ISOLATION AND PURIFICATION OF PROTEINASE INHIBITORS FROM DEVELOPING EMBRYOS OF *Hyalomma dromedarii**

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The changes in the level of trypsin, chymotrypsin, subtilisin and papain inhibitors were examined during embryonic development of *H. dromedarii.* The high level of trypsin and chymotrypsin inhibitors in the oviposited eggs did not change during embryogenesis and was followed by a sharp drop after hatching. On the other hand, the papain inhibitor exhibited very low level throughout embryonic development and was followed by a significant increase in the newly hatched larvae. A purification scheme for trypsin and chymotrypsin inhibitors which involved chromatography on DEAE-cellulose, cellulose phosphate and Ultrogel AcA 54 was established. Using synthetic substrates six multiple forms of proteinase inhibitors with different molecular weights and properties were isolated. They were classified into three specific trypsin inhibitors F_{1a}, F_{4a}, and F_{4b}, one specific chymotrypsin inhibitor F_{1b} and to mixed trypsin–chymotrypsin inhibitors F_2 and F_3. Although treatment that may cause fragmentation of the native inhibitors were avoided during purification, multiple forms of trypsin and chymotrypsin inhibitor were isolated. The significancy of such multiplicity is not understood.

Proteinase inhibitors have been extensively studied in a wide variety of avian and turtle eggs[1], however, information about egg proteinase inhibitors from other sources are still fragmentary. Four types of proteinase inhibitors have been reported from avian eggs, ovoinhibitor[2,3], ovomucoid[4-6], ovomacroglobulin[7], and cystatin[8-10]. Also turtle egg whites contain two proteinase inhibitors named, chelonianin and testudin[1,11,12].

Kang and Fuchs[13] were the first to note the presence of inhibitory activity against trypsin in *Drosophila* eggs. However, the first report on the purification and characterization of such inhibitors from *Hemileuca oliviae* eggs was presented by Kucera and Turner in 1981 (ref.[14]). During the progress of this investigation, Willadsen and McKenna[15] reported the isolation of two proteolytic enzyme inhibitors from the cattle tick *Boophilus microplus* eggs. The present papei describes the isolation and partial characterization of proteinase inhibitors from *Hyalomma dromedarii* developing embryos.

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EXPERIMENTAL

Tick Material

Engorged *Hyalomma dromedarii* females were collected from camels in the market near Cairo and held at 28°C and 85% relative humidity. Eggs were collected on the day of oviposition and kept in plastic tubes under the same conditions until the appropriate age. Eggs and newly hatched larvae (24 day) were stored at −40°C.

Bovine pancreatic trypsin (12 000 BAEE unit/mg protein), cytochrome C; azocasein; benzoylarginine *p*-nitroanilide (BAPNA); N-benzoyl L-tyrosine ethylester (BTEE) were obtained from Sigma Chemical Co., bacterial lysozyme was from DIFCO laboratories, Detroit Michigan, and bovine albumin was from BDH. Chymotrypsin (1 200 NEE unit/mg protein) was product of Fluka and Ultrogel AcA 54 of LKB. Dialysis sacks, cellulose tubing with average flat width of 25 mm (retain proteins with molecular weight greater than 12 000) was purchased from Sigma Chemical Co. All other chemicals were of the highest purity commercially available.

