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A chymotrypsin-like proteinase from the midgut of *Tenebrio molitor* larvae

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Abstract

A chymotrypsin-like proteinase was isolated from the posterior midgut of larvae of the yellow mealworm, *Tenebrio molitor*, by ion-exchange and gel filtration chromatography. The enzyme, TmC1, was purified to homogeneity as determined by SDS-PAGE and postelectrophoretic activity detection. TmC1 had a molecular mass of 23.0 kDa, p*I* of 8.4, a pH optimum of 9.5, and the optimal temperature for activity was 51 °C. The proteinase displayed high stability at temperatures below 43 °C and in the pH range 6.5–11.2, which is inclusive of the pH of the posterior and middle midgut. The enzyme hydrolyzed long chymotrypsin peptide substrates SucAAPFpNA, SucAAPLpNA and GlpAALpNA and did not hydrolyze short chymotrypsin substrates. Kinetic parameters of the enzymatic reaction demonstrated that the best substrate was SucAAPFpNA, with $k_{cat\ app}$ 36.5 s^{-1} and K_m 1.59 mM. However, the enzyme had a lower K_m for SucAAPLpNA, 0.5 mM. Phenylmethylsulfonyl fluoride (PMSF) was an effective inhibitor of TmC1, and the proteinase was not inhibited by either tosyl-L-phenylalanine chloromethyl ketone (TPCK) or N_α-tosyl-L-lysine chloromethyl ketone (TLCK). However, the activity of TmC1 was reduced with sulfhydryl reagents. Several plant and insect proteinaceous proteinase inhibitors were active against the purified enzyme, the most effective being Kunitz soybean trypsin inhibitor (STI). The N-terminal sequence of the enzyme was IISGSAASKGQFPWQ, which was up to 67% similar to other insect chymotrypsin-like proteinases and 47% similar to mammalian chymotrypsin A. The amino acid composition of TmC1 differed significantly from previously isolated *T. molitor* enzymes.

Keywords: Chymotrypsin; Digestive proteinase; Midgut; *Tenebrio molitor*; Yellow mealworm

1. Introduction

The primary digestive proteinases of many insects are serine proteinases, such as trypsin (EC 3.4.21.4) and chymotrypsin (EC 3.4.21.1) [1–4]. While trypsins from a wide range of insect species have been purified and characterized, chymotrypsins have been less studied, but some appear to differ from their mammalian counterpart in the binding region. The main digestive chymotrypsins of lepidopteran and orthopteran insects have an extended binding site and do not hydrolyze short peptide substrates commonly used in the detection of mammalian enzymes [5–8]. It is unclear whether such enzymes are characteristic for other groups of insects.

The yellow mealworm *Tenebrio molitor* (Coleoptera: Tenebrionidae) was among the first insect species in which larval digestive proteinases were studied (trypsin and exopeptidases) [9]. Later the major larval cationic trypsin-like proteinases were purified and characterized [10,11], and the mode

Abbreviations: Ac, acetyl; AM, anterior midgut; Bz, benzoyl; DMF, dimethyl formamide; E-64, L-*trans*-epoxysuccinyl-L-leucylamido(4-guanidino) butane; Glp, pyroglutamyl; PM, posterior midgut; PMSF, phenylmethylsulfonyl fluoride; pNA, *p*-nitroanilide; STI, Kunitz soybean trypsin inhibitor; Suc, succinyl; TLCK, N_α-tosyl-L-lysine chloromethyl ketone; TPCK, tosyl-L-phenylalanine chloromethyl ketone; Z, *N*-benzoyloxycarbonyl.

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of larval trypsin secretion was studied [12]. The major imaginal trypsin-like enzyme differed in many characteristics from the larval enzyme [1].

There are no published references regarding *T. molitor* chymotrypsin-like proteinases, but several characteristics of isolated larval and imaginal yellow mealworm chymotrypsin-like proteinases were provided by Applebaum [1]. Differences were found in the amino acid composition of these enzymes and also with bovine chymotrypsin.

Two additional midgut proteinases were purified and characterized from *T. molitor* adults. One of these proteinases, β-protease, had an unusually high molecular mass (Mm) of 60 kDa and hydrolyzed a trypsin ester substrate but lacked the typical cleavage specificity of bovine trypsin on the B-chain of oxidized insulin, although the enzyme was inhibited by a range of synthetic and natural trypsin inhibitors [13,14]. Another enzyme, α-protease, had a Mm 23.4 kDa, did not hydrolyze trypsin and chymotrypsin ester substrates, was not inhibited by either trypsin or chymotrypsin specific ketonic inhibitors N_{α}-tosyl-L-lysine chloromethyl ketone (TLCK) and tosyl-L-phenylalanine chloromethyl ketone (TPCK), respectively, but was sensitive to the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF) and two plant trypsin inhibitors from soya and lima beans [13]. These early data led to the conclusion that adult and larval digestive proteinases are expressed differently, although both developmental stages of *T. molitor* inhabit the same milieu and have the same diet [1].

