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# BIOCHEMICAL EVIDENCE OF SPECIFIC TRYPSIN–CHYMOTRYPSIN INHIBITORS IN THE RHYNCHOBDELLID LEECH, *THEROMYZON TESSULATUM*

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The presence of two specific trypsin–chymotrypsin inhibitors from head parts of the rhynchobdellid leech *Theromyzon tessulatum* is reported. Two proteins, anti-trypsin–chymotrypsin A (ATCA; 14636.6 ± 131 Da) and anti-trypsin–chymotrypsin B (ATCB; 14368 ± 95 Da) were purified by size exclusion and anion-exchange chromatography followed by reversed-phase HPLC. Based on amino-acid composition, N-terminal sequence determination (MELCELGQSCSRDNPQPSNM), matrix assisted laser desorption-time of flight measurement (MALDI-TOF), trypsin mapping comparison, inhibition constant determination ($K_i$), and influence on amidolytic activity of different serine proteases, it is demonstrated that ATCA and ATCB are novel and highly potent serine-protease inhibitors of trypsin and chymotrypsin (ATCA: 350 fM towards trypsin and chymotrypsin; ATCB: 400 and 75 fM towards trypsin and chymotrypsin, respectively). It is further surmised that ATCA and ATCB are linked, in that ATCB would lead to the formation of ATCA after loss of few amino acid residues.

*Keywords:* Trypsin–chymotrypsin inhibitors; Rhynchobdellid leech; Anti-trypsin–chymotrypsin A and B

*Abbreviations:* ATC, anti-trypsin–chymotrypsin; FPLC, fast protein liquid chromatography; HPLC, high pressure liquid chromatography; MALDI-TOF, matrix assisted laser desorption-time of flight; TBS, tris-buffered saline; PTH, phenylthiohydantoin

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## INTRODUCTION

Recent evidence indicates that proteases play a crucial role in numerous pathological processes. For example, destruction of extracellular matrix of articular cartilage and bone which occurs in arthritic joints is thought to be mediated by excessive proteolytic activity.[1] In other inflammatory conditions, it is suggested that tissue destruction is caused by proteolytic enzymes released from leukocytes.[2] This may occur in emphysema, gingivitis and tumor invasion.[2,3] In emphysema, a chronic lung disease characterized by the enlargement and destruction of the alveoli, the discovery of $\alpha$1-antitrypsin deficiency offered a completely new perspective for understanding this disease.[3] The deficiency of $\alpha$1-antitrypsin disturbs the alveolar protease–antiprotease balance, leading to elastase mediated tissue destruction and chronic pulmonary emphysema.[1] Given this data, it may be possible to alleviate these conditions by finding specific inhibitors of these enzymes while leaving other proteases unaffected.

In this regard, "nature" may have provided some answers to this intriguing question. In haematophagous leeches, two groups of serine protease inhibitors can be found. The first is related to specific inhibitors of proteases interfering with the activation of blood clotting system, i.e., inhibitors of thrombin (hirudin,[4] haemendin[5]) or inhibitors of factor Xa (antistasin[6] and ghilanten[7]). The second one is a class of inhibitors of proteases which are known to act on the extracellular matrix e.g. elastase, cathepsin G. These inhibitors are bdellin,[8] eglin,[9] gelin[10] and hirustasin.[11]

In the rhynchobdellid leech *Theromyzon tessulatum*, serine protease inhibitors have not been isolated. We recently characterized a novel chymotrypsin inhibitor, closely related to the CI-2 from barley seeds[13] but different in structure to other chymotrypsin inhibitors isolated in jawed leeches (unpublished data). In the present study, we demonstrate for the first time the presence and characterization of two novel trypsin–chymotrypsin inhibitors from the head of the rhynchobdellid leech *T. tessulatum*. This result demonstrates that during evolution these enzymes are quite important and may represent the need in these animals and mammals to modulate serine proteases, enzymes known to have potential to cause damage.