Assays of Enzymes and Inhibitors

Unless otherwise stated, trypsin, chymotrypsin, subtilisin and papain were routinely assayed in 1 ml reaction mixture using azocasein as substrate. The reaction mixtures were as follows: *a*) trypsin: 100 μmol Na-phosphate buffer pH 7·8 and 20 μg of trypsin; *b*) chymotrypsin: 100 μmo tris-HCl buffer pH 8·0 and 50 μg of chymotrypsin; *c*) subtilisin: 100 μmol Na-phosphate buffer pH 7·6 and 25 μg of subtilisin; *d*) papain: 100 μmol Na-phosphate buffer pH 7·6, 5 μmol β-mercaptoethanol, 2 μmol ethylenediaminetetraacetic acid (EDTA) and 0·4 mg of papain. The reaction was started by introducing 6 mg of azocasein and stopped after incubation for 15 min at 37°C by the addition of 2 ml of 8% trichloroacetic acid (TCA). The precipitate formed was removed by centrifugation at 5 000 *g* for 15 min. The supernatant was neutralized by adding 0·5 ml of 2·5 M-NaOH and measured at 428 nm (ref.[16]). Sample and control blanks were included routinely. One enzyme unit was defined as that amount of enzyme producing 1 μmol tyrosine per min at 37°C.

The amidase activity of trypsin was assayed using benzoyl arginine-*p*-nitroanilide as substrate according to the method of Erlanger et al.[17]. The standard assay reaction mixture contained in 3 ml volume: 3 μM-BAPNA (dissolved in dimethyl sulfoxide), 50 mM-tris-HCl buffer pH 8·1, 10 mM-$CaCl_2$ and 3 μg of trypsin containing 18 BAPNA units (one BAPNA unit = A_{410} of 0·001 with BAPNA as substrate at pH 7·6 per min at 25°C). Activity was measured by following the increase in absorbance at 410 nm at 25°C. The esterolytic activity of chymotrypsin was assayed by the method of Hummel[18] using N-benzoyl L-tyrosine ethyl ester hydrochloride (BTEE) as a substrate. The standard assay reaction mixture contained in 3 ml volume: 2 μg of chymotrypsin, containing 12·7 BTEE unit (one BTEE unit = A_{254} of 0·0075 per min at pH 7·6 at 25°C), 50 mM-tris-HCl pH 8, 10 mM-$CaCl_2$ and 0·3 mM-BTEE. Activity was measured by following the increase in absorbance at 254 nm at 25°C.

The inhibitory activity was assayed by meeasuring the residual acitivity of the enzyme tested after pre-incubation with known amount of test sample. Unless otherwise stated the enzyme was routinely preincubated with the inhibitor for one minute at 25°C before starting the reaction by the substrate. One inhibitory unit was defined as that amount of inhibitor which inhibited one unit of proteolytic activity and specific activity was expressed as units per mg of protein.

Molecular Weight and Protein Determination

Ultrogel AcA 54 was used for molecular weights determination of *H. dromedarii* proteinase inhibitors. Ultrogel AcA 54 column (86 × 1·5 cm i.d) in 0·05 M-Na-phosphate buffer pH 7·0 was calibrated with blue dextran (2 000 000), amylase (30 000), trypsinogen (24 500), ovalbumin (46 000), lysozyme (14 300), and cytochrome C (11 700). Protein solution (2 ml) was applied to the same column and eluted at a flow rate of 8 ml/h.

Protein was determined according to the method of Lowry et al.[19], using bovine serum albumin as a standard.

Purification of *H. dromedarii* Trypsin and Chymotrypsin Inhibitors
Unless otherwise stated all steps were performed at 4—7°C.

Preparation of crude extract: Crude extract was prepared by homogenizing 10 g of 3 day-old *H. dromedarii* eggs in 0·05 M-Na-acetate buffer pH 5·6. The homogenate was centrifuged at 5 000 *g* for 20 min and the supernatant was saved for further purification.

Chromatography on DEAE cellulose: The crude extract was dialyzed overnight against 0·05 M Na-phosphate buffer pH 7·0. The dialyzed extract was applied on the top of DEAE cellulose column (60 × 1·6 cm) previously equilibrated with 0·05 M-Na-phosphate buffer pH 7·0. Fractions exhibiting inhibitory activity against trypsin and chymotrypsin which did not bind to the column and appeared in the starting buffer were pooled, dialyzed against distilled water overnight, and lyophilyzed.

