



## Biochemical characterization of a native group III trypsin ZT from Atlantic cod (*Gadus morhua*)

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### ARTICLE INFO

#### Article history:

Received 15 June 2018

Received in revised form 4 December 2018

Accepted 11 December 2018

Available online 12 December 2018

#### Keywords:

Cold-adapted

Trypsin

Protease

### ABSTRACT

Atlantic cod trypsin ZT is biochemically characterized for the first time in this report in comparison to a group I trypsin (cod trypsin I). To our knowledge, trypsin ZT is the first thoroughly characterized group III trypsin. A more detailed understanding of trypsin ZT biochemistry may give insight into its physiological role as well as its potential use within the biotechnology sector. Stability is an important factor when it comes to practical applications of enzymes. Compared to trypsin I, trypsin ZT shows differences in pH and heat stability, sensitivity to inhibitors and sub-site substrate specificity as shown by multiplex substrate profiling analysis. Based on the analysis, trypsin ZT cleaved at arginine and lysine as other trypsins. Furthermore, trypsin ZT is better than trypsin I in cleaving peptides containing several consecutive positively charged residues. Lysine- and arginine-rich amino acid sequences are frequently found in human viral proteins. Thus, trypsin ZT may be effective in inactivating human and fish viruses implying a possible role for the enzyme in the natural defence of Atlantic cod. The results from this study can lead to multiple practical applications of trypsin ZT.

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### 1. Introduction

The global seafood industry produces large amounts of waste with huge potential for utilization [1]. As an example, the Norwegian and Icelandic seafood industries produced 1.246.000 tons of by-products in 2013 combined [2]. A portion of this material is used for low value products whereas a large share becomes waste with concurring strain on the environment [2]. Of the underutilized by-products from the seafood industry, fish viscera is a rich source of proteolytic enzymes such as trypsin, chymotrypsin and elastase [3]. A variety of industrial applications for fish trypsins have recently been reviewed [4]. Trypsin from Atlantic cod (*Gadus morhua*) gets special attention in the review [4] due to the thorough basic knowledge available on different cod trypsin isoenzymes and their potential use in high-value products for therapeutic applications for humans [5]. Notably, recent studies have shown that cod trypsin has efficacy against human respiratory viruses and herpes

simplex virus *in vitro* [5]. The epidermal mucus of fish contains trypsin like serine proteases that play a significant role in the natural defence of fish against pathogens such as viruses and bacteria [6–9]. Atlantic cod trypsin is produced from viscera as production of recombinant trypsin is not yet economically feasible [10–12]. Two main trypsin groups have been identified in Atlantic cod, termed group I and group III, based on their deduced amino acid sequence identities [13–16]. Different Atlantic cod group I trypsin isoenzymes have been isolated, purified and characterized [14,17]. Cod trypsin ZT belongs to group III trypsins. To our knowledge, cDNA sequences for group III trypsins have thus far only been described in fish [18]. Prior to this report, the group III trypsins have not been thoroughly characterized. Therefore, it was difficult to contemplate the physiological role or potential utilization of cod trypsin ZT, due to lack of biochemical data on the group III trypsins. In that context, cleavage specificity, sensitivity to inhibitors and stability is of interest. Stability analysis of proteins that have possible therapeutic applications or industrial use is important [4,19–21].

Here we present the first thorough biochemical characterization of a native group III trypsin, termed cod trypsin ZT. This enzyme was characterized in comparison to cod trypsin I, the best characterized group I cod

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trypsin [13,17]. Multiplex substrate profiling analysis and stability studies along with other methods were used for characterization of cod trypsin ZT. The aim was to search for novel properties of cod trypsin ZT different from those of cod trypsin I. The findings provide basic scientific information on the characteristics of a group III trypsin essential to identify possible practical uses of cod trypsin ZT.