## MATERIALS AND METHODS

### Materials

Starved *T. tessulatum* were maintained and breed under constant laboratory conditions as described elsewhere in detail.[14] Chromogenic substrates

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S-2765, S-2238, S-2586 were purchased from Kabi Diagnostica (Saint Quentin en Yvelines, France). Acetonitrile (HPLC grade) was obtained from Baker. Trifluoroacetic acid (TFA) was from Pierce (Strasbourg, France), porcine pancreatic elastase (EC 3.4.21.11), chymotrypsin (EC 3.4.21.1), trypsin (EC 3.4.21.4), cathepsin G (EC 3.4.21.20), thrombin (EC 3.4.21.5) and the chromogenic substrates, (benzoyl-arginine-*p*-nitroanilide, *N*-succinyl Ala-Ala-Ala-*p*-nitroanilide, *N*-succinyl Ala-Ala-Pro-Phe-*p*-nitroanilide) were obtained from Sigma (Saint Quentin Fallavier, France). Mono Q-Sepharose, Superdex G75 and molecular weight calibration markers for SDS-PAGE were purchased from Pharmacia (Saint Quentin en Yvelines, France) and trypsin sequencing grade from Boehringer (Meylan, France). All other reagents were of analytical grade.

## Isolation of the Trypsin–Chymotrypsin Inhibitors

### *Chromatography on Superdex G75 Column*

After anaesthesia in 0.01% chloretone, head parts of *T. tessulatum* were excised, immediately frozen in liquid nitrogen and stored at −70°C. Five hundred head parts were homogenized and extracted with 5 ml of TBS (20 mM Tris/HCl, pH 8.8 containing 200 mM NaCl) at 4°C. After centrifugation at 12,000 rpm for 30 min at 4°C, the pellet was re-extracted twice. Supernatants were filtered on nitrocellulose membrane (0.45 µm pore size, Millipore, Saint Quentin en Yvelines, France). The extract was applied onto a FPLC (fast protein liquid chromatography) column (Superdex G75, 16/60) pre-equilibrated with TBS at a flow rate of 1 ml/min and eluted with the same buffer. The column effluent was monitored by its absorbance at 280 nm. All column fractions were assayed for protease inhibitor activity against trypsin.

### *Chromatography on Mono Q*

Fractions containing trypsin inhibitors were pooled before being loaded onto a Mono Q (FPLC, HR 5/5, Pharmacia) column, equilibrated in TBS. The column was washed with the same buffer and eluted by a combination of linear and step gradients using TBS and TBS containing 0.15 M NaCl at a flow rate of 1 ml/min.

### *Chromatography on Reversed-Phase HPLC*

Pooled fractions containing the trypsin inhibitors were separated on a $C_8$ Lichrosphere Rp100 column (125 × 4.6 mm), equilibrated in water. Elution

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was performed with a discontinuous linear gradient of acetonitrile in water from 0% to 50% in 15 min and from 50% to 80% in 10 min at a flow rate of 1 ml/min. The column effluent was monitored by absorbance at 226 nm and presence of trypsin inhibitor material was detected by chromogenic assay.

### *Final Purification*

Fractions containing the trypsin inhibitors were concentrated 20-fold in a vacuum centrifuge (Savant, Saint Quentin en Yvelines, France) before being applied to a $C_8$ Lichrosphere Rp100 column (125 × 4.6 mm) and eluted with a linear gradient of acetonitrile in water acidified with 0.1% TFA from 0% to 60% in 60 min at a flow rate of 1 ml/min. All HPLC purifications were performed with a Beckman Gold HPLC (Gagny, France) system equipped with a photodiode array detector Beckman 168.

