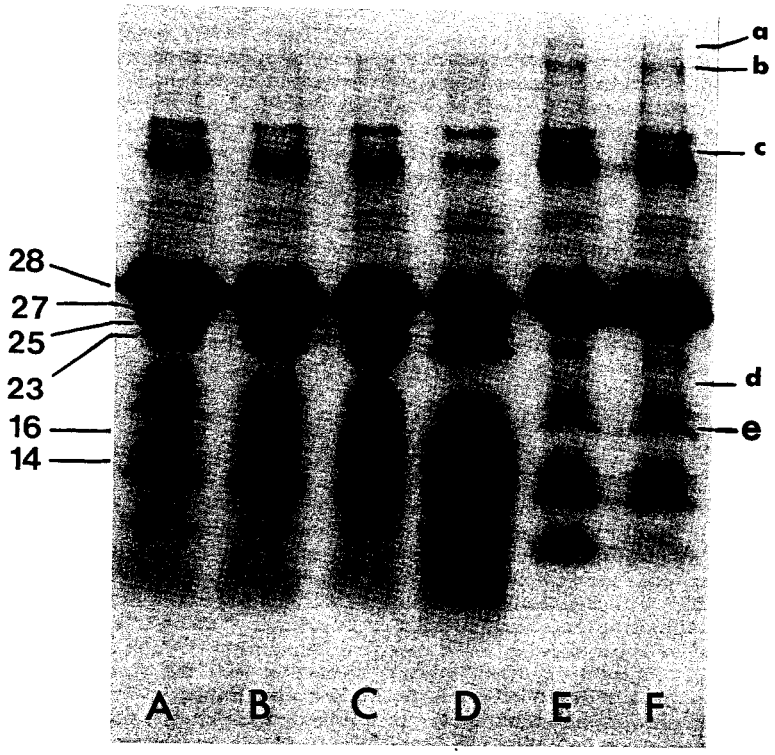

FIG. 1. *SDS-polyacrylamide gel electrophoresis of carbonate-treated GV. Purified GV was incubated in 0.1 M sodium carbonate for various periods of time. The treated GV was precipitated by 10% trichloroacetic acid and prepared for electrophoresis by boiling in 2% SDS, 5% 2-mercaptoethanol, and 10% glycerol. Incubation in carbonate for: (A) 30 min; (B) 1 h; (C) 2 h; (D) 6 h. (E) GV heated at 65°C for 30 min before incubation in carbonate for 6 h. (F) GV treated with 67% acetic acid (8), precipitated with 10% trichloroacetic acid, and prepared for electrophoresis as described above.*

for 6 h resulted in markedly reduced amounts of granulin. Cleavage products of molecular weights ranging from 10,000 to 20,000 accumulated, with the predominant bands having molecular weights of 14,500 and 16,000 (Fig. 1D). No proteolytic activity was observed when the GV was heated at 60°C for 30 min and then incubated in carbonate for 6 h (Fig. 1E) or if the granulin was solubilized under acid conditions (67% acetic acid) before electrophoresis (Fig. 1F).

Quantitation of GV protease activity. To study the protease further, an assay was developed to quantitate the proteolytic activity. Initially, a method using heat-denatured casein as substrate was employed. Whereas GV that had been heat treated was unable to degrade the casein, active preparations of GV were observed to hydrolyze casein at alkaline pH (Fig. 2A). However, for significant hydrolysis to be measured, the assay had to be incubated for 12 to 24 h (Fig. 2A), and at least 100 μg of GV was required in the assay mixture (Fig. 2B). A more rapid and more sensitive assay for the protease was developed using radioactively labeled, heat-inactivated GV as the substrate. In this assay, proteolytic activity was monitored by the release of acid-soluble radioactive material from the substrate. The hydrolysis of labeled GV was proportional to the time of incubation in carbonate over a period of 4 h (Fig. 3A). Because significant hydrolysis was detected after only 2 h of carbonate exposure, this time was adopted as the standard incubation period for the [^{14}C]-GV assay. The release of radioactivity from the labeled substrate was directly related to the concentration of protease source (GV in the reaction mixture) and was observed when as little as 10 μg (protein) of GV was present (Fig. 3B).