## 2. Materials and methods

### 2.1. Chemicals

Dimethyl sulfoxide (DMSO), ethylenediaminetetraacetic acid (EDTA), Bis-Tris, glycine, sodium dodecyl sulphate (SDS), dithiothreitol (DTT), benzamidine, 2-mercaptoethanol, phenyl methyl sulphonyl fluoride (PMSF), soybean trypsin inhibitor, tosyl-lysyl-chloromethyl ketone (TLCK), *N*-*p*-Tosyl-L-phenylalanine chloromethyl ketone (TPCK), 2-Amino-2-(hydroxymethyl)-1,3-propanediol (TRIS) and bovine albumin protein standard were from Sigma Aldrich, St. Louis, MO, USA. Calcium chloride dihydrate, sodium chloride, sodium hydroxide, isopropanol and hydrochloric acid were from Merck, Darmstadt, Germany. Carboxybenzyl-Gly-Pro-Arg *p*-nitroanilide acetate salt (CBZ-GPR-pNA) was from Bachem AG, Bubendorf, Switzerland. ECL Plex goat Anti-rabbit IgG Cy5, ECL Plex goat Anti-mouse IgG Cy3 were from GE Healthcare, Chalfont St. Giles, UK. Micro BCA Protein Assay Kit was from Thermo Scientific. Benzamidine purified cod trypsin was from Zymetech (Reykjavik, Iceland).

### 2.2. Anion exchange chromatography

Cod trypsin ZT, previously termed trypsin 8 in Stefansson et al. [14] was purified for mass spectrometry analysis as described before [14]. Cod trypsin isoenzymes CTX-V5, CTX-V7 and cod trypsin I were purified as previously described [17].

For characterization of pH and temperature dependence and stability of cod trypsin ZT, the enzyme was isolated by applying benzamidine purified cod trypsin to a MonoQ HR 5/5 ion exchange column linked to an Äkta Purifier instrument from Pharmacia Biotech equipped with Unicorn software. The column was equilibrated with 25 mM Tris, 25 mM CaCl<sub>2</sub>, pH 7.5. After applying the sample, the column was washed with a linear 0–400 mM NaCl gradient in 5 column volumes and the enzymes eluted with a linear 400–1000 mM NaCl gradient in 20 column volumes at a flow rate of 1 mL/min. The sample was diluted 1:1 using equilibration buffer before being applied to the column. The MonoQ purification steps were conducted at 4 °C. Cod trypsin ZT for multiplex substrate profiling was purified with the MonoQ column using a buffer with 25 mM Tris, 25 mM CaCl<sub>2</sub>, 15% (vol/vol) glycerol, pH 7.5. The enzymes were eluted with a linear 0–150 mM NaCl gradient in 5 column volumes and linear 150–550 mM NaCl gradient in 20 column volumes at a flow rate of 1 mL/min.

### 2.3. Enzyme assays

Measurements of cod trypsin activity were made using a 12.5 mM stock solution of CBZ-GPR-pNA (in DMSO) as the substrate and 20 mM Tris, 1 mM CaCl<sub>2</sub>, pH 8.0 buffer. Final substrate concentration in the cuvette was 125 μM and each enzymatic unit was defined as the hydrolysis of 1 μmol/min using a molar extinction coefficient at 410 nm of 8800 M<sup>-1</sup> cm<sup>-1</sup> [22]. The contents of the cuvette (970 μL of buffer and 10 μL substrate) were mixed thoroughly with a plastic stirrer and allowed to equilibrate for at least 3 min in a 25 °C water bath before 20 μL of enzyme sample containing 0.25–1.1 U/mL were added. More concentrated samples were diluted to fall within this range. The enzymatic assays were performed using Ultraspec 6300 pro spectrophotometer.

### 2.4. Generation of phylogenetic tree

Similar fish trypsins were collected using a translated BLAST (TBLASTN) search in the nucleotide (nt) database at National Center for Biotechnology Information (NCBI) with cod trypsin ZT-4 using >99% Query cover and identity >62. Aligning of sequence data was performed using ClustalX 2.1. The phylogenetic tree was generated using the *seqinr* (version 3.1-3) and *ape* (version 3.4) packages in R (version 3.1.3). The tree was rooted to human trypsin (Uniprot accession number: P07477). Data on environmental/climate regions of the fish was gathered from [www.fishbase.org](http://www.fishbase.org) [23].

### 2.5. Determination of pH and temperature dependence and stability

To determine the relative activity of cod trypsin ZT at different pH and the effect of pH on the stability of cod trypsin ZT the following buffer solutions were used at 25 °C: 100 mM Bis-Tris (pH 4.0–7.5), 100 mM Tris-HCl (pH 7.5–9.0) and 100 mM glycine (pH 9.0–12.0). In the pH stability assay, a final concentration of 10 mM CaCl<sub>2</sub> was accomplished as was previously done for cod trypsin I [14]. For pH dependence determination, activity measurements were conducted using the different buffers, containing 10 mM CaCl<sub>2</sub>, using the method described above (2.3 Enzyme assays). The effect of pH on the stability of cod trypsin ZT was determined by measuring residual CBZ-GPR-pNA activity, compared to the activity in the sample at pH 8.5 at time zero, after incubation for 1 h and 24 h at 25 °C in the different buffer solutions described above.

