Biochimica et Biophysica Acta, 662 (1981) 72–76
Elsevier/North-Holland Biomedical Press

BBA 69389

PURIFICATION AND PROPERTIES OF PROTEASE INHIBITORS FROM DEVELOPING EMBRYOS OF *HEMILEUCA OLIVIAE* (Ckl) *

MILOS KUČERA ** and RALPH B. TURNER ***

Department of Entomology and Plant Pathology, Agricultural Experiment Station, New Mexico State University, Box 3BE, Las Cruces, NM 88003 (U.S.A.)

(Received March 19th, 1981)

Key words: Protease inhibitor; Differentiation; Embryogenesis; (Insect)

A perchloric acid extract of eggs of *Hemileuca oliviae* inhibits bovine trypsin, kallikrein and papain, as well as the native proteolytic activity (pH 7.0) of the developing embryo. Specificity is indicated by the lack of inhibition of other proteases. The amount of inhibitory activity changes during embryological development, reaching a maximum around 23 days, when the larva is fully developed. The inhibitory activity was lost by dialysis and was destroyed by ashing (450°C, 18 h) but was unaffected by exposure to 97°C for 3 min. The presence of two protease inhibitors was detected in the perchloric acid extract. The principal component has a molecular weight of approx. 9000 and its heat sensitivity is affected by pH. At the present time the role of these inhibitors in the developing embryo is unknown. Some trypsin-like native protease activity occurs in the egg during embryogenesis and may thus be the target enzyme in vivo.

Introduction

As the significance of tissue proteases in the regulation of physiological functions has become more evident, there has been a concomitant increase of interest in naturally-occurring protease inhibitors. Such endogenous inhibitors provide an efficient, direct mechanism for preventing undesirable proteolysis, and thereby obviate the need for metabolically-expensive regulation through substrate-specificity. The precise biochemical role of such protease inhibitors is unknown in most cases. Their presence in tissues of insect egg, larvae and adult is well established [1–7] and suggests the extent and variability of their function in both development and differentiation of insects. Although the role of protease inhibitors in insect embryogenesis has not yet been demonstrated, the well-documented roles of proteases, and their inhibitors, in many other developing and differentiating systems (see review, Ref. 8) suggests that proteases and inhibitors may well play a significant role in the regulation of development and differentiation of insects.

Our earlier studies of the changes in proteolytic activity during embryogenesis of insects [9,10] stimulated our interest in a possible involvement of naturally-occurring inhibitors in such changes. This is the first report of purification of protease inhibitors from eggs of insects, although Kang and Fuchs [6] noted the presence of inhibitory activity against trypsin in the eggs of *Drosophila*. This paper describes the purification of the perchloric acid-soluble protease inhibitors found to occur during embryogenesis of *Hemileuca oliviae* (*Ckl.*) (Lepidoptera, Saturniidae). Some characteristics of the principal inhibitor are reported.

---

* Journal Article 834, Agricultural Experiment Station, New Mexico State University, Las Cruces, NM 88003, U.S.A.

** Present address: Department of Insect Pathology, The Institute of Entomology CSAV, Flemingovo n. 2, 166 09 Prague 6, Czechoslovakia.

*** To whom correspondence should be addressed.

Materials and Methods

All chemicals used were of reagent grade or better and were purchased from the following sources: trypsin, elastase, chymotrypsin, pepsin, papain, cathepsin, kallikrein, ficin, bromelain, collagenase, glucagon, α-*N*-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) from Sigma Chemical Co.; CNBr-Sepharose, from Pharmacia; azocoll, from Calbiochem-Behring Corp.; Bio-Gel P-30 from BioRad Laboratories; and insulin was a gift from Eli Lilly.

Protein concentrations were estimated by the method of Warburg and Christian [11]. Inhibition of proteolytic activity of commercial proteases and of the endogenous egg protease was assayed with azocoll and at appropriate pH optima, 7.0, for the native protease.

