ISSN 0006-2979, Biochemistry (Moscow), 2011, Vol. 76, No. 5, pp. 581-589. © Pleiades Publishing, Ltd., 2011.
Original Russian Text © N. V. Bulushova, E. N. Elpidina, D. P. Zhuzhikov, L. I. Lyutikova, F. Ortego, N. E. Kirillova, I. A. Zalunin, G. G. Chestukhina, 2011, published in Biokhimiya, 2011, Vol. 76, No. 5, pp. 713-723.

Complex of Digestive Proteinases of *Galleria mellonella* Caterpillars. Composition, Properties, and Limited Proteolysis of *Bacillus thuringiensis* Endotoxins

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Received December 27, 2010
Revision received January 31, 2011

Abstract—The complex of digestive proteinases in caterpillars of the greater wax moth *Galleria mellonella* was studied. Using chromogenic substrates and inhibitor analysis, it was found that serine proteinases play a key role in this complex. Three anionic and two cationic forms of trypsin and one anionic and one cationic form of chymotrypsin were identified by zymography in the midgut extract of *G. mellonella*. The most active trypsin was purified to electrophoretic homogeneity, and its N-terminal amino acid sequence was shown to be identical to that of mature trypsin from *Plodia interpunctella*. Midgut extract from *G. mellonella* was capable of processing Cry-proteins from *Bacillus thuringiensis* ssp. *galleriae*. Enzymes with tryptic and chymotryptic activities participate in this process, and activation of protoxin Cry9A is not the rate-limiting stage in the toxic action of this protein on the greater wax moth.

DOI: 10.1134/S0006297911050087

Key words: *Galleria mellonella*, insect proteinases, serine proteinases, δ-endotoxins of *Bacillus thuringiensis*, processing of Cry-toxins

Extensive studies of insect digestive proteinases are motivated by the severe impact of phytophagous insects and stored product pests on economics and by the importance of evolutionary opposition of these enzymes and proteinase inhibitors from plants for development of insects, especially from the order Lepidoptera, as well as angiosperms. Results of these studies are summarized in reviews [1].

Trypsin-, chymotrypsin-, and elastase-like serine proteinases are usually present in the midgut of lepidopteran caterpillars. They are similar to analogous mammalian proteins, but there are some differences [1]. Their activity optimum is in the strongly alkaline pH range (9.5-11.0) typical of the midgut contents of caterpillars [1].

The necessity for efficient consumption of various foodstuffs results in simultaneous synthesis of several isoforms of some proteolytic enzymes differing in substrate specificity and resistance to various plant proteinase inhibitors [2]. Peculiarities of the diet are reflected in the set of proteinase isoforms synthesized by the insect [3]. Most lepidopteran insects whose proteolytic enzymes

Abbreviations: AM, anterior midgut; Bz, benzoyl; DFP, diisopropylfluorophosphate; DNP, dinitrophenyl; DTT, dithiothreitol; E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; For, formyl; Glp, L-pyroglutamyl; PM, posterior midgut; pNA, *p*-nitroanilide; STI, Kunitz soya trypsin inhibitor; Suc, succinyl; TLCK, tosyllysine chloromethyl ketone; TPCK, tosylphenylalanine chloromethyl ketone; Z, N-benzyloxycarbonyl.

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have been studied are phytophagous. Proteases of caterpillars with other types of nutrition are less studied. In this work, we for the first time studied a complex of proteolytic enzymes in caterpillars of the greater wax moth *Galleria mellonella*, a parasite of beehives.

The digestive system of insects plays an important role in the pathological effect of δ-endotoxins of *Bacillus thuringiensis* (*Bt*) Cry- and Cyt-proteins. Various *Bt* strains and subspecies synthesize more than 100 Cry-proteins with similar molecular organization but differing in primary structure and spectrum of sensitive insects [4, 5]. Cry-proteins act efficiently against Diptera, Coleoptera, and Lepidoptera larvae and also against nematodes. They are not toxic for mammals and most invertebrates, and that is why they are constituents of many modern bioinsecticides [4]. Most Cry-proteins are protoxins that are proteolytically activated in the insect midgut [6, 7]. Sensitivity of insects to toxins is partially determined by the ability of their digestive proteinases to perform such activation efficiently [6, 7].

In this work we studied the ability of midgut proteinases of *G. mellonella* caterpillars to perform limited proteolysis of Cry-proteins synthesized by *Bt* ssp. *galleriae* efficient against this insect [8].

MATERIALS AND METHODS

Preparation of midgut extract from *G. mellonella* caterpillars. Caterpillars were reared on a mixture of wax, honey, maize flour, wheat bran, and baker's yeast [9]. Caterpillars grown to the third or fourth instar were dissected, and the midguts were cut off; in some experiments the latter was divided into anterior midgut (AM) and posterior midgut (PM). The resulting material was washed in Ringer solution and homogenized in the same solution using a Potter–Elvehjem homogenizer. The homogenate was centrifuged twice at 20,800*g* for 30 min using a 5417 R centrifuge (Eppendorf AG, Germany), and the centrifugate was stored at −80°C (1 ml of preparation contained an extract from the whole midguts of 50 caterpillars or the same amount of AM or PM).