To determine the residual activity of cod trypsin ZT and cod trypsin I at different temperatures the activity (see Section 2.3 Enzyme assays) was measured in a buffer (150 mM Tris, pH 8.0) in a thermostated cuvette preheated at the assay temperature before addition of the enzyme. The effect of temperature on the stability of cod trypsin ZT and cod trypsin I was determined by measuring residual CBZ-GPR-pNA activity (see Section 2.3 Enzyme assays) after incubation for 0, 15, 30, 45 and 60 min at different temperatures in a final concentration of 100 mM Tris, 10 mM CaCl<sub>2</sub>, pH 8.0. The pH of the buffers was corrected to pH 8.0 at each temperature that was tested. For the temperature stability assay, cod trypsin ZT samples were concentrated 3.5-fold using centrifugal filters Ultracel (10,000 MWCO, Millipore) for 2 × 30 min at 3000 rpm before being diluted into the incubation buffer. A T<sub>50</sub> value of trypsin ZT and trypsin I was calculated based on residual activity of the enzymes after incubation at different temperatures. The T<sub>50</sub> value is the temperature where half of the initial activity remains after 15 min incubation. The sigmoidal Boltzmann function was used to fit the curve resulting from the data using Origin 2019 software [24]. The equation of the fitted curve was used to calculate the temperature where half of the initial activity (0.5) remains.

### 2.6. Mass spectrometry

MALDI-TOF analysis on trypsin 8 was performed as previously described [14]. *In silico* protein digests were performed using the ProteinProspector v 5.6.2 Peptide/Protein MS Utility Program MS-Digest (<http://prospector.ucsf.edu/prospector/mshome.htm>).

### 2.7. Western blot analysis

For Western blot analysis, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was done according to Laemmli as previously described [17]. SDS-PAGE gels were immunoblotted in a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell from Bio-Rad according to the manufacturer's instructions. Protran BA 83 Nitrocellulose membranes from Whatman were used. After transfer, membranes were washed with 1 × TBS-T buffer (×3) for 5 min before being incubated with 3% BSA for 1 h to block the membrane. The membranes were incubated with primary antibodies for 2–5 h at room temperature or

overnight at 4 °C with 0.05% sodium azide to prevent microbial growth. After the primary antibody incubation, the membranes were washed with 1× TBS-T (×3) for 5 min and incubated with secondary antibodies for 1 h. After incubation, the membranes were washed with 1× TBS-T (×3) for 5 min, 1× PBS for 5 min and finally dried. Immunoblots were developed using a Typhoon fluorescent laser scanner (GE Healthcare, UK).

Antibodies against cod trypsin I and cod trypsin X were prepared against a peptide ((NH<sub>2</sub>—) CVLSGWVVRTMA (—COOH)) corresponding to residues 228–239 at the extreme C terminus of cod trypsin I and cod trypsin X, affinity purified from rabbit serum, and used at a 1:4000 dilution. Polyclonal antibodies against cod trypsin ZT-4 were produced in mouse ascitic fluid according to the method of Overkamp [25]. Mice were injected with recombinant cod trypsin ZT-4 (produced in *P. pastoris*), sacrificed on day 34 and the ascitic fluid collected. The antibodies were used at a 1:1000 dilution. The secondary antibodies ECL Plex goat anti-rabbit IgG Cy5 and ECL Plex goat Anti-mouse IgG Cy3 were purchased from GE Healthcare, and used at a 1:2000 dilution.

### 2.8. Inhibition studies

The serine protease inhibitors TPCK, PMSF, TLCK, soybean trypsin inhibitor, EDTA and 2-mercaptoethanol were used for inhibition studies. Cod trypsin ZT was incubated for 30 min with the inhibitors and residual CBZ-GPR-pNA activity measured.