In the present study one of the main serine digestive proteinases from the midgut of *T. molitor* larvae was purified to homogeneity. This enzyme, like imaginal α-protease [13], was insensitive to ketonic inhibitors, and was characterized as a chymotrypsin-like proteinase with an extended binding site.

2. Materials and methods

2.1. Reagents

Pyroglutamyl-alanyl-alanyl-leucine *p*-nitroanilide (GlpAALpNA) and other substrates used for characterization of enzyme substrate specificity were synthesized at the Department of Chemistry of Natural Compounds, Chemical Faculty, Moscow State University, Moscow, Russia [15] except for succinyl-alanyl-alanyl-prolyl-phenylalanine *p*-nitroanilide (SucAAPFpNA) and succinyl-alanyl-alanyl-prolyl-leucine *p*-nitroanilide (SucAAPLpNA), purchased from Sigma (St. Louis, MO, USA); L-*trans*-epoxysuccinyl-L-leucylamido(4-guanidino) butane (E-64) was from ICN (Aurora, OH, USA), TLCK and TPCK were from Fluka (Buchs, Switzerland); PMSF was from Serva (Heidelberg, Germany) and Kunitz soybean trypsin inhibitor (STI) was from Reanal (Hungary).

2.2. Animals

Actively feeding fourth instar *T. molitor* larvae were used in the experiments. Larvae were reared on a mixture of wheat flour, bran and brewer's yeast at 26 °C and were transferred to milled oat flakes (Raisio, Finland) 1–1.5 weeks prior to dissection. Oat flakes processed at high temperature were devoid of active proteases and proteinase inhibitors (data not shown).

2.3. Preparation of enzyme extract

Larvae were chilled in ice-cold water, the posterior and anterior tips of the larvae were removed in 0.75% NaCl, and the gut was removed from one end. The midgut was separated into anterior (AM) and posterior (PM) sections. Fifty PM were collected in 300 μl of ice-cold NaCl solution, homogenized in a glass–glass homogenizer and centrifuged for 10 min at 15,000 × *g*. The supernatant was stored at –70 °C.

2.4. Enzyme purification

An extract from 200 pooled PM was dialyzed for 24 h, 4 °C against 20 mM potassium sodium phosphate buffer, pH 6.9, containing 0.02% sodium azide, clarified with centrifugation and subjected to batch chromatography on DEAE-Sephadex A-50 in the same buffer. The filtrate containing hydrolytic activity with the substrate GlpAALpNA was concentrated and subjected to gel filtration on a Superdex-75 column in FPLC in the same buffer. Aliquots of the purified enzyme were stored at –10 °C.

2.5. Enzyme activity assays

Activity with *p*-nitroanilide (pNA) substrates was assayed spectrophotometrically at 410 nm according to Erlanger et al. [16]. The substrates were initially dissolved in dimethyl formamide (DMF) and further dissolved in 20 mM Tris–HCl buffer, pH 8.0. The reaction mixtures with hydrophilic substrates contained 2.5% DMF, while hydrophobic substrates were solubilized in 10–20% DMF. Reactions were terminated by the addition of acetic acid to a 5% final concentration. The routine concentration of GlpAALpNA was 0.5 mM. The enzyme concentration was chosen so that after 15–40 min of incubation at 37 °C the absorbance at 410 nm was in the interval of 0.3–0.55 absorbance U. One U of enzyme activity was defined as the amount of enzyme hydrolyzing 1 μmol of a substrate in 1 min at 37 °C. All measurements were performed in triplicate.

2.6. Kinetic studies

Kinetic studies were performed with an Hitachi 557 (Japan) spectrophotometer at 30 °C with substrate concentrations ranging from 0.025 to 2.0 mM in 20 mM Tris–HCl buffer, pH 8.0, containing 2.5% DMF. The kinetic parameters were determined by the Lineweaver–Burk method.

2.7. Protein determination and concentration

Protein concentration was measured according to Lowry et al. [17] as well as spectrophotometrically at 280 nm. Diluted

protein solutions were concentrated by ultrafiltration in filtration units using YM10 filters (Amicon, USA) or evaporated in a Speedvac concentrator (Savant, USA).

2.8. pH-Optimum and stability

The optimum pH for activity with 0.5 mM GlpAALpNA was assayed using 200 mM universal buffer, with pH values ranging from 2.0 to 11.0 [18].

The pH-stability of the proteinase was characterized in universal buffers, pH 2.0–11.2. One volume of enzyme was added to five volumes of 100 mM universal buffer solutions with various pH values and preincubated for 1 h at room temperature. The mixture was adjusted to pH 7.7 by addition of 42 volumes of 50 mM phosphate buffer, pH 7.7, and after 15 min incubation, activity with 0.5 mM GlpAALpNA was measured as described earlier.

2.9. Temperature optimum and stability

To determine the optimum temperature for activity with 0.5 mM GlpAALpNA, assays were conducted at 22, 25, 30, 38, 44, 51 and 55 °C after a 2 min incubation as described previously.