## Chromogenic Assay

During the purification procedure, inhibitory activity was assessed by measuring the inhibition of the release of *p*-nitroaniline from synthetic substrate, S-2765, catalyzed by trypsin. Samples were incubated with a known amount of trypsin in TBS for 15 min at 25°C. The reaction was started by addition of the chromogenic substrate. The released nitroaniline was monitored photometrically at 405 nm for 3.5 min using a Dynatec™ MR-5000 at room temperature. A control reaction, in the absence of trypsin inhibitor, was carried out under identical conditions. The reaction was stopped by the addition of 0.2 ml 50% acetic acid. The unit of protease inhibitory activity (IU) is calculated as μg of protease inhibited at 100% and specific activity as units per mg protease inhibitor.[12]

## Protein Assay

Protein concentrations were determined by the Bradford procedure[15] using γ-globulin as a standard.

## Electrophoresis

Electrophoretic analysis of reduced and denatured proteins was performed in 10–25% SDS/polyacrylamide gradient gels as described by Laemmli.[16]

## Structural Analysis of Antitrypsin Inhibitors

### *Amino Acid Composition*

Quantitative amino acid analysis was carried out with a Beckman 6300. Samples of purified trypsin–chymotrypsin inhibitors were hydrolyzed at

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110°C for 24 h in 50 µl 6 M HCl in a tube sealed under vacuum. Cysteine was determined as cysteic acid and methionine as methionine sulphone.

### *Reduction and S-β-Pyridylethylation*

The inhibitor (400 pmol) was dissolved in 40 µl 0.5 M Tris/HCl pH 7.5, 2 mM EDTA containing 6 M guanidine hydrochloride to which 2 µl 2 M dithiothreitol were added in the dark. The sample was flushed with nitrogen and incubated at 45°C for 1 h. Freshly distilled 4-vinylpyridine (2 µl) was added and the mixture incubated for 10 min at 45°C in the dark. The pyridylethylated peptide was separated by reversed-phase HPLC prior to microsequencing.

### *Microsequence Analysis*

Automated Edman degradation of the reduced and alkylated peptide and detection of phenylthiohydantoin (PTH-Xaa) derivatives were performed on a pulse-liquid automatic sequenator (Applied Biosystems™, model 473A).

### *Matrix Assisted Laser-Desorption-Time of Flight (MALDI-TOF)*

MALDI-TOF-MS was performed on a Bruker laser desorption time of flight mass spectrometer (Bruker™, Bremen, Germany) operating in the positive mode. The analyses were performed as described previously.[17]

### *Enzymatic Digestions and Separation of Products of Digestion*

Trypsin digestion: 5 nmol of pure inhibitors were treated with trypsin (Boehringer) at an inhibitor/trypsin ratio of 1–100 for 17 h at 37°C in 100 µl of 100 mM Tris–HCl, pH 8.5. The digestion was stopped by adding 100 µl of acidified water (0.1% TFA). The peptide mixture produced by enzymatic digestion was applied on an aquapore OD 300 C18 column (250 × 4.6 mm, Browlee™ Associates) equilibrated with acidified water. Elution was performed with a linear gradient 0–80% of actonitrile in acidified water over 80 min at a flow rate of 1 ml/min.

## Determination of Equilibrium Constants

To determine the specificity of trypsin inhibitors, their action to different serine proteases were compared using established chromogenic assay methods (see above). Equilibrium dissociation constants ($K_i$) for the complexes of each inhibitor with individual proteases were determined essentially as described by Bieth.[18] Briefly, increasing concentrations of the

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inhibitor were incubated with a constant concentration of the enzyme during 30 min at 37°C. Substrate was added and the residual enzyme activity was measured. Apparent $K_i$ values were calculated by fitting the steady-state velocities to the equation for tight-binding inhibitors[19] using nonlinear regression analysis.