### 2.9. Multiplex substrate profiling by mass spectrometry (MSP-MS)

Cod trypsin ZT or cod trypsin I was incubated (5, 15 and 60 min) with a library of peptides with an extensive physicochemical diversity as described in O'Donoghue, Eroy-Reveles et al. [26] and O'Donoghue, Knudsen et al. [27]. After incubation, cleavage sites were identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis [26]. To obtain similar amount of cleavages in the peptide library between the two enzymes, a concentration of 30 nM for cod trypsin ZT and 7.5 nM for cod trypsin I was used in the assay. The incubation of cod trypsin ZT or cod trypsin I with the peptide library was conducted in 20 mM TRIS, 10 mM CaCl<sub>2</sub> at pH 8.5 in a total reaction volume of 150 µL. To compare the substrate specificity of cod trypsin ZT and cod trypsin I, an iceLogo software was used to generate substrate specificity logos for amino acids at ±4 positions adjacent to the identified cleavage sites [28].

Cod trypsin ZT and cod trypsin I were purified as described above and supplied in 60 mM Tris, 30 mM CaCl<sub>2</sub>, 15% (vol/vol) glycerol at pH 8.5. The protein concentration in the samples was determined using the Micro BCA Protein Assay Kit.

## 3. Results

### 3.1. Identification of cod trypsin ZT

To identify novel cod trypsin isoenzymes a Basic Local Alignment Search Tool (BLAST) search with previously identified cod trypsins was conducted in GeneBank, codgene.ca and codgenome.no databases. Four expressed sequence tags (ESTs) were identified and the translated amino acid sequences were termed cod trypsinogen ZT-1, cod trypsinogen ZT-2, cod trypsinogen ZT-3 and cod trypsinogen ZT-4 (Supplementary file 1). The sequence identity between the four cod trypsinogen ZT isoenzymes is in the range of about 92% to 97% and based on their amino acid sequence they all belong to group III trypsins.

The four cod trypsinogen ZT amino acid sequences encode for a preproenzyme of 241 to 242 amino acids with a putative signal sequence of 15 amino acids based on SignalP 4.1 prediction [29], an activation peptide of 7 to 8 amino acids and a mature trypsin of 227 amino acids. Furthermore, the cod trypsinogen ZT isoenzymes contain the three highly conserved residues (Asp, His, Ser) involved in catalysis in

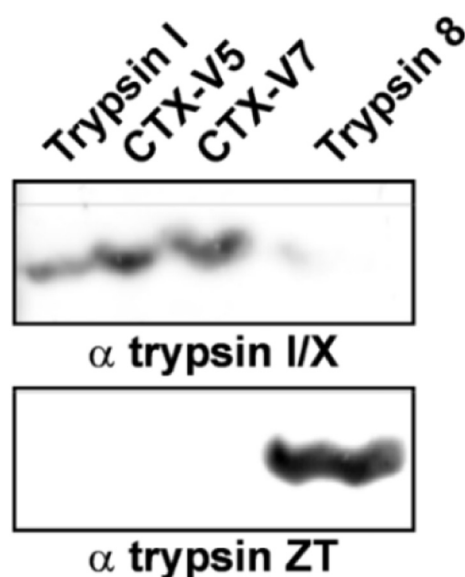
serine proteases. The mature forms of cod trypsinogen ZT-1 to ZT-4 are called cod trypsin ZT-1 to cod trypsin ZT-4, respectively.

An *in silico* digestion was performed on the cod trypsin ZT amino acid sequences and the masses obtained were compared to the observed masses from the MALDI-TOF analysis on cod trypsin 8 [14] (see Section 2.6 Mass spectrometry and Supplementary files 2 and 3). The *in silico* digest on all of the four cod trypsin ZT isoenzymes gave fragments that matched masses obtained from MALDI-TOF analysis on cod trypsin 8 (Supplementary files 2 and 3). Based on the number of matching masses, the best match to cod trypsin 8 was the amino acid sequence of cod trypsin ZT-3 with nine matching masses in the non guanidilated and the guanidilated sample of cod trypsin 8. Based on the analysis, the sequence coverage for cod trypsin ZT-1 to cod trypsin ZT-4 was 37%, 20%, 41% and 25%, respectively. Seven of the matching masses (both in the guanidilated and non guanidilated samples) found for cod trypsin 8 are unique to cod trypsin ZT-3 and cod trypsin ZT-1 compared to cod trypsin ZT-4 and cod trypsin ZT-2. Three (non guanidilated sample) and four (guanidilated sample) of the matching masses are only found in cod trypsin ZT-4 and cod trypsin ZT-2 but not in cod trypsin ZT-3 and cod trypsin ZT-1.