Inhibition of the hydrolysis of BAPNA by trypsin was used to follow purification of the inhibitors [12]. One unit (U) of inhibitory activity is defined as that amount of inhibitor which inhibits one unit of trypsin activity, i.e., ΔA_{405} of 3.32 per min at 25°C in 3 ml assay mixture. The inhibition of a wide spectrum of commercially-available proteases, and of the native egg proteolytic activity, was evaluated using azocoll as substrate. The azocollysis assay routinely contained 10 mg azocoll in 1.5 ml of appropriate buffer and 0.5 ml enzyme-inhibitor mix (0.1 ml enzyme preparation, having activity with azocoll of 0.5 ΔA_{520} per 20 h plus 1.2 ml neutralized perchloric acid extract, preincubated 5 min at 22°C); 2 drops of toluene were added and the mixture was incubated 20 h at 35°C. After incubation the assay mixtures were filtered and absorbancy of the filtrate was measured at 520 nm.

Trypsin-Sepharose 4B for affinity chromatography was prepared according to the method described by Pharmacia [13].

Eggs of *Hemileuca oliviae*, collected from rangeland near Capitan, NM, U.S.A. were crushed in 5% perchloric acid (1 g: 3.5 ml) in an ice-cold mortar; the perchloric acid extract was heated to 60°C for 3 min to destroy any trypsinogen, and then clarified by centrifugation for 10 min at 5000 × *g*. The extract was then adjusted to pH 6 with 5 M K_2CO_3 and centrifuged to separate the perchlorate. The supernate was concentrated in vacuo, loaded onto a column of Sephadex G-50 (1.5 × 6.0 cm), and the flow rate adjusted to 0.5 ml/min while fractions (0.5 ml) were collected.

Results and Discussion

Variation in the amount of total inhibitory activity occurs during embryogenesis, as shown in Fig. 1. Developmental stages indicated are based on the studies of Matolín, S. (personal communication).

Chromatography of the perchloric acid extract on Sephadex G-25 indicated the molecular weight of the inhibitory activity to be >5000 since all activity emerged in the void volume. It is of some interest that a distinctive yellow-colored material emerged near the end of the fractionation volume of Sephadex G-50, after the emergence of inhibitory activity. Emergence of both the inhibitor and yellow material coincided with the emergence of material which absorbs at 280 nm (Fig. 2).

The protease inhibitory activity in the effluent from the Sephadex G-50 column was fractionated into two protease inhibitors by affinity chromatography on trypsin-Sepharose 4B (Fig. 3). The principal inhibitor was eluted with 1.0 M NaCl, while another inhibitor was eluted with 0.1 M sodium-acetate at pH 4.5. The first peak of inhibitory activity represents excess load of inhibitor which failed to bind the trypsin-Sepharose 4B.

The inhibitory activity is extremely heat-stable. Holding the perchloric acid extract at 97°C for 3 min

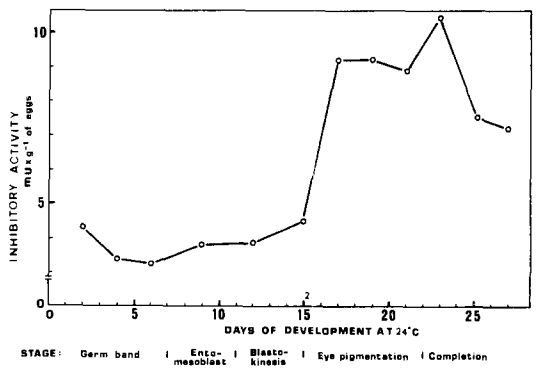

Fig. 1. Fluctuation in trypsin-inhibiting activity of perchloric acid extract during embryogenesis of *Hemileuca oliviae*. Eggs were crushed and homogenized in 5% perchloric acid, then the extract was clarified by centrifugation (12 000 × *g* for 10 min) and the supernate was assayed for inhibition of trypsin.