Estimation of total proteolytic activity of midgut extract via azocasein hydrolysis [10, 11]. Universal 30 mM acetate-phosphate-borate buffer, pH 8.5 (buffer A [12]), was added to 1-10 μl of midgut extract preparation to the total volume 500 μl, and 100 μl of 2% substrate solution was added to this mixture. The resulting mixture was incubated for 15-30 min at 37°C. The reaction was stopped by addition of 150 μl of 35% trichloroacetic acid (TCA). The suspension was kept in a refrigerator for 20 min, centrifuged at 20,800*g* for 7 min, and then 650 μl of the supernatant was added to the same volume of 1 M NaOH. Optical absorption was measured at 450 nm in 1-cm cuvette using a Shimadzu spectrophotometer (Japan). Reaction mixture with TCA added before the substrate was used as a control. The amount of extract causing 0.1 increase in optical absorption per minute was taken as the unit of total proteolytic activity. Total proteolytic activity of midgut extract was calculated per insect.

Table 1. Estimation of proteolytic activity in extracts from *G. mellonella* AM and PM (U per insect) with the substrates of various proteolytic enzymes

Substrate	AM	PM	AM	PM
	pH 8.5		pH 10.7	
Azocasein	0.528	0.275	0.851	0.481
Azocasein + 5 mM DTT*	0.321	0.180	–	–
Bz-R-pNA	0.114	0.068	0.157	0.106
Bz-R-pNA + 5 mM DTT*	0.089	0.054	–	–
For-AAF-pNA	0.011	0.005	0.014	0.006
Glp-AAL-pNA	0.018	0.015	–	–
Suc-AAPF-pNA	0.068	0.066	–	–
DNP-AALR-NH_2	0.016	0.015	–	–
Z-AAP-pNA**	0.0018	0.00044	–	–

* Incubation was performed in the presence of 5 mM DTT.
** Activity was estimated at pH 8.0.

Activity of trypsin, chymotrypsin, and post-proline-cleaving peptidases was estimated with specific *p*-nitroanilide (pNA) substrates (Table 1) according to Erlanger et al. [13]. Chymotrypsin activity was determined using For-AAF-pNA (For, formyl), Glp-AAL-pNA (Glp, L-pyroglutamyl) [14], and Suc-AAPF-pNA (Suc, succinyl). Tryptic activity was estimated via hydrolysis of Bz-R-pNA (Bz, benzoyl), and activity of post-proline-cleaving peptidases via hydrolysis of Z-AAP-pNA (Z, N-benzyloxycarbonyl). For activity estimation, 1-20 μl of extract was diluted to 1 ml with buffer A, and the substrate solution was added to this mixture to the final concentration 0.4 mM (in a case of Z-AAP-pNA to 0.1 mM). The reaction mixture was incubated for 10-15 min (in a case of Z-AAP-pNA for 40 min) at 37°C. The amount of enzyme catalyzing conversion of 1 μmol of substrate to product per minute was taken as the activity unit (U). Enzyme activity was calculated per insect.

Effect of inhibitors on the activity of proteinases was studied with the following substrates: azocasein, Bz-R-pNA, For-AAF-pNA, Glp-AAL-pNA, and DNP-AALR-NH_2 (DNP, dinitrophenyl). An aliquot of the extract was added to the corresponding volume of buffer A containing one of the inhibitors listed in Table 2 at concentrations also given there. The samples were incubated for 30 min at room temperature. Then the substrate was added, and proteolytic activity was measured according to the above-mentioned procedures. The residual enzyme activity was expressed as a percentage of the control value. All inhibitors used in this study were from Sigma (USA).

Estimation of post-electrophoretic activity of the proteinases by zymography. Electrophoresis was performed under native conditions in 10% polyacrylamide gel in buffer containing 43 mM imidazole, 35 mM Hepes, pH 7.4, according to McLellan [15], cathode- or anode-directed (cationic or anionic electrophoretic systems, respectively). An extract aliquot was mixed with an equal volume of the same buffer containing 20% glycerol.

Proteolytic activity in gels was estimated by two different methods. The total proteolytic activity was determined via hydrolysis of 0.075% gelatin placed in 12.5% indicator polyacrylamide gel polymerized in 100 mM universal buffer, pH 8.5, as described earlier [11]. Specific proteolytic activity was estimated against *p*-nitroanilide substrates. After electrophoresis the separating gel was washed with buffer A for 10 min, then the buffer was removed and a nitrocellulose membrane preincubated for 40 min in buffer containing the corresponding 0.25 mM substrate was applied onto the gel. Subsequent treatment was performed as described in [16]. The location of proteolytic activity was visualized as lilac-colored bands.