For temperature stability testing, the purified enzyme was incubated at 23, 26, 32, 39, 43, 51 and 54 °C for 30 min in 20 mM Tris–HCl buffer, pH 8.0, and quickly cooled on ice. The solution of GlpAALpNA was added to the final concentration of 0.5 mM and the residual activity was measured as described earlier.

2.10. Inhibition studies

Active site inhibitors included PMSF, TPCK, TLCK, iodoacetamide, E-64 and EDTA. Sulfhydryl reagents, 2-mercaptoethanol and dithiothreitol, were also used. For inhibition studies, the proteinase was preincubated with different inhibitors for 30 min at room temperature in 20 mM Tris–HCl, pH 8.0, and residual activity with 0.5 mM GlpAALpNA was assayed as previously described. Proteinaceous proteinase inhibitors included STI, anionic and cationic trypsin inhibitors from buckwheat seeds, anionic trypsin inhibitors from triticale seeds and subtilisin inhibitor SI1 from a cockroach, *Nauphoeta cinerea*, midgut. Purification of trypsin inhibitors from buckwheat and triticale seeds was performed by means of affinity chromatography on trypsin-Sepharose followed by ion-exchange chromatography on Mono Q [19]. Insect subtilisin inhibitor SI1 was isolated by gel filtration on Sephadex G-50 [20]. Enzyme samples were preincubated with different concentrations of inhibitors for 20 min at room temperature in 50 mM phosphate buffer, pH 7.9. Residual activity was determined with 0.5 mM GlpAALpNA and expressed as % activity relative to control incubations in the absence of inhibitor. The obtained values were plotted versus inhibitor concentrations. Molar ratio inhibitor: enzyme at 50% inhibition was calculated using linear regression analysis.

2.11. Chromatofocusing

Chromatofocusing was performed on a PBI-94 column (Pharmacia, Sweden). A pH gradient of 6.0–8.7 was formed with Polybuffer-96.

2.12. Amino acid composition

The amino acid composition was determined by a standard procedure with a Hitachi 835 (Japan) amino acid analyzer. To determine sulfur containing amino acids, the protein was oxidized with a mixture of hydrogen peroxide and 88% performic acid (1:9, v/v), followed by hydrolysis with 5.7 M HCl (110 °C, 22 h) (cysteine was determined as cysteic acid). Tryptophan was determined after hydrolysis of the protein with 4 M methanesulfonic acid containing 0.2% tryptamine.

2.13. Electrophoretic studies and postelectrophoretic activity detection

SDS-PAGE was performed in a 14% gel in Tris-glycine buffer with 0.5% cross link as described by the manufacturer (Hoeffer, San Francisco, USA). Molecular mass markers were cellulase (94.6 kDa), BSA (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa).

For in-gel activity detection, 0.026% gelatin was copolymerized with the gel. The samples were loaded onto the gel without heating, and electrophoresis was performed at 4 °C at a constant current of 25 mA. Following electrophoresis, the slab was washed twice with 2.5% Triton X-100, twice with 20 mM Tris–HCl buffer, pH 8.0, and incubated for 2 h in the same buffer at 37 °C. Finally, the gel was stained with 0.1% Coomassie brilliant blue R-250 in 4:1:5 methanol/acetic acid/water (v/v/v) solution and destained in the same solution without the dye. The proteinases were visualized as clear bands on a dark background.

2.14. N-terminal sequence analysis

After SDS-PAGE the protein was electroblotted to Immobilon P membrane (Millipore, USA) for 1.5 h at 20 °C and 250 mA using Tris-glycine electrode buffer, pH 8.6, with 20% methanol and visualized by staining in 0.1% Coomassie brilliant blue R-250 with subsequent washing in methanol solution. N-terminal amino acid sequence was determined by automated Edman degradation procedure using a Model 492 Procise Protein Sequencer (Applied Biosystems, USA).

3. Results

3.1. Purification of a chymotrypsin-like proteinase

The isolation and purification of a chymotrypsin-like proteinase from the PM of *T. molitor* larvae (TmC1) was moni-

tored by hydrolysis of a chymotrypsin substrate GlpAALpNA [15]. The procedure consisted of two main stages: anion exchange batch chromatography of the dialyzed extract on DEAE-Sephadex A-50 and subsequent gel filtration of the nonadsorbed GlpAALpNA activity on a Superdex-75 column in 20 mM potassium sodium phosphate buffer, pH 6.9 without salt (Fig. 1). During the latter procedure, a chymotrypsin-like proteinase activity was retarded on the column, providing separation from trypsin-like activity. The results of the purification are summarized in Table 1 and Fig. 2. The enzyme was purified 288-fold, with a yield of 23% and was homogenous according to SDS-PAGE (Fig. 2, lane 4). The enzymatic purity and ability to hydrolyze protein substrates was confirmed by activity electrophoresis in the presence of copolymerized gelatin (Fig. 2, lane 5).