Chromatography on cellulose-phosphate: The lyophilyzed material was dissolved in 0·05 M-Na--acetate buffer pH 5·6 and placed on the top of a cellulose phosphate column (60 × 1·6 cm). The protein was eluted stepwise with NaCl ranging from 0 to 0·3M prepared in 0·05 M-Na-acetate buffer pH 5·6. Peaks with inhibitory activity towards trypsin or chymotrypsin were pooled separately and designated F_1, F_2, F_3, and F_4 according to their elution order from the column in 0·05 M-Na-acetate buffer pH 5·6, 0·05, 0·1, 0·2 M-NaCl, respectively.

Chromatography on Ultrogel AcA 54: Pooled cellulose phosphate fractions: F_1, F_2, F_3, and F_4 were dialyzed overnight against distilled water and lyophilized. The lyophilized materials were dissolved each in 2 ml of 0·05 M-Na-phosphate buffer pH 7·0 and placed on top of Ultrogel AcA 54 (acrylamide agarose) column (86 × 1·5 cm) equilibrated with 0·05 M-Na-phosphate buffer pH 7·0. The columns were developed with the same buffer at a flow rate of 8 ml/h. Fractions containing inhibitory activity toward trypsin or chymotrypsin were pooled separately.

RESULTS AND DISCUSSION

Changes in the Levels of Trypsin, Chymotrypsin, Subtilisin and Papain Inhibitors During Embryogenesis of H. dromedarii

The level of trypsin, chymotrypsin, subtilisin and papain inhibitor activity was followed during embryogenesis of *H. dromedarii* (Fig. 1). The high level of trypsin and chymotrypsin inhibitors in the oviposited eggs ($9·45 \pm 0·89$ and $2·3 \pm 0·19$ unit/mg protein) exhibited statistically insignificant ($P > 0·1$) changes during embryonic development which was followed by a highly significant decrease in the newly hatched larvae ($P > 0·01$). Similar decrease in the level of trypsin and chymotrypsin inhibitor activity after hatching have been reported in the eggs of the nematods

Ascaris lumbricoides[20], the tick *Boophilus microplus*[21] and the insect *Hemileuca oliviae*[14].

Although the presence of a papain inhibitor has been reported in chicken egg white[8,9] and the perchloric acid extract of the insect *H. oliviae*[14]; this activity of papain inhibitor was hardly detected during early stages of embryogenesis in *H. dromedarii*. Unlike trypsin, chymotrypsin, and subtilisin inhibitors, the specific activity of the papain inhibitor significantly increased by hatching (0·8 ± 0·09 unit/mg protein). In comparison the activity of subtilisin was low throughout embryonic development of *H. dromedarii*.

The ratio between trypsin, chymotrypsin, subtilisin, and papain inhibitors in the newly laid eggs and the hatched larvae was found to be 100 : 24 : 3·3 : 0·36 and 100 : 30 : 3·7 : 17·6, respectively. The ratio between the inhibitors examined with the exception of papain inhibitor did not change during embryogenesis or after hatching. However, it is not easy to conclude from these results whether this behaviour is due to the presence of one inhibitor with different active sites for the three enzymes (multiheaded) or due to presence of more than one inhibitor for each enzyme.

Purification of Trypsin and Chymotrypsin Inhibitors from the Developing Embryos of H. dromedarii

A typical example for the different purification steps of *H. dromedarii* trypsin and chymotrypsin inhibitors is given in Table I. The procedure involved chromatography