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## RESULTS AND DISCUSSION

### Purification of Trypsin Inhibitors

The purification of the trypsin inhibitors is summarized in Table I. After a TBS extraction, filtered supernatants were subjected to a gel permeation chromatography on Superdex G75 column. The inhibitory action on trypsin was assayed on eluted fractions. A single zone at a retention time of 18–22 min corresponding to proteins of molecular mass ranging between 20–14.4 kDa, was detected (data not shown). The inhibitory active fractions were pooled and subjected to anion exchange chromatography on a Mono Q column using a stepwise gradient of NaCl from 0–1.5 M. Two inhibitory active zones eluted between 0.46–0.75 M NaCl for Z1 and 0.75–1 M NaCl for Z2 were found (Figure 1). Finally, the inhibitors were purified to homogeneity on a C8 Lichrosphere column with a linear gradient of 1%/min of acetonitrile in acidified water. A single peak presenting the inhibitory activity was obtained at a retention time of 33 min and at 35 min for the trypsin inhibitors A (ATCA) and B (ATCB) contained in Z1 and in Z2, respectively (Figure 2). Purity control of the isolated inhibitors was performed by mass spectrometry measurement with MALDI-TOF-MS on native proteins (Figure 3). Molecular monoisotropic masses of 14636.6 ± 131 Da for ATCA (Figure 3A) and 14368 ± 95 Da for ATCB (Figure 3B) were obtained despite the low level of the isolated inhibitors.

TABLE I Purification of anti-trypsin–chymotrypsin inhibitors (A and B)

| *Step* | *Inhibitor* (μg) | | *Specific activity* (IU/mg) | | *Total protein* (mg) | | *Purification Factor* | |
|---|---|---|---|---|---|---|---|---|
| Leech extract | 266 | | 8.72 | | 450 | | 1 | |
| Superdex G75 | 14 | | 164 | | 95 | | 19 | |
| Mono Q | ATCA | ATCB | ATCA | ATCB | ATCA | ATCB | ATCA | ATCB |
| | 0.48 | 0.42 | 4833 | ND | 0.47 | ND | 554 | ND |
| HPLC1 | 0.34 | 0.34 | 7363 | 5590 | 0.2 | 0.09 | 844 | 641 |
| HPLC2 | 0.25 | 0.35 | 9580 | 6628 | 0.17 | 0.07 | 1098 | 760 |

1 g leech extract was used as starting material. One inhibition unit (IU) is defined as the amount (μmol) of protease inhibited at 100% (see Materials and Methods section). ND: Not determined.

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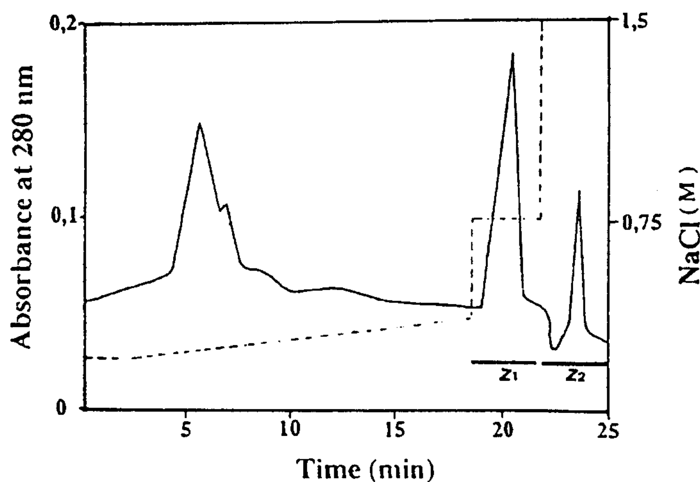


FIGURE 1 Anion exchange chromatography of *T. tessulatum* trypsin–chymotrypsin inhibitors. Solid bars indicate the active fractions, Z1 and Z2.