Effect of pH on GV protease activity. The

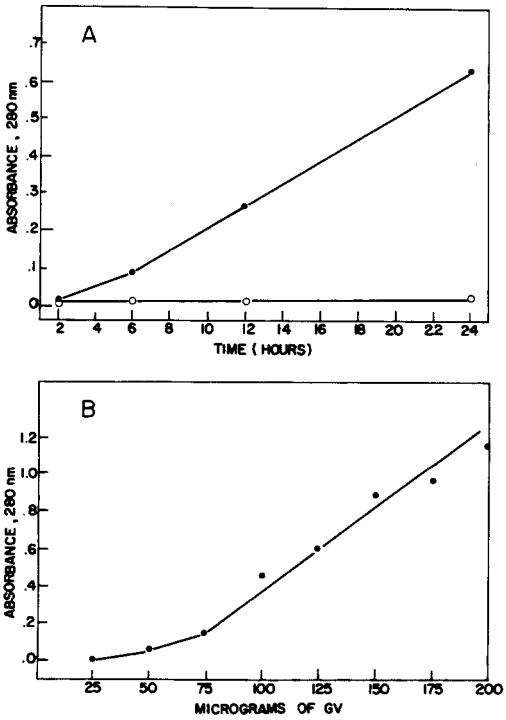

FIG. 2. *Hydrolysis of heat-denatured casein by GV-associated alkaline protease. A mixture of casein (1 ml of 1% casein in 0.1 M sodium carbonate, pH 10.6) and 0.1 ml of purified GV was incubated at 37°C. Cold trichloroacetic acid was added to a final concentration of 10% (vol/vol), and the absorbance at 280 nm of acid-soluble material was determined. (A) Reaction mixture incubated for various times at 37°C: (●) control GV; (○) heat-treated GV (65°C for 30 min). (B) Effect of GV concentration on hydrolysis.*

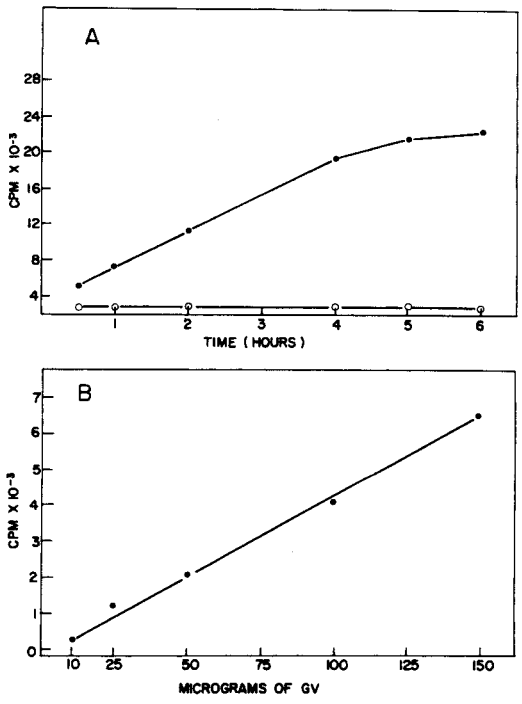

FIG. 3. *Hydrolysis of radioactively labeled GV by GV-associated alkaline protease. The reaction mixture consisted of 0.1 ml of GV, 50 μg of heat-inactivated [^{14}C]GV as substrate, and 0.3 ml of 0.1 M sodium carbonate, pH 10.6. After incubation at 37°C, cold trichloroacetic acid was added to a final concentration of 10%, and the radioactivity of acid-soluble material was determined. (A) Reaction mixture incubated for various times at 37°C: (●) control GV; (○) heat-treated GV (65°C for 30 min). (B) Effect of GV concentration on hydrolysis.*

radioactive-GV assay was used to determine the effect of pH, temperature, and various inhibitors on the virus-associated protease activity. Protease activity as a function of pH is shown in Fig. 4. The optimum pH for proteolysis of GV was 10.5, and little activity was observed below pH 9.5 or above pH 11.5. This pH optimum was obtained whether sodium glycinate, sodium carbonate, sodium borate, or universal buffer (5) was used as the reaction buffer. Proteolytic cleavage of casein also occurred to a maximum extent at pH 10.5 (data not shown).

Effect of temperature on GV protease activity. The effect of temperature on the GV protease was determined by incubating purified GV at various temperatures before assaying for protease activity (Fig. 5A). Whereas preincubation at 40 to 50°C had no influence on the protease, heating at 55 and 60°C resulted in reductions of 15 and 54%, respectively, in proteolytic activity. Exposure to 65°C for 30 min completely inactivated the protease activity in the GV preparation. The sensitivity of the [^{14}C] GV assay was demonstrated by the temperature variation experiments. When casein was used as substrate, all activity appeared to be destroyed by preincubation of the GV at 60°C. It is possible that the heat treatment reduced the concentration of active protease to less than 50 μg of GV, at which level little proteolysis was observed (Fig. 2B). On the other hand, the residual activity remaining after treatment of GV at 60°C was readily observed using the [^{14}C]GV assay (Fig. 5A).