Table 2. Inhibitor analysis of proteolytic activity with various substrates of extracts from *G. mellonella* caterpillar AM and PM

Inhibitor	Concentration, mM	Azocasein		Bz-R-pNA		For-AAF-pNA		Glp-AAL-pNA		DNP-AALR-NH_2	
		AM	PM	AM	PM	AM	PM	AM	PM	AM	PM
DFP	1	17.5	9.5	1.4	0.0	0.9	0.0				
–"–	3							0.18	0.0	29.5	28.0
STI	0.01	20.7	12.8	6.8	0.0	40.4	0.0				
TLCK	0.135	57.6	45.0	4.5	0.0	102		94.9	94.7	83.9	
TPCK	0.285	95.7	101	99.6	99.5	87.5		79.7	89.2	90.6	
DTT	5	60.8	65.6	75.9	75.4						
E-64	0.001	104		108	90.0						
–"–	0.01	101	103	122	98.3	92.9	106.8			93.1	
Iodoacetamide	0.1	103	101								
–"–	1	97.2									
EDTA	10	147	123							92.2	
EGTA	10	141	106								
o-Phenanthroline	2.5	107	90.0					51.8	59.1	66.7	65.3

Note: Residual activity at pH 8.5 is expressed as percentage of the control activity with the given substrate.

Electrophoresis under denaturing conditions. SDS-PAGE (SDS from Sigma) was performed in 10% gels according to Laemmli [17]. To determine molecular masses, a standard kit (Fermentas, Lithuania) containing proteins of 170, 130, 95, 72, 55, 43, 34, 26, 17, and 11 kDa was used.

Purification of trypsin. Midgut extract (12.5 ml) was diluted threefold with the Ringer solution and centrifuged at 15,000*g* for 30 min using a J2-21 centrifuge (Beckman, Germany). The supernatant was applied onto a HiTrap Q column (GE Healthcare, Sweden) in FPLC equilibrated with 0.05 M Tris-HCl, pH 7.45 (buffer B). The sample was eluted with a gradient of NaCl concentration (0-1 M) in buffer B. Fractions forming a peak with maximal activity against Bz-R-pNA were pooled, diluted fourfold with buffer B, and applied onto a Mono Q column (GE Healthcare) equilibrated with the same buffer. The sample was eluted under the same conditions. Fractions with tryptic activity were pooled, and after addition of NaCl to 1 M concentration the sample was applied onto a column with benzamidine-Sepharose (GE Healthcare) equilibrated with buffer B containing 1 M NaCl. The sample was eluted with 0.05 M glycine-HCl buffer, pH 3.0, into test tubes containing 200 µl of 0.1 M sodium carbonate buffer, pH 11.0.

Purification of chymotrypsin. Fractions with activity against Glp-AAL-pNA eluted from the HiTrap Q column during the above-described chromatography of midgut extract were applied onto a Mono Q column equilibrated with buffer B. The sample was eluted with a gradient of NaCl concentration (0-1 M) in the same buffer. Chymotrypsin-containing fractions were pooled, concentrated on a DIAFLO PM10 membrane (Amicon Corporation, USA) in an Amicon cell (Millipore, USA), and applied onto a column with Superdex-75 (GE Healthcare) equilibrated with buffer B containing 0.3 M NaCl. The sample was eluted with the same buffer.

Determination of N-terminal amino acid sequence. Eluate (0.5 ml) from benzamidine-Sepharose was concentrated by precipitation with TCA, subjected to SDS-PAGE, and electrotransferred onto an Immobilon-P^{SQ} membrane (Millipore) in a Trans-Blot SD apparatus (BioRad, USA) for 1.5 h at current 150 mA. The membrane was stained with 0.15% solution of Coomassie R-250 (Serva, Germany) in 50% methanol. The band corresponding to trypsin was cut out and stored at −80°C. The N-terminal amino acid sequence was determined using a model 470A automatic gas-phase sequencer (Applied Biosystems, USA). The N-terminal amino acid sequences were compared with those deposited in the Non-redundant (nr) protein sequences database (NCBI) using the BLAST package (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Isolation of δ-endotoxins. *Bt* B-1757 (ssp. *galleriae*) strain from the Russian Collection of Industrial Microorganisms (VKPM) was used for toxin isolation. The microorganisms were grown on a liquid medium containing 1% trypcasin (Oltoanyagtermelo es Kutato Intezet, Hungary), 0.2% yeast extract (Serva), and 0.6% glucose at 28°C to complete lysis of sporangia [18]. Crystals of endotoxin were separated from other components of the cell autolysate in a biphasic xylene/water system [18].

To obtain entomocidal proteins, the crystals were treated with 0.1 M sodium carbonate buffer, pH 10.3, containing 0.01 M dithiothreitol (DTT; Serva), 0.005 M EDTA, and 0.001 M diisopropylfluorophosphate (DFP) for 1 h at 20°C with intensive stirring. The mixture was centrifuged at 15,000*g* for 20 min. DTT was separated by gel filtration on a column PD-10 (GE Healthcare) equilibrated with 0.1 M carbonate buffer, pH 10.3. The sample was eluted with the same buffer.

Limited proteolysis of entomocidal proteins. To study the proteolysis of *Bt* ssp. *galleriae* entomocidal proteins, a solution of crystals was treated with midgut extract and preparations of purified trypsin and partially purified chymotrypsin from *G. mellonella* larvae. Mixtures with various enzyme/substrate ratios were incubated for 1 h at 37°C, and the products of proteolysis were characterized by SDS-PAGE.

Immunoblotting was performed as described in [19] using polyclonal rabbit antiserum against Cry9A endotoxin [8] in dilution 1 : 500 and conjugate of secondary antibodies with horseradish peroxidase.