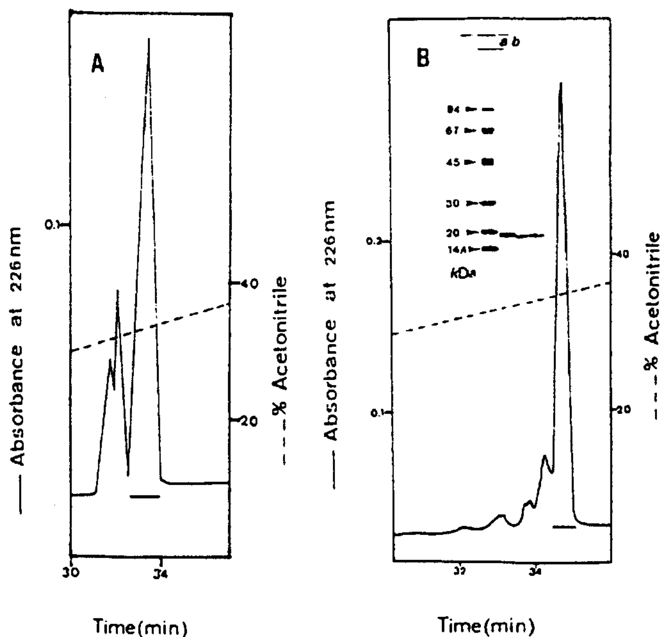


FIGURE 2 HPLC profiles of ATCA (A) and ATCB (B) at the final step of purification. Inset photographs correspond to ATCA (lane a) and ATCB (lane b) after SDS-PAGE. Gels are stained with silver. Arrows to the left correspond to molecular mass standards.

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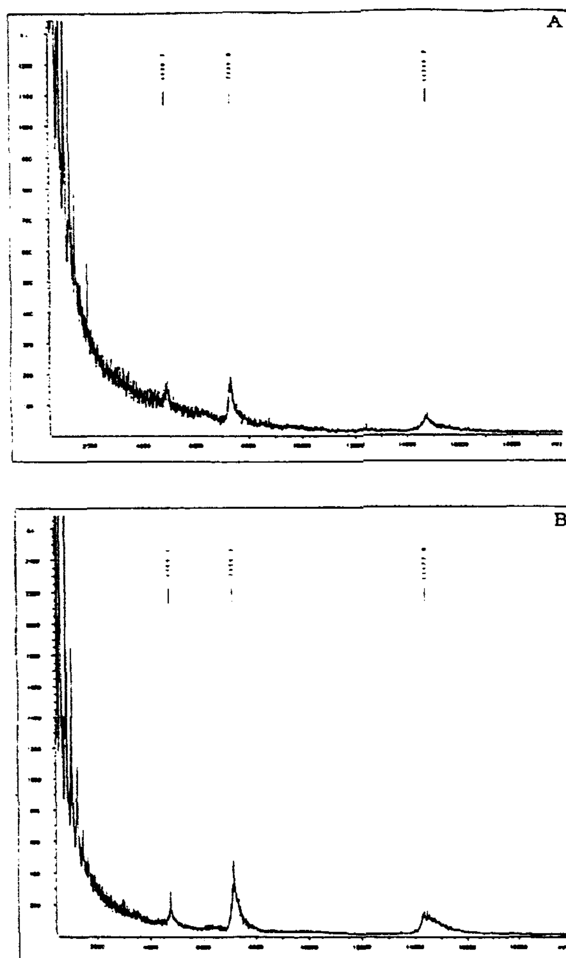


FIGURE 3 MALDI-TOF measurement of ATCA (A) and ATCB (B) at the final step of purification. Peaks at $m/z = 4830.7$ and $m/z = 7334$ for ATCA and at $m/z = 4769.1$ and $m/z = 7159.3$ for ATCB are multiple charged ions with three or two charges corresponding to a mass of $14{,}636.6 \pm 131$ Da for ATCA and of $14{,}368 \pm 95$ Da for ATCB.