3.2. Characteristics of the enzyme

The molecular mass (Mm) of the purified enzyme was 23.0 ± 1.4 kDa according to SDS-PAGE (Fig. 2, lanes 1, 4). The p*I* of TmC1, as determined by chromatofocusing, was 8.4 (data not shown). The maximum activity of the enzyme with GlpAALpNA was observed in buffer of pH 9.5 (Fig. 3) although the activity was also high at pH values ranging from 7.5 to 10.0. No enzymatic activity was detected in buffers with a pH below 4.0. The proteinase was stable for 1 h at 22 °C (with relative activity more than 80%) in the pH range 6.5–11.2 (Fig. 4). In buffers below pH 4.0, the residual activ-

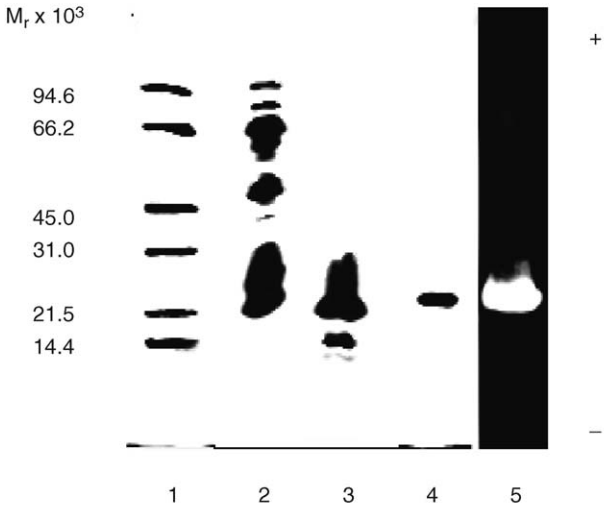

Fig. 2. SDS-PAGE profile of *T. molitor* larvae midgut chymotrypsin-like proteinase purification. Lane 1: molecular weight markers. Lane 2: midgut extract after dialysis. Lane 3: proteins after chromatography on DEAE-Sephadex A-50. Lane 4: purified proteinase obtained after FPLC Superdex-75 gel filtration. Lane 5: zymogram of the purified proteinase in the same gel with 0.026% gelatin.

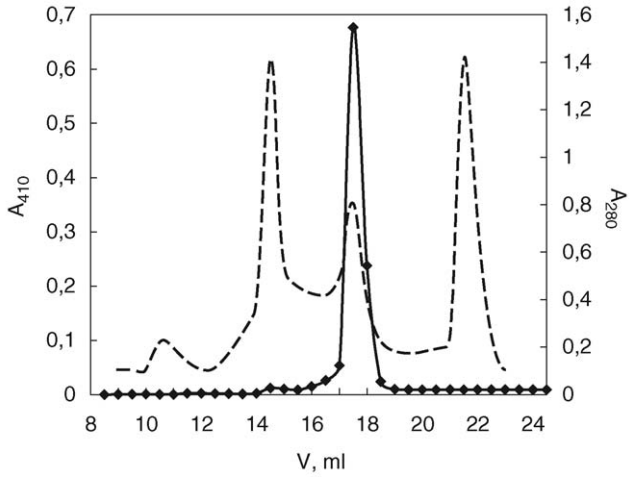

Fig. 1. Purification of chymotrypsin-like proteinase from *T. molitor* larvae PM on Superdex-75 column (FPLC). Solid line: profile of proteolytic activity assayed at 410 nm with 0.5 mM GlpAALpNA; dashed line: profile of protein determined spectrophotometrically at 280 nm.

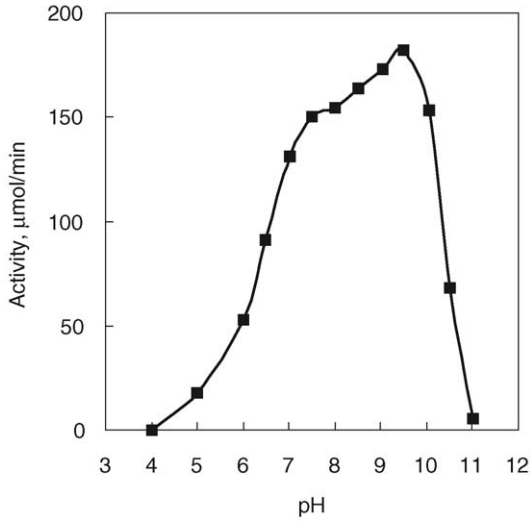

Fig. 3. Effect of pH on the activity of the *T. molitor* midgut proteinase with 0.5 mM GlpAALpNA.