RESULTS

Total proteolytic activity of the midgut extracts from *G. mellonella* caterpillars was determined via their ability to hydrolyze protein substrate azocasein. This activity in AM and PM extracts was maximal at pH 10.7 (Fig. 1). Activity decreased at pH values lower than 8.0 and was

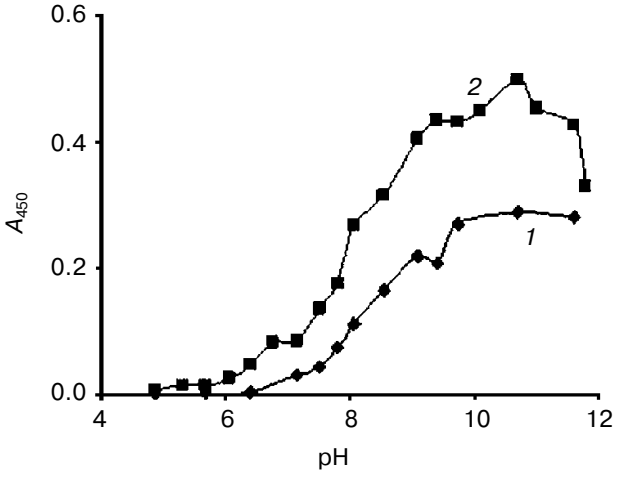

Fig. 1. Effect of pH on the total proteolytic activity with azocasein in extracts of *G. mellonella* AM (*1*) and PM (*2*). The pH values were adjusted using 30 mM universal buffer.

almost zero at pH 6.0. It was shown that pH of the midgut contents of *G. mellonella* caterpillars was 8.5, and at this pH the activity was 62 and 58% of the maximal for AM and PM, respectively. As seen in Table 1, at pH 8.5 the activity of the extract from AM with azocasein is ~0.53 U per insect. The activity of the extract from PM is significantly lower (0.28 U per insect), that is, ~65% of the total activity of *G. mellonella* midgut (the sum of AM and PM activities per insect) at the physiological pH value (8.5) was detected in AM and 35% in PM. AM activity was higher than that of PM at all pH values.

Inhibitor analysis of azocaseinase activity indicated that serine proteinases mainly contribute to the proteolysis of proteins in caterpillars: when the serine proteinase inhibitor DFP was added at pH 8.5, 17.5 and 9.5% of the initial activity was retained in AM and PM, respectively (Table 2). At pH 10.7 the efficiency of this inhibitor was even higher (residual activity was 2.1 and 6.3%, respectively). Kunitz soya trypsin inhibitor (STI) (0.01 mM) is close to DFP in inhibitory efficiency. However, cysteine proteinase inhibitors E-64 (*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane) (0.01 mM) and iodoacetamide (1 mM), and also metallopeptidase inhibitors *o*-phenanthroline (2.5 mM), EDTA, and EGTA (both 10 mM) had no effect on hydrolysis of azocasein by midgut extracts. So, the latter enzymes are either absent from midgut contents or do not significantly contribute to their proteolytic activity at pH 8.5. The data on decrease (not increase) in azocaseinase activity in the presence of DTT, an activator of cysteine proteinases, indicate the same.

Analysis of the spectrum of *G. mellonella* proteolytic complex with specific peptide substrates. Activity against Bz-R-pNA [13], a specific substrate of trypsin-like and some cysteine proteinases, in extracts from AM and PM as well as the total proteolytic activity was significantly higher at pH 10.7 than at pH 8.5 and was mainly localized in AM – 63% of the sum of activities in AM and PM at physiological pH (Table 1). Activity against Bz-R-pNA in AM was almost completely suppressed by DFP, was less than 7% of the control in the presence of STI or tosyllysine chloromethyl ketone (TLCK), was by 24% inhibited by DTT (an activator of cysteine proteinases), and was insensitive to E-64 and iodoacetamide, inhibitors of cysteine proteinases. The same activity in PM was completely inhibited by DFP and trypsin inhibitors and in a similar manner responded to reagents for cysteine proteinases; this indicates that the activity against Bz-R-pNA belongs to trypsin-like proteinases in both parts of *G. mellonella* midgut. The earlier conclusion that digestive cysteine proteinases are absent from *G. mellonella* caterpillars is also supported by these data.

Of three chymotrypsin substrates, For-AAF-pNA, Glp-AAL-pNA, and Suc-AAPF-pNA, the latter was hydrolyzed by midgut proteinases of *G. mellonella* with significantly higher rate (Table 1). Activities with this substrate in AM and PM were approximately equal, whereas the distribution of activity against For-AAF-pNA between AM and PM corresponded with the data on the total tryptic activity. Hydrolysis of both substrates by extracts from AM and PM was completely suppressed by DFP and was not inhibited by E-64; this indicates that they are hydrolyzed by serine proteinases (Table 2). Hydrolysis of these substrates was significantly inhibited by STI, but it was inhibited only by 12-20% by tosylphenylalanine chloromethyl ketone (TPCK), a mammalian chymotrypsin inhibitor. This is typical of insect chymotrypsin-like proteinases since it was shown that they prefer longer substrates than mammalian chymotrypsins [14, 20].