Maximum proteolytic cleavage of viral substrate was observed when the assay was conducted at 40°C. From the temperature-activity curve shown in Fig. 5B, it can be seen that at reaction temperatures above this optimum the protease activity rapidly decreased.

Effect of inhibitors on GV protease. No inhibition of protease activity was observed when iodoacetate (up to 50 mM) or EDTA (up to 25 mM) was preincubated for 1 h with intact GV or was included in the assay reaction (Table 1). Enzyme activity was partially inhibited by 10 mM TPCK and 10 mM $HgCl_2$. Although PMSF extensively inhibited the protease (73% inhibition) at a concentration of 25 mM, DFP was the

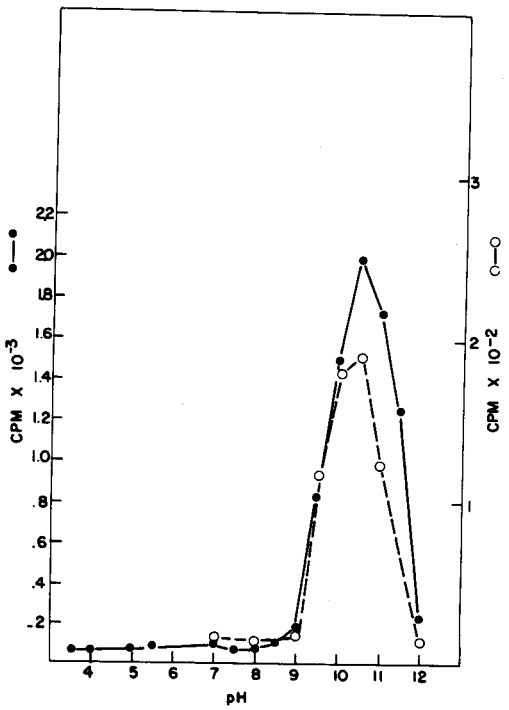

FIG. 4. *Effect of pH on GV protease. Proteolytic activity was measured as described in the legend to Fig. 3 in 0.1 M sodium glycinate adjusted to various pH values. Symbols: (●) GV-associated protease; (○) purified protease.*

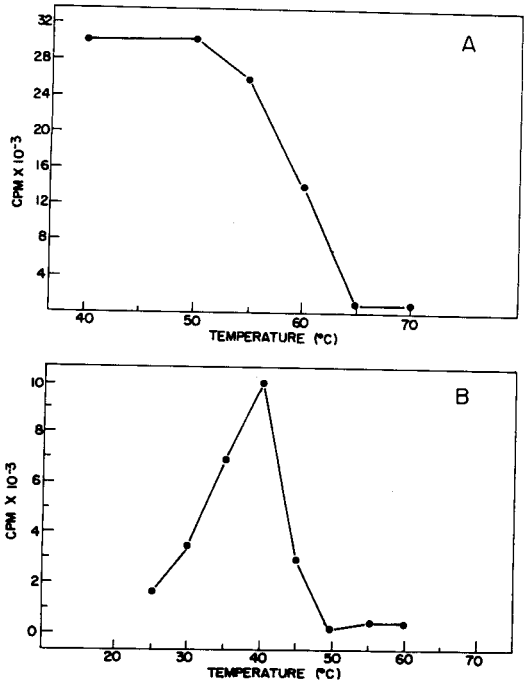

FIG. 5. *Effect of temperature on GV protease. (A) Heat sensitivity of protease. Purified GV was incubated in a water bath at temperatures ranging from 40 to 70°C for 30 min. Protease activity was then measured as described in the legend to Fig. 3. (B) Optimum temperature for protease. Proteolytic activity was measured as described in the legend to Fig. 3 except for incubation of the reaction mixture over the temperature range of 25 to 60°C.*

TABLE 1. *Effect of inhibitors on GV-associated alkaline protease*

Inhibitor	Concn (mM)	% of control activity	
		1-h preincubation with inhibitor[a]	Inhibitor in assay mixture[b]
None (control)		100	100
DFP	0.5	76	0
DFP	0.1	100	53
PMSF	25.0		27
TPCK	10.0	54	33
$HgCl_2$	50.0	68	12
$HgCl_2$	10.0	88	45
EDTA	25.0	94	100
Iodoacetate	50.0	100	100

[a] GV (100 μg) was preincubated for 1 h at room temperature with each inhibitor. Unreacted inhibitor was removed by pelleting the virus at 10,000 rpm (HB-4 rotor) for 30 min and washing it twice. Protease activity was then measured as described in the legend to Fig. 3.