Table 1
Purification of a chymotrypsin-like proteinase from *T. molitor* larvae posterior midgut. Enzyme activity was determined with 0.5 mM GlpAALpNA. Protein was determined spectrophotometrically at 280 nm

Purification stage	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (-fold)	Yield (%)
Dialyzed midgut extract	138.0	776.0	5.64	1	100
DEAE-Sephadex A-50, (nonadsorbed fraction)	1.15	329.0	286.0	50.6	42.4
FPLC Superdex-75	0.112	182.0	1620.0	288.0	23.4

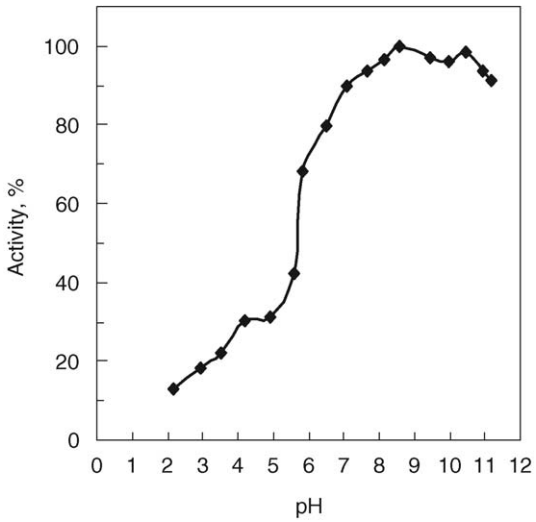

Fig. 4. pH-Stability of the *T. molitor* midgut proteinase. Maximal activity with 0.5 mM GlpAALpNA was assumed as 100%.

ity was less than 30%. A sharp drop in enzyme stability was observed between pH 5.8 and 5.6, from 68% to 42% of residual activity, respectively. The optimum temperature for the activity of TmC1 towards GlpAALpNA at pH 8.0 was 51 °C (Fig. 5). The proteinase was stable at least for 30 min at temperatures not exceeding 43 °C (Fig. 6).

A study of TmC1 activity towards a range of different *p*-nitroanilide substrates (Table 2) revealed the highest activity with the two chymotrypsin substrates containing four amino acid residues with Phe and Leu in P1 position, SucAAPFpNA and SucAAPLpNA. TmC1 had lower activity with only one substrate type containing three amino acid residues—Ala–Ala–Leu—and did not hydrolyze chymotrypsin and trypsin substrates containing one amino acid residue. Substitution of the Glp protective group in GlpAALpNA to a much more hydrophobic *N*-benzoyloxycarbonyl (Z) resulted in almost a fivefold decrease in the activity of the enzyme at equal DMF concentrations. With all tested substrates the activity was maximal at low DMF concentration

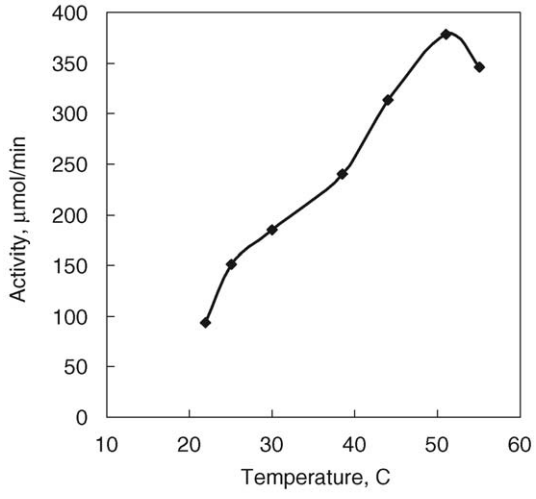

Fig. 5. Effect of temperature on the activity of *T. molitor* midgut proteinase with 0.5 mM GlpAALpNA.

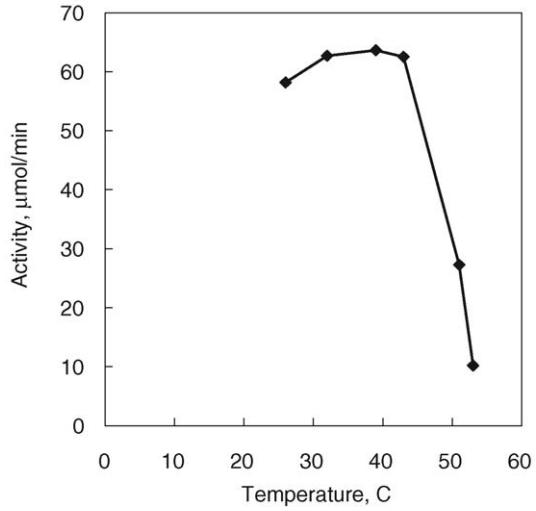

Fig. 6. Temperature stability of the *T. molitor* midgut proteinase. Residual activity was measured with 0.5 mM GlpAALpNA.