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## Structure Identification of the Trypsin Inhibitors

MALDI-TOF measurement of reduced and s-$\beta$-pyridylethylated ATCA (16,869 Da) and ATCB (16,545 Da) and the comparison with mass measurements of the native inhibitors revealed that they possess 20 cysteines. Furthermore, amino acid compositions (Table II) show that these inhibitors are rich in the dicarboxylic amino acids, cysteine and glycine. A slight

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TABLE II Amino acid composition of the anti-trypsin–chymotrypsin inhibitors from the rhynchobdellid leech *T. tessulatum* and the bdellin c from *Hirudo medicinalis*

| *AA* | *ATCA* (Mol%) | *ATCB* (Mol%) | *Bdellin c* (Mol%) |
|---|---|---|---|
| Asp | 16.2 | 14 | 15.2 |
| Thr | 9.3 | 6.4 | 8.6 |
| Ser | 5.4 | 4 | 4.3 |
| Glu | 13.9 | 13.8 | 13 |
| Pro | 6.6 | 5.8 | 0 |
| Gly | 11 | 9.5 | 6.5 |
| Ala | 2.3 | 7.4 | 8.6 |
| Cys/2 | 10 | 12.5 | 11 |
| Val | 1.2 | 2 | 8.6 |
| Met | 1.1 | 0.5 | 0 |
| Ile | 1.5 | 1.5 | 0 |
| Leu | 3.9 | 4.6 | 6.5 |
| Tyr | 4 | 4.2 | 2.2 |
| Phe | 1.7 | 2.6 | 0 |
| His | 1 | 0.8 | 13 |
| Lys | 4.1 | 5.3 | 0 |
| Arg | 3.5 | 5.3 | 2.17 |

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difference exists between the two proteins; ATCB seems to be richer in alanine. These data demonstrate that the inhibitors are different from bdellins, isolated from the jaw leech *H. medicinalis* (Table II). In fact, bdellin c is rich in cysteine and histidine but is devoid of proline in contrast to the *T. tessulatum* trypsin inhibitors.[8] The hypothesis that *T. tessulatum* trypsin–chymotrypsin inhibitors are novel leech trypsin inhibitors is also demonstrated by the N-terminal sequence (MELCELGQSCSRDNPQPSNM) obtained on each (30 pmol) of the s-$\beta$-pyridylethylated purified inhibitors with a repetitive yield of 95%.

## Specificity of ATCA and ATCB

Characterization of their activity and their specificities towards various serine proteases e.g. chymotrypsin, trypsin, cathepsin G, elastase, thrombin and factor Xa reveals that these inhibitors are specific for trypsin and chymotrypsin (Table III). ATCA and ATCB present the same high affinity towards trypsin ($K_i$ of around 400 fM). However, a difference exists between these inhibitors in regard to chymotrypsin inhibition. ATCB is more potent than ATCA towards this enzyme (75 fM vs 400 fM, respectively). In order to determine if a link between the two inhibitors exists, a trypsin mapping with an excess of trypsin was performed on these enzymes (Figure 4). After

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TABLE III Trypsin–chymotrypsin inhibitors specificities

| *Enzymes* | $K_i$ (fM) ATCA | $K_i$ (fM) ATCB |
|---|---|---|
| Chymotrypsin | $350 \pm 30$ | $400 \pm 35$ |
| Trypsin | $360 \pm 28$ | $75 \pm 12$ |
| Cathepsin G | n.i. | n.i. |
| Thrombin | n.i. | n.i. |
| Elastase | n.i. | n.i. |
| Factor Xa | n.i. | n.i. |

n.i.: no inhibition.