[b] Protease activity was measured as described in the legend to Fig. 3 in the presence of the specified concentrations of inhibitor.

most effective inhibitor. Complete inhibition resulted when DFP was present in the alkaline dissolution mixture at concentrations of 0.5 mM or greater (Table 1). DFP at 0.1 mM provided for partial inhibition of 47%. Similar effects of iodoacetate, EDTA, DFP, and $HgCl_2$ on proteolytic activity were observed when casein was used as substrate (data not shown).

When GV was preincubated with $HgCl_2$, TPCK, or DFP and the unbound inhibitor was removed before assaying for protease, the extent of protease inhibition was less than when the inhibitors were added directly to the assay mixture. The latter inhibition was 88% for 50 mM $HgCl_2$, 67% for 10 mM TPCK, and 100% for 0.5 mM DFP. Preincubation with these inhibitors resulted in reduction of activity by 32, 46, and 24%, respectively (Table 1). This observation was investigated further by exposing GV to the inhibitor DFP for various periods of time, removing unreacted DFP, and assaying for protease activity. When GV was preincubated for 1 h with 10 mM DFP, the majority of the protease in the virus preparation was still active. After exposure to DFP for 4 to 8 hours, GV contained 32 to 35% of its original protease activity. After 24 h of preincubation with 10 mM DFP, the enzyme activity was minimal. A similar, although less rapid, reduction in protease activity was obtained when GV was preincubated with 1 mM DFP. Whereas 75% of the activity remained after 8 h of exposure to DFP, 24 h of preincubation with the inhibitor resulted in 27% activity. These results were in marked contrast to the complete loss of activity observed when DFP (1 mM) was added directly to the assay mixture.

Localization of protease in GV. The above results suggested that the protease was an integral component of the occluded GV, and experiments were conducted to further localize the enzyme activity in the virus. The GV was separated into granulin and enveloped nucleocapsid fractions by carbonate dissolution and velocity sedimentation in glycerol gradients. The granulin was confirmed to be free of envelope contamination by SDS-polyacrylamide gel electrophoresis (results not shown). The protease activity in each fraction was compared to that of intact GV, and the results are shown in Table 2. The majority of the protease activity was recovered in the granulin fraction; only 1.3% of the activity was found to be associated with the enveloped nucleocapsids.

Purification of GV protease. The protease was isolated from the granulin fraction, which was separated from enveloped nucleocapsids by velocity sedimentation in glycerol gradients. The protease activity eluted from a DEAE-Sepharose column in a single peak, which was separated from the bulk of granulin and its degradation products (Fig. 6). The protease eluted at a NaCl concentration of approximately 0.1 M, whereas 0.35 M NaCl provided for elution of the granulin. SDS-polyacrylamide gel electrophoresis of the pooled protease fractions (no. 50 through 110) revealed a major band of protein having a molecular weight of 14,000 and a few minor bands consisting of granulin and degradation products (Fig. 8B). These contaminants were removed from the protease by chromatography on Sephadex G-100. When fractions constituting the major peak of protease activity were pooled (fractions 66 through 73, Fig. 7) and

TABLE 2. *Localization of alkaline protease in GV*

Viral component[a]	Protein (mg)	Protease activity (acid-soluble cpm)	Sp act (cpm/mg protein)	Total protease activity (%)
Occluded (intact) virus	11.00	1.21×10^6	1.10×10^5	100.0
Granulin	9.45	1.06×10^6	1.12×10^5	88.0
Enveloped nucleocapsid	0.17	1.60×10^4	9.30×10^4	1.3

[a] Purified GV was incubated for 10 min at 37°C in 0.05 M Na_2CO_3–0.05 M NaCl (pH 10.6). The dissociated virus was layered on a 30 to 70% glycerol gradient, which was centrifuged at 25,000 rpm (SW41 rotor) for 30 min. Granulin and enveloped nucleocapsids were recovered from the gradient, and the protease activity was determined as described in the legend to Fig. 3.