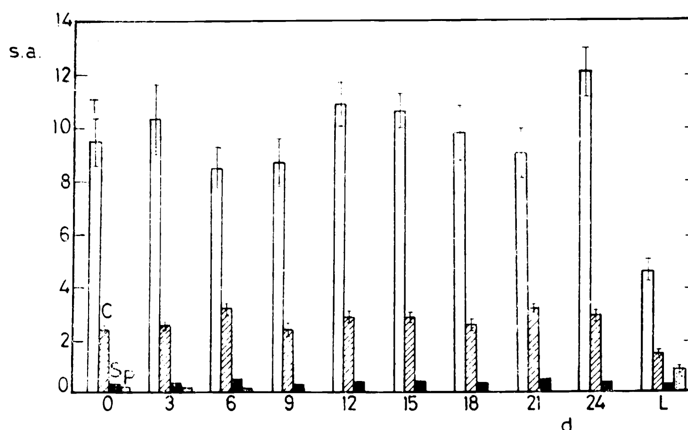

FIG. 1

Changes in the specific activity (s.a.) of trypsin (T), chymotrypsin (C), subtilisin (S) and papain (P) inhibitors during embryonic development of *H. dromedarii*, d days after oviposition, s.a. specific activity in units per mg protein

TABLE I

Purification of trypsin (T) and chymotrypsin (C) inhibitors from *H. dromedarii* eggs

Fraction	Total protein (mg)	Total units		% Activity in crude homogenate		Specific activity units/mg protein		Ratio[a] T/C
		T	C	T	C	T	C	
Crude egg homogenate	233·0	2 175	402	100	100	9·33	1·72	5·41
DEAE-cellulose eluate	123·3	1 722	345	79·2	85·8	14·00	2·80	5·00
Cellulose-phosphate eluate F_1	21·0	593	176	27·3	44·0	28·20	8·40	3·36
F_2	36·0	491	82·5	22·6	20·5	13·60	2·29	5·94
F_3	7·0	311	76·0	14·3	18·9	44·40	10·86	4·10
F_4	5·8	207	—	9·5	—	35·70	—	—
Dialyzed concd. (F_1)	13·80	468	176	21·50	44·0	33·90	12·75	2·660
Ultrogel AcA 54 eluate F_{1a}	1·28	120	—	5·52	—	93·75	—	—
F_{1b}	2·87	74	191	3·40	47·5	25·78	66·00	0·388
Dialyzed concd. (F_2)	34·00	408	67	18·08	16·70	12·00	1·97	6·100
Ultrogel AcA 54 eluate	4·60	253	73	11·60	18·16	55·00	15·87	3·470
Dialyzed concd. (F_3)	6·50	309	75·5	14·20	18·78	47·50	11·60	4·100
Ultrogel AcA 54 eluate F_{3a}	4·12	119	41·5	5·47	10·32	28·90	10·10	2·870
F_{3b}	1·77	118	24·8	5·42	6·17	66·70	14·00	4·760
Dialyzed concd. (F_4)	5·50	170	—	7·82	—	30·90	—	—
Ultrogel AcA 54 eluate F_{4a}	1·58	64	—	2·94	—	40·50	—	—
F_{4b}	1·67	30	—	1·38	—	18·00	—	—

[a] Specific activity of trypsin inhibitor divided by specific activity of the chymotrypsin inhibitor.

on DEAE cellulose, cellulose phosphate and Ultrogel AcA 54. Initial specific activities in the crude extract of the three days old eggs were 9·3 and 1·72 for trypsin (T) and chymotrypsin (C) inhibitory activities, respectively, with a T/C ratio of 5·4. Protein fractions with both trypsin and chymotrypsin inhibitory activities were not retained on DEAE cellulose and appeared in the void volume (Fig. 2) with a T/C ratio of 5·0. The specific activity of trypsin and chymotrypsin inhibitors increased 1·5 and 1·6 fold over the crude extract with 79·2 and 85·8% recovery, respectively. Chromatography of the DEAE cellulose pooled fractions on cellulose-phosphate (Fig. 3) revealed the presence of four peaks of protein (F_1, F_2, F_3, and F_4). All the fractions except F_4 exhibited inhibitory effect toward both trypsin and chymotrypsin. F_1, F_3, and F_4 but not F_2 were resolved into two fractions when chromatographed on Ultrogel AcA 54 (Fig. 4). They were classified according to their inhibitory effect toward trypsin and chymotrypsin into: *a*) specific trypsin inhibitors (F_{1a}, F_{4a}, and F_{4b}), *b*) specific chymotrypsin inhibitor (F_{1b}), *c*) double headed trypsin–chymotrypsin inhibitors (F_2 and F_3).