Activity with the substrate DNP-AALR-NH_2 usually used for testing of metallopeptidases was low. It was by 70-72% suppressed by DFP and by 33-35% by *o*-phenanthroline. This suggests that such activity is mainly caused by serine chymotrypsin-like proteinases (hydrolyzing a bond after leucine by analogy with Glp-AAL-pNA) and also by metallopeptidases. It should be noted that hydrolysis of Glp-AAL-pNA was also partially inhibited by *o*-phenanthroline. However, it was completely inhibited by DFP and consequently was performed by serine proteinases. Possibly, *o*-phenanthroline affects a hydrophobic pocket of the substrate-binding site of chymotrypsins, thus hindering binding of precisely this substrate.

The detected activity against Z-AAP-pNA (0.018 U per insect, Table 1) suggests the presence of post-proline-cleaving peptidases in the midgut of the greater wax moth.

The role of trypsin-like proteinases in hydrolysis of protein substrates can be evaluated via the action of the specific inhibitor TLCK. It seems likely that about a half of the total activity against azocasein is due to trypsin-like proteinases, because TLCK almost completely inhibits these proteinases of *G. mellonella* caterpillars (tested with Bz-R-pNA), and hydrolysis of azocasein is approximately half-inhibited by TLCK (58% residual activity in AM and 45% in PM, see Table 2). We failed to evaluate a contribution of chymotrypsin-like proteinases using TPCK because this reagent poorly inhibits chymotrypsin-like activity of midgut extracts from *G. mellonella* (Table 2) and some other insects, as mentioned above. However, it is believed that the remaining ~50% of azocaseinase activity inhibited by DFP is due to chymotrypsin-like proteinases.

Study of the spectrum of *G. mellonella* midgut proteinases by zymography. The total post-electrophoretic activity of peptidases was analyzed in gelatin-containing gel replicas incubated in contact with the separating gel. As can be seen in Fig. 2a, there are three components with gelatinolytic activity migrating to the anode at pH 7.4 in the course of electrophoretic fractionation of the extracts from AM (anionic forms G1-G3). G1 is the major one and probably consists of several different proteins. Among proteins positively charged at pH 7.4 (cationic forms, Fig. 2b) one can see two slowly migrating

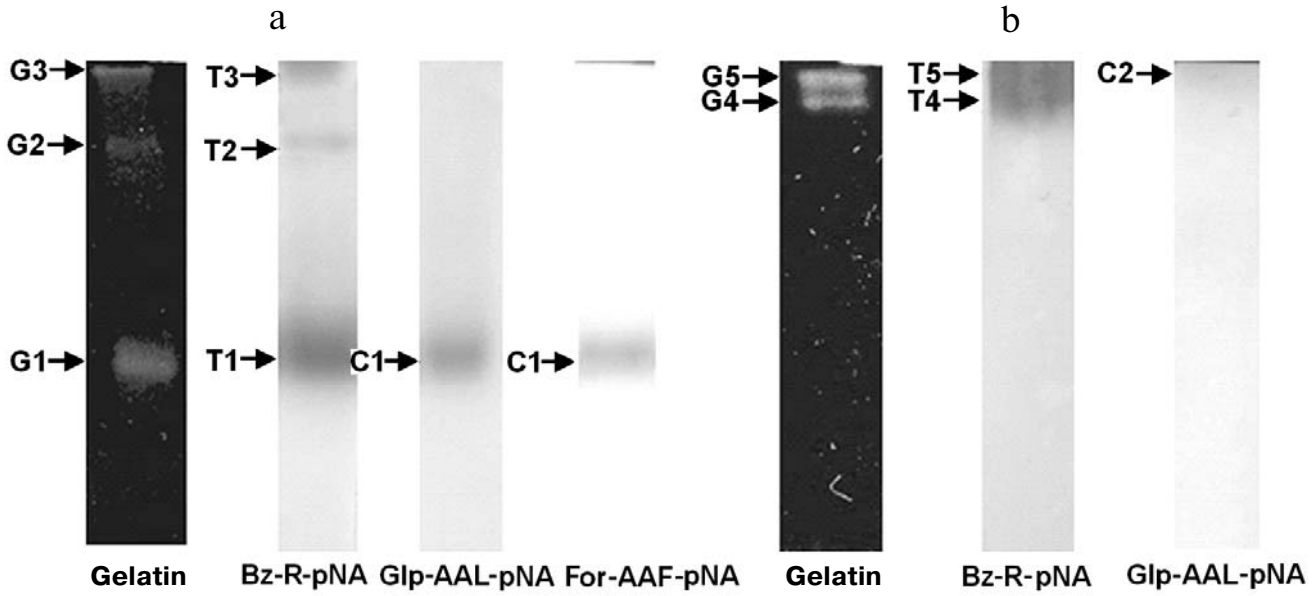

Fig. 2. Post-electrophoretic anionic (a) and cationic (b) activities of the proteolytic complex from AM of *G. mellonella* caterpillars at pH 8.5. Activity was evaluated in an indicator gelatin-containing gel or on nitrocellulose membranes saturated with one of the substrates indicated in the figure, which were laid on the separating gel and incubated for 1 h at 37°C. Bands of substrate hydrolysis were detected as described in "Materials and Methods". Positions of bands in which gelatinolytic (G1-G5), trypsin-like (T1-T5), or chymotrypsin-like (C1, C2) activities were detected are indicated by arrows.

components able to hydrolyze gelatin (G4, G5). This indicates the presence of at least five proteolytic enzymes in the extract from *G. mellonella* midgut.