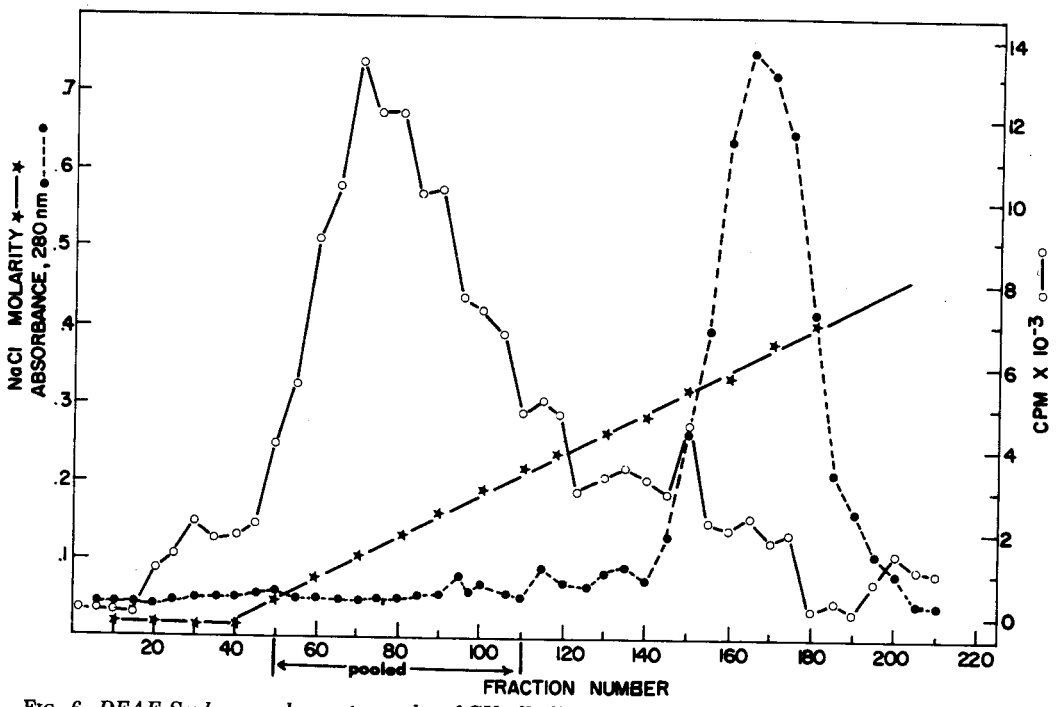

FIG. 6. *DEAE-Sepharose chromatography of GV alkaline protease. Granulin preparation was applied to the column (2 by 30 cm) and was eluted with a linear salt gradient (0.01 to 0.5 M NaCl) in 0.01 M Tris (pH 8.5) as indicated (★). Fractions of 3 ml were collected, protein was measured by absorbance at 280 nm (●), and protease activity (○) was determined as described in the legend to Fig. 3. Fractions 50 through 110 were pooled and used for further purification.*

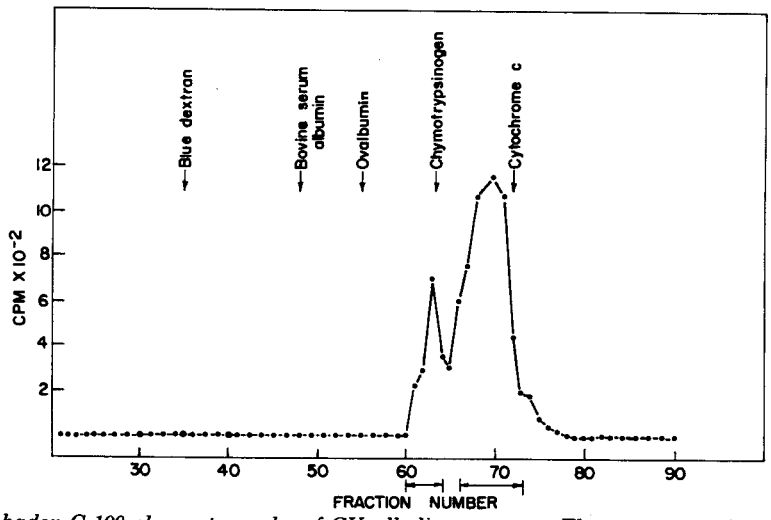

FIG. 7. *Sephadex G-100 chromatography of GV alkaline protease. The protease-active eluate from the DEAE-Sepharose column was applied to a Sephadex G-100 column (1.7 by 80 cm) and was eluted with 0.01 M Tris–0.1 M NaCl (pH 8.0). Fractions of 1 ml were collected, and protease activity (●) was determined as described in the legend to Fig. 3. For molecular weight determinations, the column was calibrated with the designated molecular weight marker proteins and the purified protease was chromatographed on it.*

subjected to electrophoresis on SDS-polyacrylamide gels, a single band of protein having a molecular weight of 14,000 was observed (Fig. 8C and E). A minor peak of protease activity (fractions 60 through 64, Fig. 7) was consistently observed when the protease-active eluate from

DEAE-Sepharose was chromatographed on Sephadex G-100. Although the presence of additional proteases in GV cannot be ruled out, SDS-polyacrylamide gel electrophoresis indicated that the minor peak of activity most probably consisted of substrate-bound protease. In addition to a major 14,000-dalton band of protein, minor bands having molecular weights of 28,000 and 10,000 to 13,000 were observed and probably represent granulin and degradation products (data not shown). The isolation procedure resulted in an approximately 50-fold purification of the protease and recovery of 8% of the enzyme activity originally present in the granulin preparation (Table 3).

