ALKALINE PROTEASE ASSOCIATED WITH THE MATRIX PROTEIN OF A VIRUS INFECTING THE CABBAGE LOOPER

Deborah A. Eppstein and John A. Thoma

Department of Chemistry, University of Arkansas, Fayetteville, Ark. 72701

Received December 4,1974

An alkaline protease was found to be tightly associated with the matrix protein of a nuclear-polyhedrosis virus infecting Trichoplusia ni. Partial purification of the protease was achieved by $CaCl_2$ precipitation of the matrix protein. The protease had a pH optimum of 9.5 using casein as substrate and behaved as a serine protease. It hydrolyzed N-benzoyl-L-tyrosine ethyl ester, and it was inhibited by Hg^{2+} and Cu^{2+}. Most of the protease was separated from the matrix protein by polyacrylamide gel electrophoresis at pH 9.5.

INTRODUCTION

The potential use of nuclear-polyhedrosis viruses (NPV)[1] of the Baculovirus genus as entomopathogenic biocontrol agents for Lepidopterous insecta has elicited a great deal of interest. As part of a program dealing with the safety and identification of these potential biocontrol agents, we are characterizing the chemical and serological nature of a matrix protein of an NPV which attacks the cabbage looper, Trichoplusia ni.

The characterization of the viral matrix protein has been greatly complicated by the presence of an endogenous protease. The cabbage looper nuclear-polyhedrosis virus matrix protein (NPV MP) preparation isolated by standard procedures (1,2) is a mixture of peptides resulting from proteolytic degradation. The endogenous protease, which hydrolyzes the intact NPV MP subunit, behaves as a serine protease and is effectively inhibited by some heavy metals. Nondegraded NPV MP can be isolated if the protease is inhibited.

There have been conflicting reports about the presence of proteases in virus matrix protein preparations (3-5). Summers (6) recently found proteases

[1]Abbreviations used are: BTEE, N-benzoyl-L-tyrosine ethyl ester; DTT, dithiothreitol; EDTA, disodium ethylenediaminetetracetate; $K_{m,app}$, apparent Michaelis constant; NPV, nuclear-polyhedrosis virus; NPV MP, nuclear-polyhedrosis virus matrix protein; TCA, trichloroacetic acid; Tris, tris(hydroxymethyl)aminomethane.

in several insect viral matrix protein preparations, and we have found a protease associated with T. ni NPV MP. The presence of these proteases leads us to suspect that the peptide mixtures isolated by other workers (7-9) from insect viral matrix proteins may have been formed by proteolytic degradation of the matrix protein(s). This report discusses some properties of the alkaline protease associated with T. ni NPV MP, a method for its inhibition, and preliminary studies on the purification of the protease from the matrix protein.

MATERIALS AND METHODS

Preparation and Isolation of T. ni NPV MP: The NPV (single embedded) was propagated in laboratory reared T. ni larvae and purified by the procedure of Scott et al. (1), excluding the ultrasonication step. The matrix protein was isolated from lyophilized NPV polyhedra by the procedure of Scott et al. (1,2).

Protease Assay: Protease activity was determined using a modification of the method of Kunitz (10). The assay was conducted in 0.2 M Na_2CO_3, pH 9.5, at 38°C by incubating 250 µl of NPV MP (4 mg/ml) with 250 µl of 1% (w/v) heat-denatured casein. After 13 minutes, 0.75 µl of 5% (w/v) TCA was rapidly added, the tube was centrifuged, the supernatant solution was filtered through a fine scintered-glass filter, and the absorbance was read at 280 nm. A blank, containing NPV MP but no casein, was run to correct for proteolysis of the NPV MP.

The pH profile of the protease was measured by assaying activity at pH 7.5, 8.3 (in 0.1 M Tris-HCl), and at pH 8.7, 8.9, 9.5, 10.2, 10.7, and 11.0 (in 0.2 M $NaHCO_3$-Na_2CO_3).