The *G. mellonella* AM proteins possessing specific trypsin-like activity (able to hydrolyze Bz-R-pNA) include three anionic (T1-T3) and two cationic (T4, T5) forms that seem to coincide with analogous components of gelatinolytic activity (Fig. 2). In *G. mellonella* AM, chymotrypsin-like activity against For-AAF-pNA and Glp-AAL-pNA is represented by one anionic form C1 and one minor cationic form C2 with low electrophoretic mobility in the system used (Fig. 2). Mobility of component C1 coincides with those of G3 and T3, whereas it is difficult to compare C2 with any other bands possessing gelatinolytic activity. Analogous results were obtained when analyzing isoforms of proteolytic enzymes in *G. mellonella* caterpillar PM.

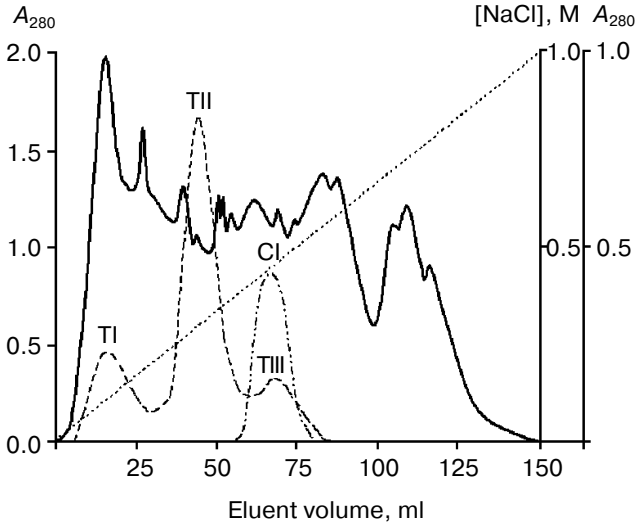

Fig. 3. Chromatography of midgut extract from *G. mellonella* caterpillars on a HiTrap Q anion-exchange column. Elution profile of protein is measured by optical absorption at 280 nm (solid line), tryptic activity is assayed with Bz-R-pNA (dashed line), and chymotryptic activity with Glp-AAL-pNA (dash-dotted line). The gradient of NaCl concentration is depicted by the dotted line.

Isolation and properties of trypsin. Midgut extract from *G. mellonella* caterpillars was chromatographed at pH 7.4 on a HiTrap Q anion-exchange column. Tryptic activity eluted in three peaks (TI, TII, and TIII), whereas chymotryptic activity eluted in one partly coinciding with the third peak of tryptic activity (C1, see Fig. 3). TII, a trypsin peak with dominating activity, was chosen for further purification. Fractions composing this peak were pooled, and the enzyme was further purified by sequential chromatography on Mono Q and benzamidine-Sepharose columns.

The results of zymography indicated that purified trypsin preparation TII contained only one component possessing both gelatinolytic activity and ability to hydrolyze Bz-R-pNA (Fig. 4). Its electrophoretic mobility corresponded to that of T3 component of AM extract from *G. mellonella* caterpillars (Fig. 2). SDS-PAGE revealed a single band with molecular mass 28 kDa.

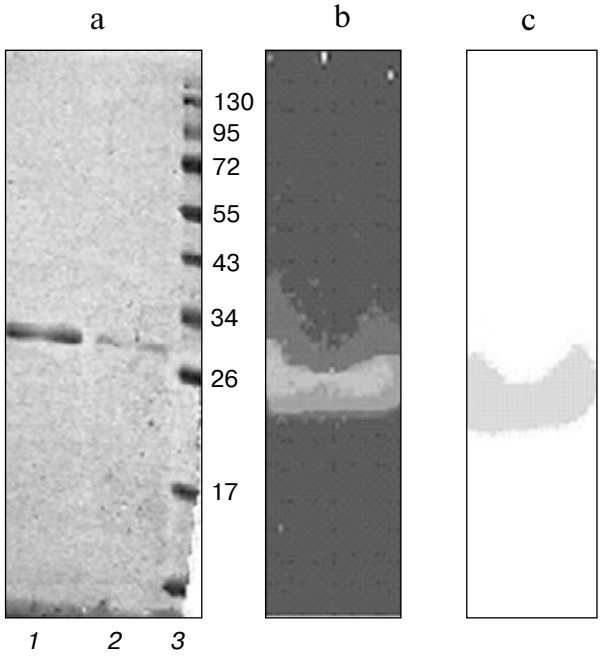

Fig. 4. Analysis of purified trypsin preparation TII by SDS-PAGE (a) and by zymography (b, c). Lanes: *1*, *2*) 5 and 1 μg of trypsin, respectively; *3*) molecular mass markers. b, c) Trypsin preparation was electrophoresed under native conditions in the anionic electrophoretic system; tryptic activity was detected with gelatin (b) or Bz-R-pNA (c).