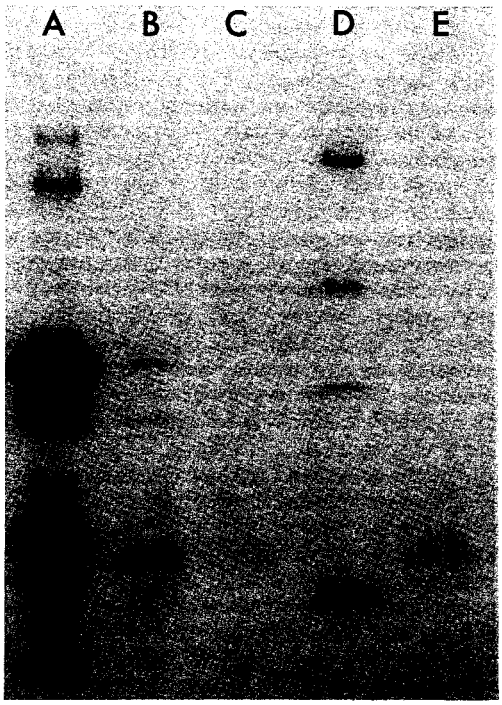

FIG. 8. *SDS-polyacrylamide gel electrophoresis of GV alkaline protease preparations. (A) Crude preparation of matrix protein or granulin. (B) Pooled, protease-active fractions from DEAE-Sepharose column. (C and E) Protease-active eluate (fractions 66 through 73) from Sephadex G-100 column, 10 and 50 μg, respectively. (D) Molecular weight markers bovine serum albumin, ovalbumin, chymotrypsinogen, and cytochrome c with respective molecular weights of 68,500, 43,000, 27,500, and 11,700.*

Properties of the isolated protease. The effect of pH on the activity of the isolated protease was similar to that observed for the GV-associated enzyme (Fig. 4). The pH optimum was 10.5, and significant activity was observed only between pH 9.5 and 11.5. The isolated protease was inhibited most effectively by DFP (1 mM, 92.5% inhibition). PMSF (25 mM) and TPCK (5 mM) were also inhibitory to the enzyme, causing 65 and 43% reductions, respectively, in proteolytic activity. Like the GV-associated protease, the purified enzyme was not affected by iodoacetate or EDTA. The molecular weight of 14,000 determined in gel filtration studies on the isolated protease (Fig. 7) corresponded to that estimated by SDS-polyacrylamide gel electrophoresis.

DISCUSSION

The results clearly demonstrate the association of alkaline protease with the GV of *P. interpunctella*. The protease was activated by the alkalinity of the carbonate system, which was used to selectively solubilize the protein matrix of the GV. Most evident was the hydrolysis of the granulin, the major constituent of the protein matrix, by the protease. In the absence of proteolytic activity, the granulin consisted of a protein having a molecular weight of 28,000. Granulin solubilized from GV containing protease activity was extensively degraded to a number of polypeptides ranging in molecular weight from 10,000 to 27,000 (Fig. 1).

The GV-associated proteolytic activity was readily detected by measuring the release of acid-soluble peptides from radioactively labeled, heat-inactivated GV. The [^{14}C]GV assay was rapid (Fig. 3A) and sensitive (Fig. 3B) and provided a method for characterizing the protease using GV proteins as substrate. The SDS-polyacrylamide gel profiles of carbonate-solubilized GV (Fig. 1A through D) demonstrated that the proteolysis of granulin was predominantly to degradation products having approximate molecular weights of 27,000, 25,000, 23,000, 16,000, and 14,500. These discrete protein bands probably resulted from cleavage of 1,000- to 10,000-

TABLE 3. *Purification of an alkaline protease from the GV of P. interpunctella*

Purification step	Total protein (mg)	Total enzyme (units)[a]	Sp act (units/mg of protein)	Purification (fold)	Yield (%)
Crude (granulin) preparation	290.00	2,620	9.03	1.0	100.0
DEAE-Sepharose eluate	16.20	1,377	85.00	9.4	52.5
Sephadex G-100 eluate	0.51	223	440.00	48.7	8.5

[a] One enzyme unit is equivalent to the release of 1,000 cpm of acid-soluble material from ^{14}C-labeled GV substrate. Conditions for protease assay were as described in the legend to Fig. 3.

molecular-weight polypeptides from the granulin. The ability of the [^{14}C]GV assay to detect the protease activity indicates that these low-molecular-weight cleavage products were further degraded to acid-soluble peptides.