Protease Inhibition: The effects of potential inhibitors on the protease were determined at pH 9.5. A series of assay mixtures prepared as described above contained respectively the following reagents: 1.0×10^{-2} M EDTA, 3.0×10^{-3} M cysteine-HCl, 1.0×10^{-2} M DTT, 5.0×10^{-3} M $HgCl_2$, 4.0×10^{-4} M $HgCl_2$, 4.0×10^{-3} M copper(II) acetate, 0.02% lima bean trypsin inhibitor (type II-L, Sigma). The protease was also assayed in 1.0 M formic acid, pH 1.9. To measure recovery of activity after removal of the Hg^{2+} inhibitor, a protease

solution (4 mg NPV MP/ml) containing 5.0 x 10^{-3} M Hg^{2+} was dialyzed against two 1 ℓ changes of 0.2 M Na_2CO_3, 0.01 M DTT, pH 9.5, or 0.2 M Na_2CO_3, 0.01 M EDTA, pH 9.5, for 24 hours at 4°C.

Esterase Assay: Esterase activity was determined, at ambient temperature (23°C), using a modification of the method of Hummel (11). A 300 µl sample containing 0.50-2.00 mM N-benzoyl-L-tyrosine ethyl ester (BTEE) (Sigma) in 50% (w/w) methanol/water was placed in a 1 cm, 1.5 ml quartz cuvette, and 300 µl of NPV MP (2mg/ml) in 0.1 M Tris-HCl, pH 8.3, was added at zero time. Two blanks were prepared; one contained 300 µl of BTEE solution + 300 µl Tris-HCl buffer and the other contained 300 µl of 50% methanol/water + 300 µl of NPV MP solution. The absorbance at 256 nm was read every 2-5 minutes for the first hour, and then at longer intervals for 19 hours. In a parallel experiment the esterase activity was determined in the presence of 0.068 M $CaCl_2$, using NPV MP stock solutions (2 mg/ml) 0.09 M in $CaCl_2$. The extinction coefficient of N-benzoyl-L-tyrosine in 25% methanol, 0.05 M Tris-HCl, pH 8.3, was determined by the method of Kumar and Hein (12), using α-chymotrypsin (bovine, type II, Sigma) to completely hydrolyze BTEE.

Apparent K_m Determination: To determine the apparent Michaelis constant for BTEE, the esterase activity was determined as described above, at substrate concentrations of 0.25, 0.375, 0.417, 0.667, and 1.0 mM BTEE. The initial velocities were calculated using an unweighted least squares fit to a plot of absorbance vs. time. The plot was linear for at least one hour at the lowest substrate concentration. The $K_{m,app}$ was calculated using a weighted least squares fit to a plot of initial velocity vs. substrate concentration (13).

Protease Assay on Polyacrylamide Gels: Polyacrylamide gel electrophoresis was performed on 6% gels at pH 9.5 by the method of Davis (14). Protein bands were located in reference gels by staining with Coomassie brilliant blue R (Sigma) (15). Proteolytic activity was determined after electrophoresis of 320 µg NPV MP. Duplicate gels were sectioned into 3 mm slices; each slice was pulverized in 200 µl of 0.2 M Na_2CO_3, pH 9.5, and assayed for proteolytic

activity, using 200 µl of 1% casein, as described above. Incubation periods ranged from 4-18 hours.

Proteolytic activity was also determined on polyacrylamide gels by including denatured, ultrasonicated hemoglobin in the gel solutions before polymerization (16). A 320 µg sample of NPV MP was electrophoresed, and the gels were incubated in 0.2 M Na_2CO_3, pH 9.5, for 16-18 hours at 38°C. After 2-4 hours incubation, the major protease bands appeared as light areas devoid of hemoglobin, and after 16-18 hours incubation the minor protease bands appeared.