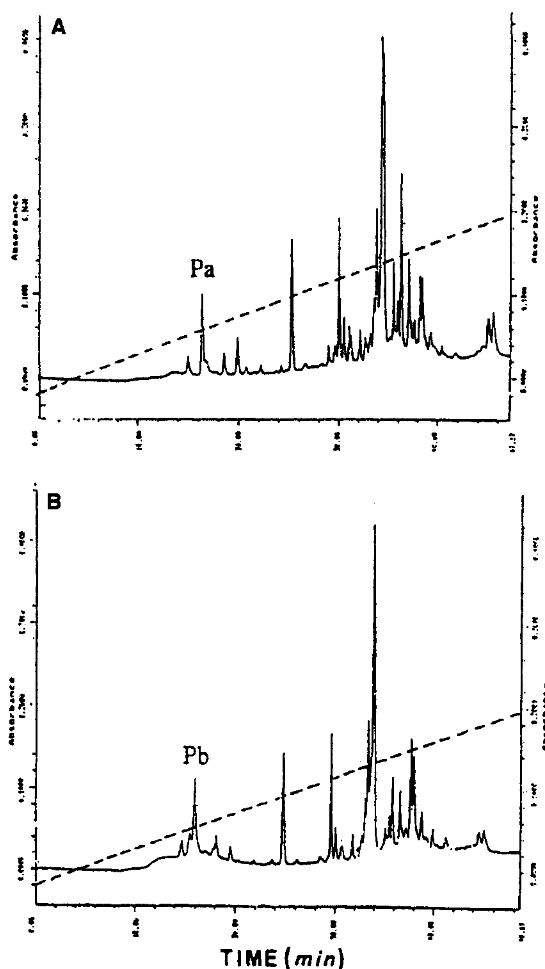


FIGURE 4 Tryptic digestion profiles of ATCA (A) and ATCB (B) after reversed-phase HPLC separation. The inhibitor/trypsin ratio used was 1–100, with digestion conducted during 17 h at 37°C. Elution position of peptides (Pa and Pb) are indicated in the graphs.

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reversed-phase HPLC separation, the same elution profile was found except for the presence of two peptides (Pa, Pb) at the retention time of respectively 16 min and 15.49 min (Pa: $m/z = 2456$ Da) in ATCA (Figure 4A) and at 16.41 min (Pb: $m/z = 1061$ Da) in ATCB (Figure 4B). This suggests that the two inhibitors are very closely related. This hypothesis was confirmed by the sequencing of Pa and Pb. They give a related primary sequence i.e. CSDWPCFQGCNRAYAGCMITCK for Pa and AYAGCMITCK for Pb. These data reveal that the Arg–Ala link in Pa was not cleaved by the trypsin enzyme. Two hypothesis could account for this, (1) the enzymatic digestion cleavage yield is different between the two inhibitors, (2) the CSDWPCFQGCNR sequence in Pa is not present in Pb. This last hypothesis allow us to postulate that ATCB results from ATCA post-translational processing or degradation. We surmise that the sequence difference between the two inhibitors may provoke a difference in the structure folding in the two inhibitors. This difference could explain why ATCB is a more active chymotrypsin inhibitor than ATCA.

## CONCLUSION

This is the first report of the isolation and the characterization of two highly specific and novel trypsin–chymotrypsin inhibitors from the rhynchobdellid leech *T. tessulatum*. Among the serine protease inhibitors found in jawed leeches, bdellins, trypsin–plasmin inhibitors found in *H. medicinalis*, are small proteins (20–30 kDa) containing a relatively large number of cysteine residues. They also contain many histidine residues but are devoid of any proline residues. These inhibitors reveal a strong homology with pancreatic trypsin inhibitors found in mammals.[8]

A hypothetical role for these inhibitors may be found in the preservation of the blood stored in the foregut.[20] In this regard, leeches are able to ingest large quantities of blood (8-fold the initial volume of their body) and store it over a period of several months. Conservation of the stored blood would require inhibition of proteases present in macrophages and granulocytes, since lysis of these cells could induce an untimely and uncontrolled digestion of their food, destroying its nutritive value. We postulate that ATCA and ATCB may also be involved in such a phenomenon. Furthermore, this action would imply that these molecules may be used to diminish the action of mammalian serine proteases activated in abnormal circumstances.[21] The involvement of proteolytic enzymes in a number of degenerative diseases e.g. pancreatitis (zymogenic forms of proteolytic enzymes

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(trypsin 1 and 2, chymotrypsin A[22,23] and elastase[24])), is becoming more evident as is the need for curtailing this activity in uncalled situations.[25-27] In this regard, aprotinin, a trypsin and kallikrein inhibitor isolated from bovine organs[28] has been used for more than three decades as an intensive care drug for acute pancreatitis,[29] demonstrating the significance of this class of enzyme.

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