Although alkaline proteases have been found in other baculoviruses (2, 3, 8, 12, 16), only partial purification of the protein having the protease activity has been achieved (2). In the present report, it was demonstrated that chromatography on DEAE-Sepharose readily provided for separation of the protease from the protein (granulin) composing the crystalline matrix of the virus. After further purification by gel permeation chromatography, the protease was observed to consist of an electrophoretically homogeneous protein having a molecular weight of 14,000 (Fig. 8E). The pH activity profile indicated that the protease was an alkaline protease (Fig. 4); the pH optimum of 10.5 was observed regardless of the buffer or substrate used, and enzyme activity was restricted to the narrow range of pH 9.5 to 11.5. The GV protease appeared to be a serine protease because it was strongly inhibited by DFP, PMSF, and TPCK (Table 1). Similar inhibition has been observed for the proteases associated with the NPVs of *T. ni* (16) and *P. dispar* (12). The enzyme was not affected by inhibitors of sulfhydryl proteases and did not require metal ions for its activity (Table 1). Although the data indicate that the proteolytic activity in GV is due to a single species of protein, the possibility that there are additional proteases associated with the virus cannot be ruled out. The assay used for detection of protease activity measured the release of acid-soluble peptides from the GV substrate. Proteases which, by endolytic cleavages, hydrolyzed granulin only to large, acid-insoluble polypeptides would not have been detected by this assay. Further experimentation is needed to determine whether such protease activity is associated with the isolated protease or with GV.

The majority of the alkaline protease was found to be associated with the crystalline matrix of the virus. Although the enveloped nucleocapsids may contain an inherent protease, the small amount of proteolytic activity detected in these preparations (Table 2) was probably due to incomplete removal of the matrix protein from them. Electrophoresis on SDS-polyacrylamide gels (results not shown) verified this, demonstrating that the enveloped nucleocapsids were contaminated, to a small extent, with matrix protein.

The inability of DFP to completely inhibit the protease in GV unless it was preincubated with the occluded virus for prolonged periods of time possibly provides evidence that the alkaline protease is an integral component of the protein matrix. If the protease was embedded in the matrix of the GV, it would not be readily available for binding by the inhibitor. It is speculated that brief exposure of intact GV to DFP resulted in inhibition of protease molecules located on or near the surface of the protein matrix. More extensive exposure of the occluded virus to DFP may have resulted in greater penetration of the matrix by the inhibitor, with subsequent inhibition of internally located protease. The complete protease inhibition observed when DFP was directly added to the reaction mixture would be expected. Under these conditions, the protein matrix was solubilized and internal protease molecules were immediately exposed to the DFP. These results may indicate that the protease is not a surface contaminant but an integral part of the protein matrix. The distribution of the protease within the matrix and whether the enzyme is host contributed or virus specified remain to be determined.

Although the most evident activity of the protease was the hydrolysis of granulin, the specificity of the protease may not be limited to this viral protein. Other viral proteins may be cleaved (Fig. 1). Gradual degradation of GV proteins a, b, c, d, and e was observed during prolonged incubation in the alkaline carbonate. Preliminary experiments (results not shown) indicate that some of these proteins are constituents of the enveloped nucleocapsids. The activity of the GV alkaline protease must, as a result, be considered with respect to these viral components as well as with respect to the granulin. In particular, the influence of the protease on the integrity of the enveloped nucleocapsids isolated by use of alkaline carbonate must be investigated. Protease activity directed against protein constituents of the enveloped nucleocapsids could substantially affect their antigenicity and the determination of their polypeptide composition. Now that the protease has been isolated (Table 3, Fig. 8), these possibilities can be critically examined.

The biological significance of the alkaline protease has not been determined. The common occurrence of this enzyme in occluded insect viruses (3, 8, 12, 16) and the preliminary observation that the protease is an integral component of the protein matrix suggest that the association of the protease with the virus is not fortuitous. In order for GV infection to proceed after ingestion of the virus by an insect, the enveloped nucleocapsid must be freed from the protein matrix. This occurs in the insect midgut, where alkaline conditions effect the disruption of the matrix (4), which results in the release of the enveloped nucleocapsid (14, 15). Preliminary

experiments in our laboratory indicate that the rate of solubilization of the protein matrix from GV is decreased if the virus-associated protease activity has been inactivated. It is possible that the enzyme, activated by the insect gut alkalinity, functions in the initiation of GV infection by aiding in the removal of the protein matrix from the occluded virus. Further in vitro and in vivo characterization of the activity of the purified alkaline protease should provide insight into the possible biological activity of this enzyme and its role in the infection process of GV.