Properties of Isolated Inhibitors

a) *Trypsin inhibitors*: *H. dromedarii* trypsin inhibitors (F_{1b}, F_{4a}, and F_{4b}) exhibited inhibitory effect toward the amidase activity of trypsin and did not affect

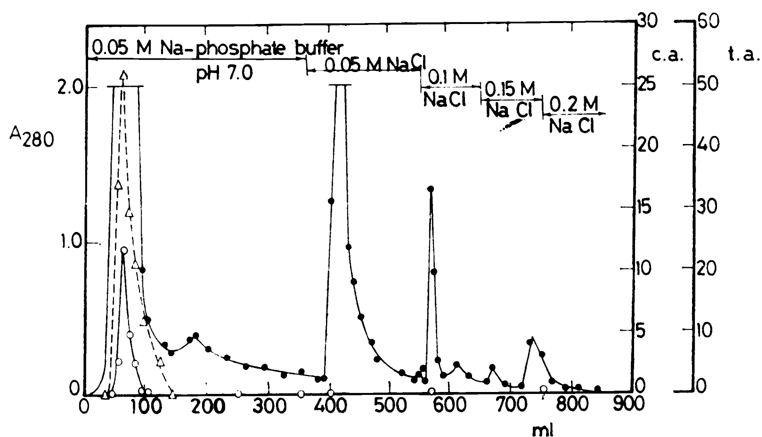

FIG. 2

Typical elution profile for the chromatography of *H. dromedarii* crude egg homogenate (3-days-old) on DEAE-cellulose column (60 × 1·6 cm i.d) previously equilibrated with 0·05 M-Na-phosphate buffer pH 7·0 at a flow rate of 60 ml/h. Absorbance at 280 nm (●——●), t.a. trypsin inhibitory activity in units per ml (△---△) and c.a. chymotrypsin inhibitory activity in units per ml (○——○)

the esterolytic activity of chymotrypsin (Fig. 5a). The apparent molecular weights of F_{1a}, F_{4a}, and F_{4b} according to their behavior on Ultrogel column were 35 000, 27 200 and 14 800, respectively.

The presence of more than one specific trypsin inhibitor (completely inactive toward α-chymotrypsin) have been reported in the perchloric acid extract of *Hemileuca oliviae*[14] and one in the larvae of *Drosophila melanogaster*[13].

b) Chymotrypsin inhibitor: Inhibitor F_{1b} (M.w. 26 300), inhibited the esterolytic activity of α-chymotrypsin and was completely inactive toward the amidase activity of trypsin (Fig. 5b). However, high concentration of this inhibitor (three times that used for complete inhibition of chymotrypsin) exhibited inhibitory effect toward the proteolytic activity of trypsin (T/C ratio = 0·38). Titration curve of F_{1b} against the esterolytic activity of α-chymotrypsin activity suggested that this inhibitor act as competitive inhibitor, which is further confirmed by Hanes plot[22].