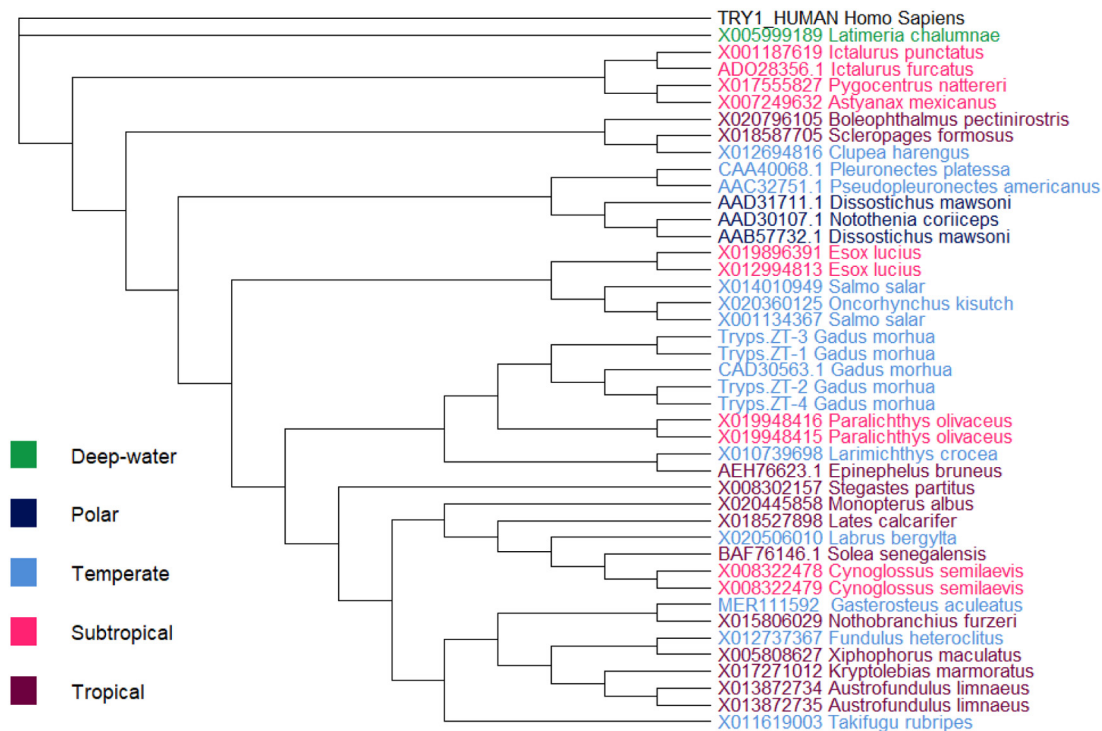
To further analyze cod trypsin 8, a fraction from the peak was subjected to Western blot analysis (Fig. 1). As can be seen in the figure, an antibody raised against cod trypsin ZT-4 recognized proteins in cod trypsin 8. On the other hand, the antibody did not recognize the previously characterized cod trypsin I or the two cod trypsin X variants CTX-V5 and CTX-V7 [14]. These three group I trypsins (cod trypsin I, CTX-V5 and CTX-V7) were recognized by an antibody directed against cod trypsin I and cod trypsin X. Conversely, the same antibody did not recognize proteins within cod trypsin 8. Based on the results from mass spectrometry and Western blot analysis, the peak termed cod trypsin 8 is composed of two or more cod trypsin ZT isoenzymes. For that reason, cod trypsin 8 is from now on called cod trypsin ZT. It was of interest to characterize cod trypsin ZT as no group III trypsins had been well characterized.

### 3.2. Phylogenetic analysis of group III trypsins

Phylogenetic analysis of group III trypsins revealed that the most closely related trypsins to the Atlantic cod trypsins are from olive flounder (*Paralichthys olivaceus*), a flatfish native to the northwestern Pacific



**Fig. 1.** Western blot analysis of trypsin 8 (trypsin ZT). Trypsin 8, cod trypsin I, CTX-V5 and CTX-V7 were subjected to SDS-PAGE and Western blot analysis. Following transfer, samples were immunoblotted with trypsin I/X (top panel) and trypsin ZT (bottom panel) reacting antibodies.