ACKNOWLEDGMENTS

K.A.T. is recipient of a research assistantship from the U.S. Grain Marketing Research Laboratory, U.S. Department of Agriculture, Agricultural Research Service.

The excellent technical assistance of Kimberly Osborne and Diane Potts is appreciated.

LITERATURE CITED

1. **Andrews, P.** 1964. Estimation of the molecular weights of proteins by Sephadex gel filtration. Biochem. J. **91**:222–233.
2. **Eppstein, D. A., and J. A. Thoma.** 1975. Alkaline protease associated with the matrix protein of a virus infecting the cabbage looper. Biochem. Biophys. Res. Commun. **62**:478–484.
3. **Eppstein, D. A., J. A. Thoma, H. A. Scott, and S. Y. Young III.** 1975. Degradation of matrix protein from a nuclear polyhedrosis virus of *Trichoplusia ni* by an endogenous protease. Virology **67**:591–594.
4. **Faust, R. M., and J. R. Adams.** 1966. The silicon content of nuclear and cytoplasmic viral inclusion bodies causing polyhedrosis in lepidoptera. J. Invertebr. Pathol. **8**:526–530.
5. **Frugoni, J. A.** 1957. A universal buffer for biochemical studies. Gazz. Chim. Ital. **87**:403–407.
6. **Harrap, K. A.** 1972. The structure of nuclear polyhedrosis viruses. I. The inclusion body. Virology **50**:114–123.
7. **Harrap, K. A., C. C. Payne, and J. S. Robertson.** 1977. The properties of three baculoviruses from closely related hosts. Virology **79**:14–31.
8. **Kozlov, E. A., N. M. Sidorova, and S. B. Serebryani.** 1975. Proteolytic cleavage of polyhedral protein during dissolution of inclusion bodies of the nuclear polyhedrosis viruses of *Bombyx mori* and *Galleria mellonella* under alkaline conditions. J. Invertebr. Pathol. **25**:97–101.
9. **Kunitz, M.** 1947. Crystalline soybean trypsin inhibitor. II. General properties. J. Gen. Physiol. **30**:291–310.
10. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
11. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
12. **McCarthy, W. J., and S. Liu.** 1976. Electrophoretic and serological characterization of *Porthetria dispar* polyhedron protein. J. Invertebr. Pathol. **28**:57–65.
13. **McMillen, J., and R. A. Consigli.** 1974. In vitro radioisotopic labeling of proteins associated with purified polyoma virus. J. Virol. **14**:1627–1629.
14. **Summers, M. D.** 1969. Apparent in vivo pathway of granulosis virus invasion and infection. J. Virol. **4**:188–190.
15. **Summers, M. D.** 1971. Electron microscopic observations on granulosis virus entry, uncoating and replication processes during infection of the midgut cells of *Trichoplusia ni*. J. Ultrastruct. Res. **35**:606–625.
16. **Summers, M. D.** 1975. Biophysical and biochemical properties of baculoviruses, p. 17–29. *In* M. Summers, R. Engler, L. A. Falcon, and P. V. Vail (ed.), Baculoviruses for insect pest control: safety considerations. American Society for Microbiology, Washington, D.C.
17. **Summers, M. D., and K. Egawa.** 1973. Physical and chemical properties of *Trichoplusia ni* granulosis virus granulin. J. Virol. **12**:1092–1103.
18. **Summers, M. D., and J. D. Paschke.** 1970. Alkali-liberated granulosis virus of *Trichoplusia ni*. I. Density gradient purification of virus components and some of their *in vitro* chemical and physical properties. J. Invertebr. Pathol. **16**:227–240.
19. **Summers, M. D., and G. E. Smith.** 1975. *Trichoplusia ni* granulosis virus granulin: a phenol-soluble, phosphorylated protein. J. Virol. **16**:1108–1116.
20. **Tweeten, K. A., L. A. Bulla, Jr., and R. A. Consigli.** 1977. Isolation and purification of a granulosis virus from infected larvae of the Indian meal moth, *Plodia interpunctella*. Appl. Environ. Microbiol. **34**:320–327.
21. **Weber, K., and M. Osborn.** 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. **244**:4406–4412.
22. **Yamafugi, K., J. Makai, and F. Yoshihara.** 1959. Deoxyribonuclease and protease in polyhedral viral particles. Enzymologia **22**:1–12.