Amino Acid Analysis: Amino acid analysis was performed using Durram IA

TABLE I

Effect of pH and Inhibitors on Protease Associated with T. ni NPV MP

pH	Addition or treatment	Per cent remaining activity	Per cent activity after dialyzing against pH 9.5, 0.01 M EDTA	0.01 M DDT
11.0	-	30		
10.7	-	65		
10.2	-	79		
9.5	-	100	100-110	100
8.9	-	79		
8.7	-	70		
8.3	-	22		
7.5	-	11		
9.5	0.02% lima bean trypsin inhibitor	70		
9.5	4.0×10^{-3} M Cupric acetate	10		
9.5	4.0×10^{-4} M $HgCl_2$	30		
9.5	5.0×10^{-3} M $HgCl_2$	0	50	40
1.9	1.0 M formic acid	0		

resin and single column methodology, with the modified ninhydrin assay of Rosen (18). Amino acid analyses were performed on NPV MP peptide bands separated by 6% polyacrylamide gel electrophoresis at pH 9.5 (14). The gels were stained with Coomassie blue, the peptide bands were sliced out, and the gel slices were prepared for amino acid analysis by the method of Spiro (17). Corresponding peptide bands from six or more gels were combined.

RESULTS AND DISCUSSION

Protease Activity: The effects of pH and various inhibitors on the protease associated with NPV MP are given in Table I. The protease had a pH optimum of 9.5 for casein hydrolysis. Hg^{2+}, the most effective protease inhibitor tested, gave complete inhibition at 5.0×10^{-3} M Hg^{2+}.

Esterase Activity: The protease hydrolyzed a synthetic substrate, BTEE, which is a specific substrate for chymotrypsin proteases (19). The $K_{m,app}$ of BTEE with the protease (1 mg NPV MP/ml, pH 8.3) was 1.1 ± 0.3 mM, whereas the K_m of BTEE with α-chymotrypsin is 3.9 mM (19). The apparent maximum velocity of the protease (1 mg NPV MP/ml, pH 8.3) was 0.028 mM BTEE hydrolyzed/hour. The esterase assay was performed at a pH below the optimum pH due to the increasingly high absorbance at 256 nm of BTEE as the pH was raised.

Partial Purification of the Protease: Selective salting-out of the NPV MP may be a useful method to purify the NPV MP from the protease. Over 50% of the total protein was precipitated by 0.09 M $CaCl_2$, whereas the solution retained 80% of its initial esterase activity.

Protease Assay on Polyacrylamide Gels: The protease apparently bound very tightly to the NPV MP, so tightly in fact that a small amount of the protease migrated with the main NPV MP peptide bands during polyacrylamide gel electrophoresis (Fig. 1). The likelihood that the proteolytic activity was an inherent property of the NPV MP may be discounted for two reasons: 1) most of the proteolytic activity (R_m 0.02-0.14 band) separated from the NPV MP peptide bands (Fig. 1); 2) the main protease peptide band contained serine in greatest molar quantities (ser:asx = 1.25) whereas the NPV MP peptide bands contained