Analysis of the N-terminal amino acid sequence of the protein composing this band showed a single N-terminal sequence: Ile-Val-Gly-Gly-Glu-Leu-Thr-Thr-Ile-Glu; this also indicates that the isolated trypsin is pure.

Preparation of partially purified chymotrypsin. Fractions with activity against Glp-AAL-pNA obtained from chromatography of midgut extract on a HiTrap Q anion exchange column (Fig. 3) were pooled and further purified on a Mono Q anion exchange column and a Superdex-75 gel-filtration column. Chymotryptic activity was eluted as one peak. SDS-PAGE demonstrated that there were at least four proteins in the studied preparation (data not presented here), so we mention this preparation as partially purified chymotrypsin from the midgut of *G. mellonella* caterpillars. This preparation did not show any tryptic activity.

Proteolysis of *Bt* ssp. *galleriae* crystal proteins. When the total solution of *Bt* ssp. *galleriae* crystals was treated with midgut extract from *G. mellonella* caterpillars, the molecular mass of initial endotoxins (130 kDa) sequentially decreased (Fig. 5a). In the course of 1 h incubation the proteolysis began at the extract (re-calculated on the tryptic activity)/crystal proteins ratio $0.05 \cdot 10^{-3}$ U/mg. At $0.25 \cdot 10^{-3}$ U/mg the extract hydrolyzed most of the initial proteins to half products with molecular masses 110-100 kDa, and at $6.25 \cdot 10^{-3}$ U/mg ratio it hydrolyzed the proteins to fragments with molecular mass 69 kDa. After treatment of *G. mellonella* midgut extract with trypsin inhibitor TLCK, the qualitative characteristics of the limited proteolysis changed negligibly, but in order to attain analogous effect more enzyme preparation was required (data not presented here).

The immunoblotting data indicate that Cry9A endotoxin is subjected to proteolysis to 65-70 kDa fragments by the action of midgut extract with $6.25 \cdot 10^{-3}$ tryptic activity units per mg total crystal protein (Fig. 5d).

Purified trypsin preparation TII is also able to cause limited proteolysis of *Bt* ssp. *galleriae* endotoxins (Fig. 5b). Hydrolysis also begins at $0.05 \cdot 10^{-3}$ tryptic activity units per mg entomocidal protein. Significant accumulation of products with molecular masses ~100 and 64 kDa occurs on increasing the ratio to $6.25 \cdot 10^{-3}$ U/mg.

Partially purified chymotrypsin preparation additionally treated with TLCK also effectively hydrolyses *Bt* endotoxins (Fig. 5c). Proteolysis is evident even at $0.05 \cdot 10^{-3}$ U/mg, and predominant accumulation of 58 kDa fragment occurs at 0.03 chymotryptic activity units (evaluated via hydrolysis of For-AAF-pNA) per mg crystal protein. In the latter case minor amounts of fragments with molecular masses 46 and 49 kDa also appear (Fig. 5c).

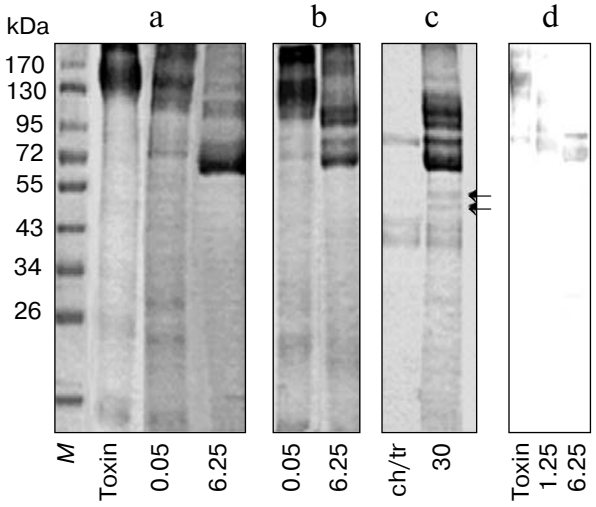

Fig. 5. Limited proteolysis of proteins of *Bt* ssp. *galleriae* crystals. Solution of crystals (20 μg) was incubated with midgut extract from *G. mellonella* (a, d), trypsin (b), and chymotrypsin (c) preparations isolated from the insect. Proteolysis was detected by gels staining with Coomassie R-250 (a-c) or Western blotting using specific polyclonal antiserum to Cry9A endotoxin (d). Midgut extract and purified trypsin preparation were added in the amount given at the figure bottom (1000 times decreased) in tryptic activity units per mg protein of δ-endotoxins; amount of chymotrypsin preparation was calculated in chymotryptic activity units per mg entomocidal proteins. Initial solution of crystals is in the "toxin" well, chymotrypsin preparation is in the "ch/tr" well. M, molecular mass markers. Fragments with molecular masses 46 and 49 kDa indicated by arrows were obtained after hydrolysis of toxins by *G. mellonella* chymotrypsin preparation.

DISCUSSION

Wax is the main food source of *G. mellonella* caterpillars [1], but protein food, e.g. bee-bread and also excrements and exuviae, is also present in beehives [21]. Our data indicate that caterpillars of this species are able to digest proteins and have a complex of digestive proteinases including mainly serine proteinases, whereas cysteine and metalloproteinases in comparable amounts are absent. In *G. mellonella* caterpillars the digestive proteolysis mainly occurs in AM.