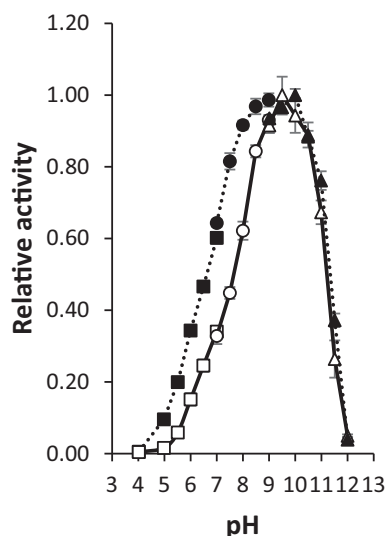


**Fig. 2.** A phylogenetic tree of fish trypsins with identity to cod trypsin ZT isoenzymes (see Section 2.4 Generation of phylogenetic tree). Species names are next to gene accession numbers, except for cod. Color indicates climate regions of fish: Green for deep water fish, dark blue for polar regions, light blue for temperate regions, red for subtropical and burgundy (dark red) for tropical fish. The tree is drawn using unscaled branches.

Ocean (Fig. 2). There appears to be no direct phylogenetic grouping of the trypsins by climate.

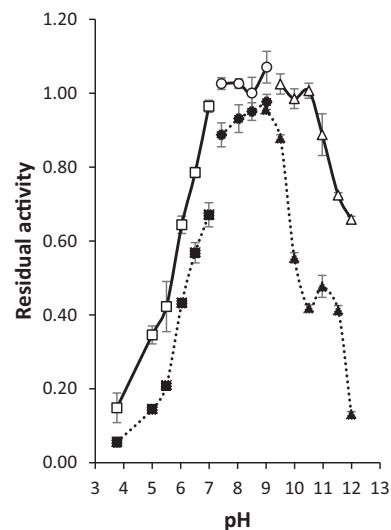
### 3.3. Biochemical characterization of cod trypsin ZT – Stability and sensitivity to inhibitors

The pH dependence and pH stability of cod trypsin ZT were determined. Activity of cod trypsin ZT and cod trypsin I (for comparison) was measured at different pH (Fig. 3). The pH stability of cod trypsin



**Fig. 3.** pH dependence of trypsin ZT and trypsin I. Activity of trypsin ZT (solid line and open symbols) and trypsin I (dotted line and filled symbols) was measured in buffers at different pH (see Section 2.5 Determination of pH and temperature dependence and stability). Boxed symbol ( $\square$  and  $\blacksquare$ ) Bis-Tris buffer (pH 5.0–7.5), circular symbol ( $\circ$  and  $\bullet$ ) Tris-HCl buffer (pH 7.5–9.0) and triangle symbol ( $\Delta$  and  $\blacktriangle$ ) glycine buffer (pH 9.0–12.0).

ZT was determined by incubating the enzymes at different pH for 1 h or 24 h and measuring the remaining activity (Fig. 4). As has been observed for CTX-V7 [14], after 1-h incubation most of the cod trypsin ZT activity is retained at pH 7.4–10.5 (Fig. 4). Cod trypsin I retained most of its activity at pH 6.0–9.0 after 1-hour incubation [14]. The change in cod trypsin ZT activity between 1-hour and 24-hour incubation is relatively small at pH 7.4–9.5 which is similar to what was observed for cod trypsin I (pH 7.5–9.0) and CTX-V7 (pH 8.0–9.5) [14]. Cod trypsin



**Fig. 4.** pH stability of trypsin ZT. Effects of pH on activity and stability of trypsin ZT. Samples of the enzymes were incubated at 25 °C for 1 h (solid line and open symbols) and 24 h (dotted line and filled symbols) in buffers at different pH (see Section 2.5 Determination of pH and temperature dependence and stability). The residual activity after incubation vs. pH is plotted. Boxed symbol ( $\square$  and  $\blacksquare$ ) Bis-Tris buffer (pH 4.0–7.5), circular symbol ( $\circ$  and  $\bullet$ ) Tris-HCl buffer (pH 7.5–9.0) and triangle symbol ( $\Delta$  and  $\blacktriangle$ ) glycine buffer (pH 9.0–12.0).