Table 2
Activity of *T. molitor* larval midgut chymotrypsin proteinase TmC1 with different substrates. All substrates were 0.5 mM, with the exception of marked ([a]) substrates, where the concentration was 0.25 mM

Substrate	Concentration of DMF (%)	Initial rates (µmol/min)
Suc–Ala–Ala–Pro–Phe–pNA	2.5	9040
	10	2870
	20	909
Suc–Ala–Ala–Pro–Leu–pNA	2.5	1760
Glp–Ala–Ala–Leu–pNA	2.5	734
	10	261
	20	85
Z–Ala–Ala–Leu–pNA	20	16.1
Z–Gly–Gly–Leu–pNA	10; 20	0
Z–Ala–Ala–Phe–pNA[a]	10	0
Z–Gly–Ala–Phe–pNA	10	0
Z–Gly–Gly–Phe–pNA[a]	10	0
Glp–Gly–Gly–Phe–pNA[a]	2.5; 20	0
Z–Ala–Ala–Ala–pNA	10	0
Ac–Leu–pNA	10	0
Glp–Phe–pNA	2.5; 10	0
Bz–Phe–pNA	10	0
Suc–Phe–pNA	10	0
N–Ac–tyr–pNA	10	0
Ac–Ala–pNA	10	0
Bz–Arg–pNA	2.5	0

(2.5%) and sharply decreased at higher DMF concentrations (10% and 20%) necessary for the solubilization of hydrophobic substrates.

The reaction kinetics with appropriate *p*-nitroanilide substrates (SucAAPFpNA, SucAAPLpNA and GlpAALpNA) were according to the Michaelis–Menten equation. The lowest K_m value of 0.5 mM was observed for SucAAPLpNA (Table 3). The highest $k_{cat\ app}$ of 36.5 s^{-1} was for SucAAPFpNA. The $k_{cat\ app}/K_m$ ratio was the highest also for SucAAPFpNA (23,040 s^{-1} M^{-1}), and was the lowest for GlpAALpNA (1920 s^{-1} M^{-1}).

Table 3
Kinetic parameters for the hydrolysis of *p*-nitroanilide substrates by *T. molitor* larval midgut chymotrypsin proteinase TmC1

Substrate	K_m (M 10^{-3})	$k_{cat\ app}$ (s^{-1})	$k_{cat\ app}/K_m$ (s^{-1} M^{-1})
SucAAPFpNA	1.59	36.5	23,040
SucAAPLpNA	0.5	3.77	7540
GlpAALpNA	3.86	7.41	1920

A study of the effect of inhibitors and activators of the active site groups demonstrated that the activity was completely inhibited by an inhibitor of serine proteases, PMSF (Table 4). Inhibitors of metalloproteinases, EDTA, and cysteine proteinases, E-64 and iodoacetamide, had little to no effect on the activity of TmC1. Specific inhibitors of chymotrypsin, TPCK, and trypsin, TLCK, also had no effect on the enzyme, while the sulfhydryl reagents 2-mercaptoethanol and dithiothreitol demonstrated substantial (51% and 75%, respectively) inhibition of activity. A study of the effect of proteinaceous proteinase inhibitors from plant seeds and a cockroach, *N. cinerea*, midgut revealed that STI was a very effective inhibitor of this enzyme (Table 5). Buckwheat, triticale and *N. cinerea* inhibitors were also potent, but less effective.

The data on the amino acid composition of TmC1 are presented in Table 6. The enzyme contained 6 Cys residues, 1 Trp and more acidic (17 + 15) than basic (6 + 3 + 5) residues. The alkaline p*I* value (8.4) of the proteinase is probably due to the high content of Asn and Gln in the protein. The N-terminal amino acid sequence of TmC1, as determined by means of Edman degradation, was IISGSAASKGQFPWQ (Table 7). The TmC1 sequence had high % of identity with other insect chymotrypsins from the orders Lepidoptera, Orthoptera and Diptera (Brachycera) (53–67%). Mammalian chymotrypsin A was more similar to TmC1 than chymotrypsin C (47% vs. 33%).

Table 4
Effect of the active site inhibitors and activators on the hydrolysis of 0.5 mM GlpAALpNA by *T. molitor* larval midgut chymotrypsin proteinase TmC1

Reagent	Concentration (mM)	Residual activity (%)
PMSF	1	2.3
TLCK	0.1	97.4
TPCK	0.1	108.5
E-64	0.01	114.0
Iodoacetamide	1	105.2
2-Mercaptoethanol	0.1	51.2
Dithiothreitol	1	74.8
EDTA	10	90.1

Table 5
Effect of proteinaceous proteinase inhibitors on the activity of chymotrypsin-like proteinase TmC1 from *T. molitor* larvae midgut. Enzyme activity was determined with 0.5 mM GlpAALpNA. E/I_{50}—molar ratio inhibitor:enzyme at 50% inhibition

Inhibitor	Mm (kDa)	I_{50}/E (mol/mol)
STI	20.1	0.35
Buckwheat cationic PI	6.0	2.18
Buckwheat anionic PI	7.8	3.68
Triticale anionic PI	17.0	3.58
N. cinerea SI1	13.0	3.35

Table 6
Amino acid composition of chymotrypsin proteinases

Amino acid residues per protein molecule	*T. molitor*			*A. ipsilon* AgiC6, [21]	Bovine α-chymotrypsin, [22]
	Larval		Imaginal		
	TmC1	[1]	[1]		
Cys	6	8	1	6	10
Asp	17	20	15	27	22
Thr	22	12	10	13	22
Ser	38	14	13	25	27
Glu	15	22	13	12	15
Pro	7	10	11	8	9
Gly	34	21	15	31	23
Ala	17	19	11	21	22
Val	13	15	11	23	23
Met	0	2	1	0	2
Ile	11	8	8	15	10
Leu	17	12	11	25	19
Tyr	11	5	3	4	4
Phe	6	7	4	9	6
Lys	6	6	16	1	14
His	3	3	2	6	2
Trp	1	nd	nd	3	8
Arg	5	7	8	11	3
Total amino acid residues	227	191	153	240	241