TABLE I

PURIFICATION OF PROTEASE INHIBITORY ACTIVITY IN RANGE CATERPILLAR, *HEMILEUCA OLIVIAE* (*CkI*), EGGS

Step of purification	Vol. (ml)	Protein		Inhibitory activity			Purification			
		mg · ml^{-1}	Total (mg)	mU · ml^{-1}	mU · mg^{-1} protein	Total (mU)	Fold		Yield (%)	
							From last step	Total	From last step	Total
1 Perchloric acid extract	205	11.4	2 335	5.1	0.45	1 050	–	–	100	100
2 Concentration (10x)	20	96.4	1 927	47.5	0.49	950	1.09	1.09	90.4	90.4
3 Gel filtration (G-50)	141	2.2	307	4.7	2.17	667	4.43	4.83	70.2	63.5
4 Concentration (14.1x)	10	25.7	257	58.9	2.29	589	1.06	5.12	88.3	56.1
5 Affinity chromatography										
Inhibitor 1	8	0.20	1.60	43.0	214.3	344	93.6	476	58.6	32.8
Inhibitor 2	8	0.079	0.63	11.0	139.2	88	60.8	309	14.9	8.4

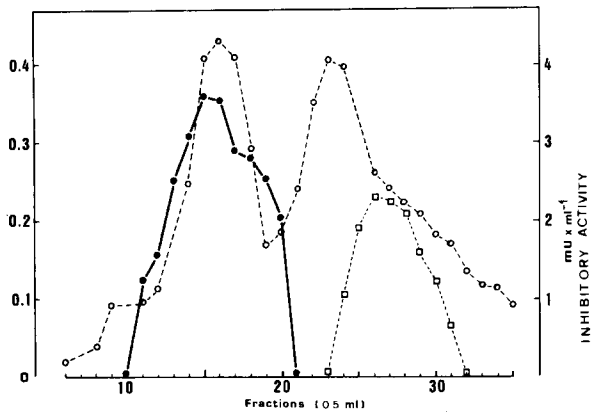

Fig. 2. Gel permeation chromatography of perchloric acid extract on Sephadex G-50. The column (2 × 30 cm) was equilibrated in Tris-HCl 0.1 M pH 7.8, and loaded with the clarified concentrated perchloric acid extract, then washed with the equilibrating buffer. Fractions (0.5 ml) were collected at a flow rate of 6 ml/h at 24°C. □ - - - - - - □, yellow band, ○———○, absorbance at 280 nm; ●———●, inhibitory activity.

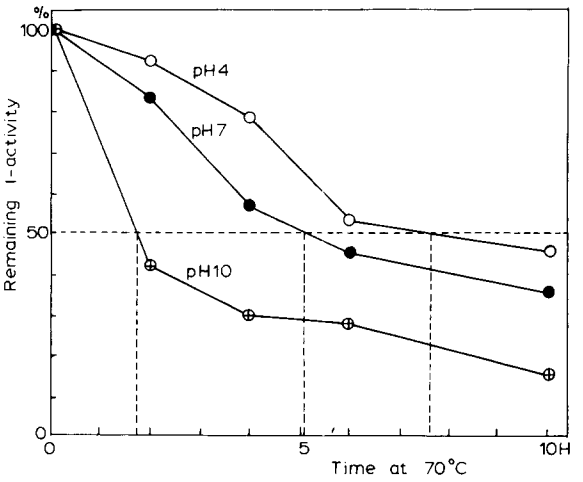

Fig. 4. Effect of pH on stability of inhibitor I-1 at 75°C. 1 ml inhibitor I-1 was mixed with 1 ml of appropriate buffer at each pH, and at each time interval 0.5 ml was removed and assayed. ○———○, pH 4; ●———●, pH 7; ⊕———⊕, pH 10.00.

did not affect the inhibitory activity. An effect of pH on the heat sensitivity of the purified inhibitor is shown in Fig. 4. At 70°C, 50% of the activity was destroyed more rapidly at pH 10 (1.75 h) than at pH 7 (5.1 h) and less rapidly at pH 4 (7.4 h). The