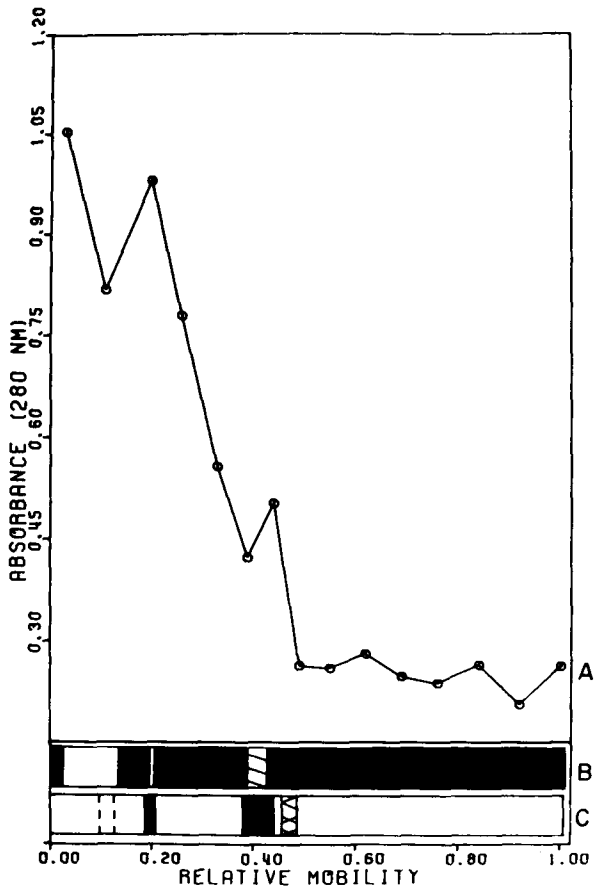

Figure 1. Localization of the protease associated with T. ni NPV MP. A 320 µg sample of NPV MP was electrophoresed on a 6% polyacrylamide gel at pH 9.5. A, 3 mm slices of the gel were assayed for proteolytic activity by incubating with casein, precipitating with TCA, then determining the absorbance at 280 nm of the hydrolyzed casein. B, gel was polymerized containing denatured hemoglobin. After electrophoresis the gel was incubated at 38°C at pH 9.5 for 18 hours. Light areas indicate presence of protease. C, gel was stained with Coomassie blue. Protein bands appear as dark areas.

---

aspartic and/or asparagine in greatest molar quantities (ser:asx = 0.48).

The inhibition of the endogenous protease in NPV MP preparations is essential for structural studies of the polyhedral protein. The similarity of the protease to chymotrypsin suggests the use of specific serine-protease inhibitors (20) instead of Hg^{2+} to inhibit proteolysis. A forthcoming article will report on the properties of the intact NPV MP.

ACKNOWLEDGMENTS

We would like to thank Dr. Howard A. Scott and Dr. Seth Y. Young III at the Virology and Biocontrol Laboratory at the University of Arkansas for the preparation and isolation of the NPV polyhedra and the protease-cleaved matrix protein. Dr. Max Summers was kind enough to discuss his unpublished work on proteases associated with viral matrix proteins with us. The University of Arkansas Computing Center made their facilities available to us. Jimmy D. Allen wrote the computer plot program for drawing polyacrylamide gels. D.A.E. was the recipient of an NDEA Title IV Research Fellowship.

REFERENCES

1. Scott, H. A., Young III, S. Y., and McMasters, J. A. (1971) J. Invert. Pathol. 18, 177-182.
2. Eppstein, D. A., Thoma, J. A., Scott, H. A., and Young III, S. Y., submitted for publication.
3. Yamafugi, K., Yoshihara, F., and Hirayama, K. (1957) Enzymologia 19, 53-58.
4. Kuzin, A. M. and Krzhevova, R. V. (1948) Biokhimiya 13, 523-529. Chem. Abstr. 43, 3051f (1949).
5. Bergold, G. H. (1963) in "Insect Pathology, an Advanced Treatise", Vol. 1, pp. 413-456, ed. Steinhaus, E. A., Academic Press, N. Y.
6. Summers, M. D. (1974) University of Texas, Austin, Tex., personal communication.
7. Summers, M. D. and Egawa, K. (1973) J. Virol. 12, 1092-1103.
8. Shigematsu, H. and Suzuki, S. (1971) J. Invert. Pathol. 17, 375-382.
9. Longworth, J. F., Robertson, J. S., and Payne, C. C. (1972) J. Invert. Pathol. 19, 42-50.
10. Arnon, R. (1970) in "Methods in Enzymology", Vol. XIX, pp. 226-244, ed. Colowick, S. P. and Kaplan, N. O. Academic Press, N. Y.
11. "Worthington Enzymes" (1972) pp. 129-131, Worthington Biochemical Corporation, Freehold, N. J.
12. Kumar, S. and Hein, G. (1969) Anal. Biochem. 30, 203-211.
13. Cleland, W. W. (1963) Nature 198, 463-465.
14. Davis, B. J. (1964) Ann. N. Y. Acad. Sci. 121, 404-427.
15. Chambach, A. (1967) Anal. Biochem. 20, 150-154.
16. Andary, T. J. and Dabich, D. (1974) Anal. Biochem. 57, 457-466.
17. Spiro, M. J. (1973) J. Biol. Chem. 248, 4446-4460.
18. Rosen, H., Berard, C., and Levenson, S. (1962) Anal. Biochem. 4, 213-221.
19. Dixon, M. and Webb, E. (1964) "Enzymes", pp. 199-258, Academic Press, N. Y.
20. Walsh, K. A. and Wilcox, P. E. (1970) in "Methods in Enzymology", vol. XIX, pp. 31-41, ed. Colowick, S. P. and Kaplan, N. O., Academic Press, N. Y.