Study of substrate specificity of the proteinases (Table 1) demonstrated that trypsin-like and chymotrypsin-like serine proteinases are present in the midgut of *G. mellonella* caterpillars. About half of the total activity against azocasein seems to be due to trypsin-like proteinases at physiological pH value 8.5. Comparison of hydrolytic activity of midgut extracts against three chymotrypsin substrates showed that Suc-AAPF-pNA is the most effective substrate. This corresponds with the literature data on substrate specificity of insect chymotrypsins, which prefer longer substrates [14, 20, 22, 23]. This preference can also explain the absence of inhibition of chymotryptic activity by TPCK (Table 2) also mentioned in [14, 20]. Activity against Z-AAP-pNA (Table 2) detected in Lepidoptera for the first time indicates that post-proline-cleaving peptidases mainly belonging to serine peptidases are present in the midgut of caterpillars [24]. The data obtained with the substrate DNP-AALR-NH_2 (Tables 1 and 2) suggest that minor amounts of metallopeptidases can be present in *G. mellonella* midgut extracts.

So, digestive proteolysis in the midgut of *G. mellonella* caterpillars is organized similar to that in phytophagous caterpillars from the order Lepidoptera, whose midgut contents usually have basic pH 10-12 [25, 26]. *Mamestra configurata* caterpillars contain only serine proteinases with trypsin, chymotrypsin, and elastase types of activity [27]. The same was shown for *Helicoverpa armigera* caterpillars [28]. This supports the earlier suggestion that a set of insect digestive enzymes (at the level of enzyme subsubclasses but not at the level of isoforms) is mainly defined by taxonomy rather than nutrition type [1, 3]. The presence of five trypsin isoforms and two chymotrypsin isoforms in *G. mellonella* caterpillars was shown by zymography. This is also typical of phytophagous caterpillars, e.g. several trypsins [29] and chymotrypsins [2] were found in *Manduca sexta*. Optimal pH value of proteinases in these insects is also very high (9.5-10.0) [1].

The combination of ion-exchange and affinity chromatographies allowed us to isolate pure anionic trypsin TII. This protein is identical to mature trypsin (AAF24227) from *Plodia interpunctella* caterpillar in its N-terminal sequence and has 70-90% coincidence with trypsins from such representatives of Lepidoptera order as *Sesamia nonagroides*, *Heliothis virescens*, *H. armigera*, and also *Aedes aegypti* and some *Drosophila* species (all Diptera).

The ability of insect midgut proteinases for limited proteolysis of Cry-proteins was mentioned by several authors. In most cases this results in activation of toxins accompanied by degradation of the C-terminal part of the molecule and transformation of initial 130 kDa protoxin into the activated toxin with molecular mass 65-70 kDa [6, 7]. We were interested in the study of the ability of midgut extract from *G. mellonella* caterpillars for limited proteolysis of Cry proteins synthesized by the highly toxic for this insect strain B-1757 *Bt* ssp. *galleriae*. Protein crystals of this strain consist of Cry9A endotoxin, which is responsible for toxicity for the greater wax moth, and several other Cry proteins with low toxicity for this insect [30]. We showed that 1 h treatment of toxins with midgut extract in the ratio 0.00625 tryptic activity units per mg crystal protein results in accumulation of fragments with molecular mass 69 kDa, which are the activated forms of the initial proteins judging by their size (Fig. 5a) [4, 31]. The immunoblotting data indicate that Cry9A is subjected to proteolysis with the same efficiency as other δ-endotoxins of *Bt* ssp. *galleriae*.

The crystal proteins were also hydrolyzed by purified trypsin as well as by partially purified chymotrypsin. Consequently, both species of serine proteinases participate in their activation. This is also supported by the fact that TLCK-treated extract is capable of limited proteolysis of the mentioned endotoxins but with less intensity than that untreated with TLCK. Proteolysis of Cry9A by the chymotrypsin preparation from the greater wax moth can be more profound than by the trypsin, up to formation of fragments with molecular masses 49 and 46 kDa (Fig. 5c) that was shown earlier with bovine chymotrypsin [32].

It was shown above that AM extract from one insect contains 0.114 tryptic activity units. Since Cry9A concentration which causes 100% inhibition of nutrition of *G. mellonella* caterpillars is 2 μg per gram of food [31] (0.6 μg recalculated per insect), we conclude that proteolytic activity present in the midgut of one insect is ~3000 times higher than that required for the activation of a lethal dose of toxin. Really this activity is even higher, because caterpillars have no time to eat all food during biotesting. Of course, appearing in the midgut, toxin cannot mix at once with all the pool of digestive enzymes. Along with this, most of the digestive proteinases must hydrolyze food proteins, because the toxin penetrates into the insect organism together with food. Nonetheless, one can state that the potential ability of the insect digestive system is enough for the activation of the amount of toxin required for its death. Thus, activation of Cry9A protoxin most probably is not the rate-limiting stage in the action of this protein in the greater wax moth.

This work was financially supported by the Russian Foundation for Basic Research (grants No. 09-04-91289-INIS-a and 09-04-01449-a).

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