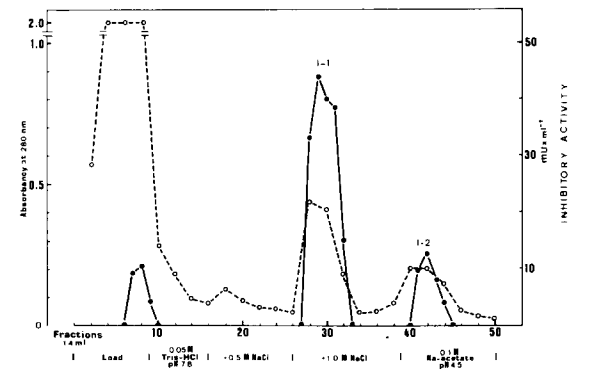

Fig. 3. Affinity chromatography of the effluent from Sephadex G-50 column on trypsin-Sepharose 4B. The column (1.5 × 2.5 cm) was equilibrated with Tris-HCl 0.05 M, pH 7.8, and loaded with 10 ml of effluent (25.7 mg protein/ml) then washed with the starting buffer (10 ml). At fraction 13 elution was begun with the starting buffer +0.5 M NaCl, and at fraction number 23, with the starting buffer +1 M NaCl, then at fraction number 39, with 0.1 M sodium-acetate pH 4.5. Fractions (1.4 ml) were collected at a flow rate of 6.5 ml/h. I-1, inhibitor 1; I-2, inhibitor 2. ○ – – – ○, absorbance at 280 nm; ●———●, mU · ml^{-1}.

relative stability at pH 4 is consistent with the findings of Kang and Fuchs [7] for a protease inhibitor from *D. melanogaster*, i.e., 50% loss of activity at pH 3.6, at 70°C in 5.7 h.

The molecular weight of the protease inhibitor was estimated to be 9000 based on chromatography on Bio-Gel P-30 using insulin and glucagon as standards.

Table I summarizes the results of the purification method.

Due to the preliminary nature of this study we are unable to establish a precise physiological role of these protease inhibitors, but the concurrent finding of trypsin-like protease activity during our other studies of proteolytic activity during embryogenesis suggests such an enzyme as a likely candidate as the target protease in vivo of these inhibitors.

Recent studies have noted that the interference with complex biological phenomena, e.g., tumorigenesis, by synthetic protease inhibitors (TLCK = *N*-α-*p*-tosyl-L-lysine chloromethyl ketone) may be more correctly attributed to inhibition of protein kinase activity [14]. If such a role is of significance for synthetic exogenous inhibitors then it seems logical to investigate such an effect by endogenous inhibitors in a similarly complex biological phenomenon such as embryogenesis.

Our future studies are planned to investigate both of these possibilities.

References

1 Greenberg, B. and Paretsky, D. (1955) Ann. Ent. Soc. Am. 78, 46–50
2 Kang, S.H. (1972) Ph.D. Thesis. University of Notre Dame
3 Yang, Y.J. and Davies, D.M. (1972) Comp. Biochem. Physiol. 43, 137–141
4 Kikawa, H. (1962) Drosoph. Inf. Serv. 36, 83
5 Kang, S.H. and Fuchs, M.S. (1974) Insect Biochem. 4, 1–8
6 Kang, S.H. and Fuchs, M.S. (1973) Comp. Biochem. Physiol. 46B, 367–374
7 Kang, S.H. and Fuchs, M.S. (1980) Biochim. Biophys. Acta 611, 379–383
8 Holzer, H. and Heinrich, P.C. (1980) Ann. Rev. Biochem. 49, 63–91
9 Kučera, M. and Turner, R.B. (1972) Nature 240, 287–288
10 Kučera, M. and Turner, R.B. (1973) Comp. Biochem. Physiol. 44(2B), 577–585
11 Warburg, O. and Christian, W. (1957) Methods Enzymol. 3, 451–454
12 Fritz, H., Trautschold, I. and Werle, E. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H.U., ed.), Vol. 2, p. 1064, Academic Press, New York
13 Pharmacia Fine Chemicals AB. (1979) Affinity Chromatography, Principles and Methods
14 Kinzel, V. and König, N. (1980) Biochem. Biophys. Res. Commun. 93, 349–353