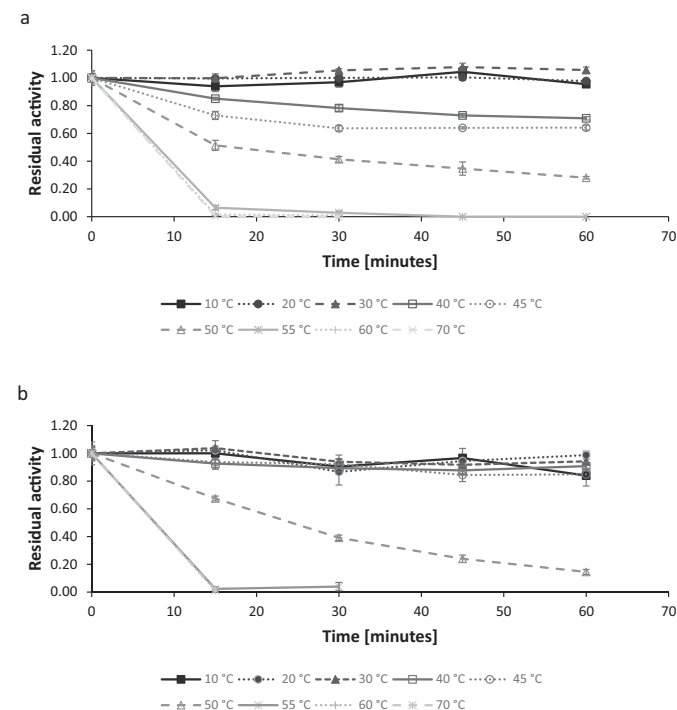


ZT has low stability at low pH that is more pronounced compared to CTX-V7 and cod trypsin I [14] but acid lability is observed in anionic trypsins from invertebrates and lower vertebrates [30–35].

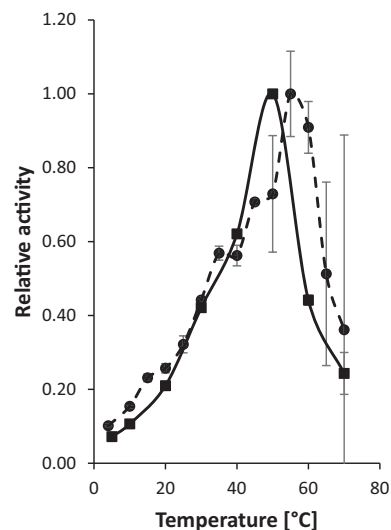
Temperature stability of cod trypsin ZT and cod trypsin I was determined by incubating the enzymes at different temperatures for 15, 30, 45 and 60 min and measuring the remaining activity (Fig. 5). In addition, temperature dependence of cod trypsin ZT and cod trypsin I was determined by measuring the activity of the enzymes at different temperatures (Fig. 6). Less than 20% change in relative activity over 60 min is observed after incubation of cod trypsin ZT at 10–30 °C and incubation of cod trypsin I at 10–45 °C (Fig. 5). Cod trypsin ZT activity drops about 30% when incubated at 40–45 °C and 70% at 50 °C over 60 min. A gradual decrease in activity of cod trypsin I is observed when incubated at 50 °C resulting in an 85% drop after 60 min. When cod trypsin ZT and cod trypsin I are incubated at 55–70 °C no or almost no activity was measured after 15 min. For that reason, the stability of the two enzymes was not measured at temperatures above 70 °C. Based on the results,  $T_{50}$  was calculated for trypsin ZT and trypsin I (see Section 2.5 Determination of pH and temperature dependence and stability). Trypsin ZT had a  $T_{50}$  value of 49.8 °C whereas trypsin I had a  $T_{50}$  value of 50.8 °C.

As the temperature is increased up to 50 °C for cod trypsin ZT and 55 °C for cod trypsin I, an increase in activity towards CBZ-GPR-pNA is observed (Fig. 6). With increasing temperature, a rapid decrease in activity is observed that is most likely due to instability and/or autolysis. The same observation was made with CTX-V7 except the highest activity was observed at 65 °C [14].

The effect of various protease inhibitors on the CBZ-GPR-pNA activity of cod trypsin ZT was measured. Table 1 shows the effect of serine protease inhibitors (PMSF, TLCK, TPCK and soybean trypsin inhibitor), 2-mercaptoethanol and EDTA on cod trypsin ZT activity after 30 min incubation at 25 °C. Cod trypsin ZT is less sensitive to the trypsin inhibitors TLCK, PMSF and soybean trypsin inhibitor (Table 1) compared to cod trypsin I. The results on cod trypsin I derive from a study on the effect



**Fig. 5.** Temperature stability of trypsin ZT and trypsin I. Effect of temperature on the stability of trypsin ZT (a) and trypsin I (b). Samples of trypsin ZT or trypsin I were incubated at different temperatures for 60 min and the activity measured at different timepoints (see Section 2.5 Determination of pH and temperature dependence and stability). The residual activity vs. incubation time is plotted for each temperature tested.