The presence of specific chymotrypsin inhibitors with different molecular weights have been reported from different sources: 21 000, 12 000, and 9 700 for winged bean seeds[23], *Drosophila melanogaster*[24], and *Oesophagostomum radiatum*[25], respectively. Also different molecular weights were reported for different species of *Ascaris*, 15 000 for *Ascaris galli*[26] and 8 200 for *Ascaris lumbricoides var suum*[27]. A function of the chymotrypsin inhibitors is still uncertain, for example

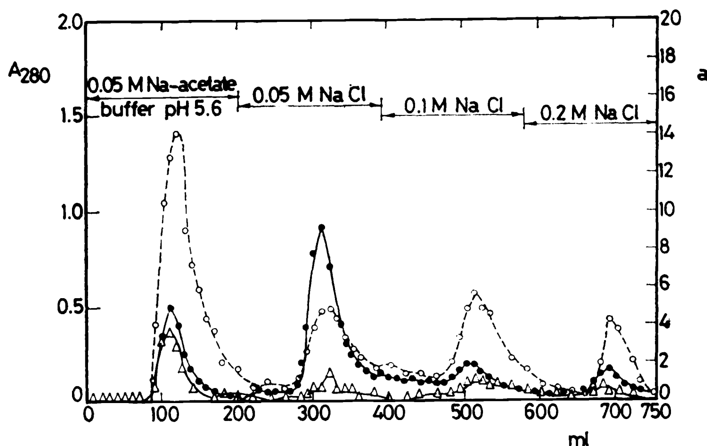

FIG. 3

Typical elution profile for the chromatography of pooled DEAE-cellulose fractions of *H. dromedarii* inhibitors on cellulose-phosphate column (30 × 1·6 cm i.d) previously equilibrated with 0·05 M-Na-acetate buffer pH 5·6 at a flow rate of 60 ml/h. Absorbance at 280 nm (●——●), a trypsin inhibitory activity (○---○) and chymotrypsin inhibitory activity (△——△), in units per ml

Drosophila larval chymotrypsin inhibitor inhibits the esterolytic and endopeptidase activities of chymotrypsin, however, no chymotrypsin activity has been detected in *Drosophila*. This inhibitor exhibited inhibitory activity toward cathepsin D from

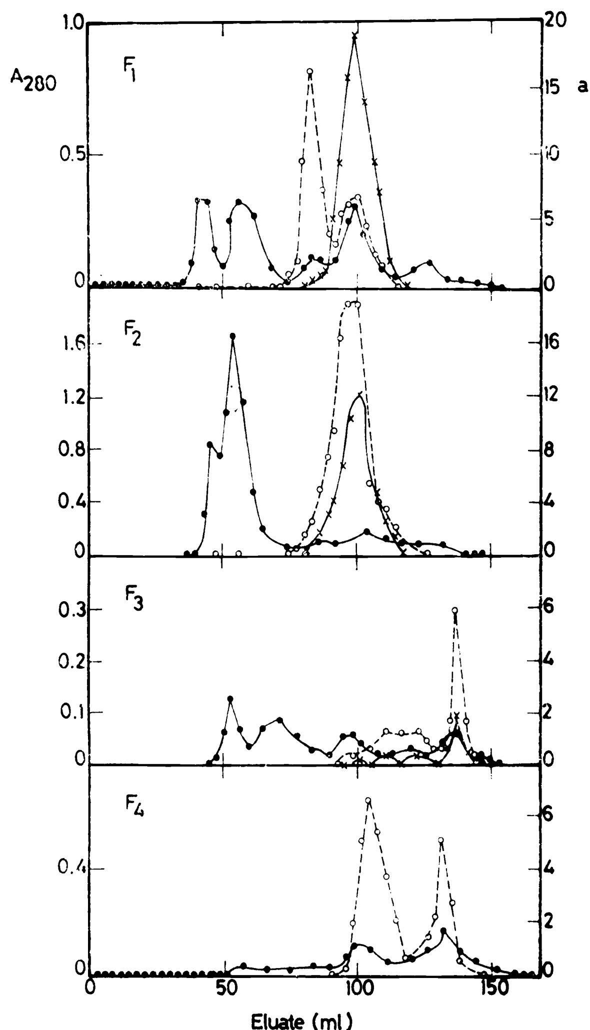

FIG. 4

Typical elution profiles for the chromatography of concentrated cellulose phosphate fractions, F_1, F_2, F_3, F_4 on Ultrogel AcA 54 column (86 × 1·5 cm i.d) previously equilibrated with 0·05 M-Na-phosphate buffer pH 7·0 at a flow rate of 8 ml/h. Absorbance at 280 nm (●—●), [a] activity of trypsin inhibitor (○---○) and activity of chymotrypsin inhibitor (×----×) in units per ml