4. Discussion

Chymotrypsin and trypsin proteinases are the major cationic proteinases in the PM of *T. molitor* [11]. Separation of these PM proteinases was achieved by gel filtration on Superdex-75 using a neutral phosphate buffer without salt. In this buffer, TmC1, unlike trypsin, was retarded on the column and eluted together with peptides of about 3 kDa. The elution profile corresponding to the actual molecular mass of TmC1 (23.0 kDa according to SDS-PAGE) was observed with 0.5 M NaCl, whereby TmC1 coeluted with a trypsin of similar mass, and they were not separated. After the two-step purification, TmC1 was homogenous according to SDS-PAGE and in-gel activity determination. The estimated molecular mass of TmC1, 23.0 ± 1.4 kDa, was similar to other mammalian and insect chymotrypsin proteinases [2,3,6,29]. TmC1 is a cationic protein with an alkaline p*I* of 8.4. Its high activity in alkaline buffers of pH 7.5–10.0 is in agreement with the average pH 7.9 reported for the PM contents [30] The high stability of TmC1 (more than 80%) in the pH range 6.5–11.2 suggests that the enzyme is very stable in the PM. Its stability in the middle midgut, where average pH is 5.9–6.5 (Vinokurov et al., manuscript in preparation), was slightly reduced (up to 68%), and in the AM, where the pH is 5.6 [30], the stability of the enzyme was reduced to 42%. A sharp reduction in pH-stability in the pH interval 5.8–5.6 suggests that enzyme compartmentalization regulates TmC1 activity. The low stability of the enzyme in buffers with acidic pH

Table 7
Alignment of N-terminal amino acid sequences of chymotrypsin-like enzymes. The abbreviations of the chymotrypsins include the first letters of the species Latin name, the first letter of the enzyme name and the number or type of the enzyme if it exists

Enzyme source	Reference	Enzyme	N-terminal sequence	Identity (%)
T. molitor	Present study	TmC1	**IISGSAASKGQFPWQ**	100
Lepidoptera:				
H. zea	[21]	HzC4	**I**VG**GS**TS**S**L**GAFPYQ**	53
H. zea	[21]	HzC20	**I**VG**GSTASLGQFPYQ**	67
H. zea	[21]	HzC21	**I**VG**GS**NANL**GQFPYQ**	60
A. ipsilon	[21]	AgiC5	**I**VG**GS**LS**S**L**GQFPHQ**	60
A. ipsilon	[21]	AgiC6	**I**VG**GSASSLGQFPYQ**	67
Manduca sexta	[7]	MseC	**I**VG**GSSSSVGQFPYQ**	60
Orthoptera:				
L. migratoria	[8]	LmC1	**IIGGTTAS**IANY**PWQ**	53
L. migratoria	[8]	LmC2	**IIGGS**NADIADY**PWQ**	53
Coleoptera:				
Rhyzopertha dominica	[23]	RdCl	**I**VG**GS**DAEEA**QFP**FI	47
Phaedon cochleariae	[24]	PcC	**I**VN**G**QEVVPHSI**PYQ**	28
Diptera:				
Drosophila melanogaster	http://www.flybase.bio.Indiana.edu	DmCG6467	**ITGGS**NAAV**GQFPYQ**	60
D. melanogaster	http://www.flybase.bio.Indiana.edu	DmCG7542	**ITNG**EPAEV**GQFPYQ**	53
Lucilia cuprina	[25]	LucC	**ITNG**QDAVM**GQFPYQ**	53
Anopheles darlingi	[26]	AndC1	VVG**GQEAEEGSAPYQ**	33
A. darlingi	[26]	AndC2	VVG**GQDAEESSAPYQ**	28
Anopheles aquasalis	[26]	AnaC1	VVG**GQEAEEGSAPYQ**	33
Mammals:				
Bos taurus	[22]	BtCA[a]	**I**VN**GEEAVPGSWPWQ**	47
Homo sapiens	[27]	HsCl[a]	**I**VN**GENAVLGSWPWQ**	47
B. Taurus	[28]	BtCC[a]	VVG**GEDAIPHSWPWQ**	33

[a] β-chain.

values below 4.0 (less than 30%) is typical for insect chymotrypsins, unlike mammalian enzymes [2,3].

The data on the substrate specificity indicate that TmC1 is a chymotrypsin-like proteinase with an extended binding site. The enzyme hydrolyzed only long peptide substrates, containing three or four amino acid residues with Phe or Leu in the P1 position, but did not hydrolyze *p*-nitroanilide substrates containing one amino acid residue, which are hydrolyzed efficiently by mammalian chymotrypsins, and Bz–Arg–pNA, a common substrate for trypsins. TmC1 had the highest affinity for SucAAPLpNA, while the highest reaction rate ($k_{cat\ app}$) and efficiency ($k_{cat\ app}/K_m$) were observed for SucAAPFpNA. The effect of the active site inhibitors and activators indicated that the purified enzyme is a serine proteinase. The proteinase was insensitive to the specific short substrate-like chymotrypsin inhibitor TPCK, and this insensitivity is in agreement with the absence of enzyme activity with the short one amino acid residue *p*-nitroanilide substrates. Inhibition of TmC1 by sulfhydryl reagents suggested that disulfide bonds are important for proteolytic activity and may be critical for active site integrity. The sensitivity of insect chymotrypsin-like enzymes to sulfhydryl reagents has not been reported. The effect of proteinase inhibitors from plant seeds and *N. cinerea* midgut demonstrated that STI was the most effective inhibitor of TmC1 and reacted with the proteinase in a ratio close to equimolar (1:0.7).

The data on the sensitivity of TmC1 to STI correlated with the results of the alignment of N-terminal amino acid sequence of TmC1 with the sequences of chymotrypsins from lepidopterans *Helicoverpa zea* and *Agrotis ipsilon* [21]. The highest percent of identity of TmC1 was with HzC20 and AgiC6 (67%). Expression of the latter two chymotrypsins was higher in insects reared without dietary STI, and these enzymes were sensitive to STI. Chymotrypsins HzC4, HzC21 and AgiC5 were predominantly expressed in insects reared on diets containing 1% STI and were insensitive to STI. Identity of TmC1 N-terminal sequence with these sequences was lower (53–60%). It is important to note that *T. molitor* larvae used for isolation of TmC1 were reared on a diet devoid of proteinase inhibitors.

The data indicate that TmC1 differs in several characteristics from mammalian chymotrypsins but is similar to several recently reported insect digestive chymotrypsin-like proteinases. Chymotrypsin-like proteinases from the Lepidoptera *Heliothis virescens* [5], *Spodoptera littoralis* [6] and *Lacanobia oleracea* [31], as well as the orthopteran *Locusta migratoria* [8] also have an extended binding site and hydrolyze only long peptide substrates. These enzymes, as well as chymotrypsins from *H. zea* and *A. ipsilon* [21] and a cockroach *Periplaneta americana* [32], also were insensitive to TPCK, although chymotrypsin-like enzymes from *L. migratoria*, which also did not hydrolyze short substrates, were sensitive to TPCK [8]. A strong inhibitory effect of STI and several other proteinaceous trypsin inhibitors on digestive chymotrypsin-like proteinases, including TmC1, is typical for insects but is not so noted for mammals [2,5,6,8]. The three

conserved disulfide bridges predicted for TmC1 by the amino acid analysis are common to most invertebrate chymotrypsins, differing from the five disulfide bridges found in bovine chymotrypsin [7,21,33,34].

The amino acid composition of TmC1 differed significantly from previously isolated chymotrypsins from *T. molitor* larvae and imago [1], although the Mm of the two larval enzymes were similar: 23.0 kDA for TmC1 and 23.4 kDa for previously described enzyme. TmC1 and chymotrypsin AgiC6 from *A. ipsilon* have the same number of cysteine amino acid residues, also different from the previously described *T. molitor* chymotrypsins as well as bovine α-chymotrypsin. TmC1 also contains much more Thr, Ser, Tyr, Gly and Leu residues. Additionally, other *T. molitor* proteinases reportedly hydrolyzed short chymotrypsin substrates and imaginal enzyme was inhibited by TPCK [1]. Therefore, TmC1 is distinct from the previously isolated chymotrypsins from *T. molitor* larvae and imago. However, TmC1 is similar to an unusual serine proteinase, named α-protease, which was found earlier in *T. molitor* adults by Zwilling et al. [14] and was originally classified separately (EC 3.4.21.18). This proteinase did not hydrolyze a common chymotrypsin substrate, *N*-acetyl-L-tyrosine ethyl ester, and was insensitive to TLCK and TPCK, but was inhibited by PMSF. The imaginal enzyme was a cationic protein consisting of one subunit with a molecular mass of 24 kDa. It was inhibited by STI, soybean Bowman–Birk trypsin and chickpea trypsin and chymotrypsin inhibitors [14]. Therefore, our data conflict with the previous conclusion of significant differences in digestive proteinases reported in imago and larvae of *T. molitor* [1].

5. Conclusion

Thus, a new proteinase from the PM of a coleopteran *T. molitor* larvae was purified to homogeneity and characterized. The results reported above indicate that the enzyme is a chymotrypsin-like serine proteinase with an extended binding site, which does not hydrolyze short chymotrypsin substrates and is not affected by TPCK. This report extends the knowledge about the distribution of chymotrypsin-like proteinases with an extended binding site, presenting the first detailed characteristics of such enzyme among the insect order Coleoptera.

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