both the parasite *Nippostrongylus brasiliensis* and rabbit liver. Since cathepsin D has been reported in *Drosophila melanogaster* as well as *Rhodnius prolixus*[28]. Kang and [24] suggested that the function of this inhibitor is associated with lysosomal mediated histolysis. One has to note that *H. dromedarii* inhibitor F_{1b} did not show any inhibitory effect when tested toward the partially purified *H. dromedarii* eggs cathepsin D (ref.[29]).

c) Trypsin–chymotrypsin inhibitors: *H. dromedarii* egg inhibitors F_2 (M.w. 28 500) and F_3 (M.w. 15 400) inhibited both the amidase and esterolytic activities of trypsin and chymotrypsin (Fig. 5c and 5d).

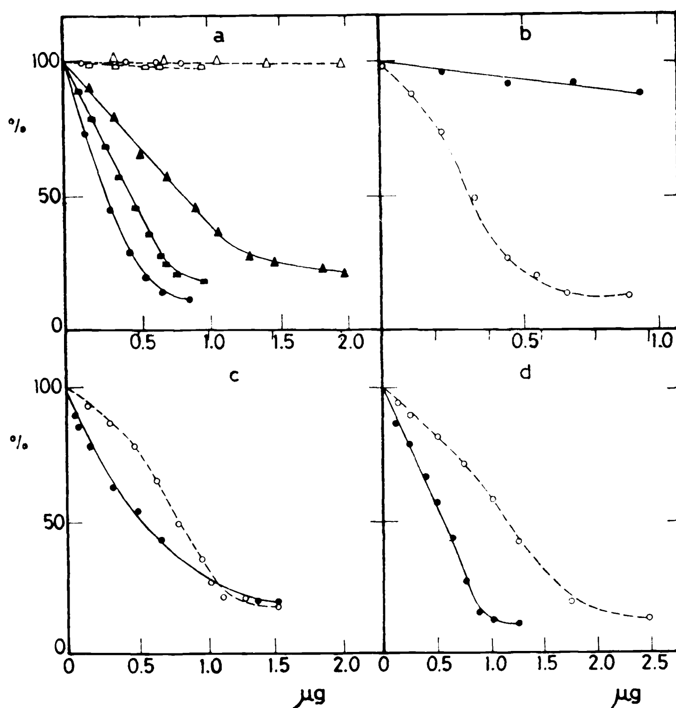

FIG. 5

Inhibition of the amidase activity of trypsin (———) and the esterolytic activity of chymotrypsin (-----) by varying amounts of *H. dromedarii* inhibitors. The residual enzyme activity (expressed in %) of trypsin and chymotrypsin was measured in 3 ml assay medium with constant BAPNA or BTEE units enzyme activity respectively, after the addition of increasing amounts of the purified inhibitor (ug): a F_{1a} (●, ○) F_{4a} (■, □), F_{4b} (▲, △); b F_{1b} (●, ○); c F_2 (●, ○); d F_3 (●, ○)

The multiplicity of proteinase inhibitors has been noted in eggs from different sources: avian[5,6,30,31], insect[14], and tick[15]. It has been demonstrated that enzymatic or acid treatment for various proteinase inhibitors not only produce low molecular weight fragments but also it changes the property of the inhibitors[32–36]. In spite of avoiding acid treatment or using the affinity chromatography technique (adsorption on trypsin or chymotrypsin sepharose) in this investigation, multiple forms of trypsin and chymotrypsin inhibitors were isolated. The significancy of such multiplicity is not understood and further investigation is needed.

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