**Fig. 6.** Temperature dependence of CBZ-GPR-pNA activity of trypsin ZT and trypsin I. Activity of trypsin ZT (solid line) or trypsin I (dotted line) was measured at different temperatures as described (see Section 2.5 Determination of pH and temperature dependence and stability). The relative activity vs. temperature is plotted.

of inhibitors on the amidase activity (BzArg-NH-Np) of cod trypsin I [36]. On the other hand, cod trypsin ZT is more sensitive to EDTA and 2-mercaptoethanol compared to cod trypsin I [36]. Cod trypsin ZT is partly inhibited by TPCK which is an irreversible inhibitor of chymotrypsin. TPCK is generally known to bring about no inhibition on the activity of trypsin [37]. The 2-mercaptoethanol has been shown to convert sulfur-containing groups of ox trypsin to —SH groups and to lower the activity of the enzyme [38]. Slightly more inhibition of cod trypsin ZT is observed with 2-mercaptoethanol at 100 mM concentration compared to cod trypsin I [36]. The effect of the metalloprotease inhibitor EDTA on cod trypsin ZT activity was much higher compared to cod trypsin I [36]. Taken together, the results signify that cod trypsin ZT is a serine protease based on its inhibition by PMSF and belongs to the trypsin family based on its susceptibility to inhibition by TLCK and soybean trypsin inhibitor.

### 3.4. Multiplex substrate profiling of cod trypsin ZT

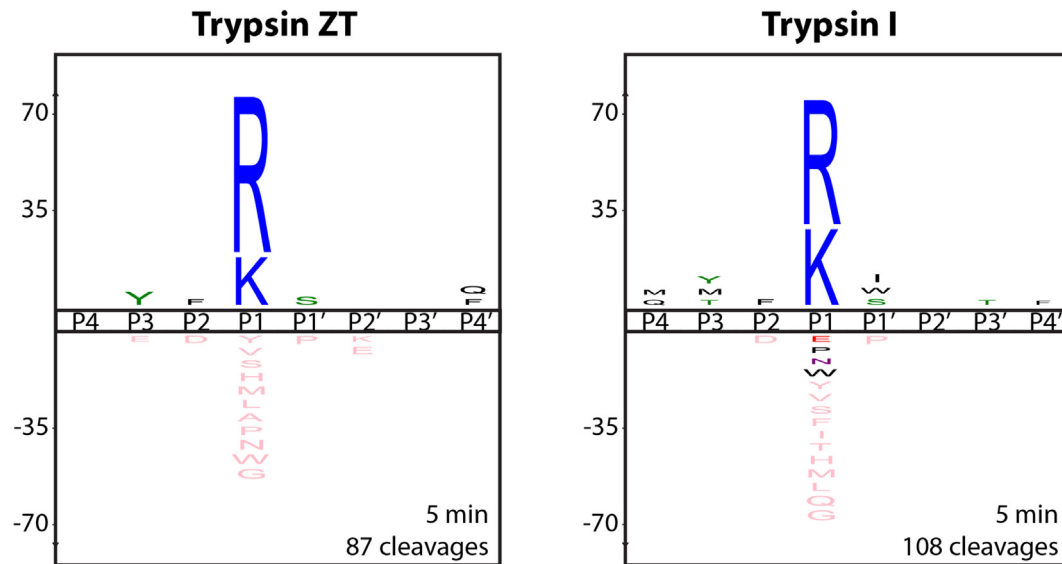
To compare the substrate specificity of cod trypsin ZT and cod trypsin I the multiplex substrate profiling by mass spectrometry (MSP-MS) method was used. The MSP-MS method uses mass spectrometry to characterize protease cleavage patterns within 14-mer peptides and provides data on the effect of different amino acids on both sides of the scissile amide bond.

The purified cod trypsin ZT isoenzymes or cod trypsin I were incubated (5, 15 and 60 min.) with a library of peptides (14-mer sequences) with an extensive physicochemical diversity (see Section 2.9 Multiplex

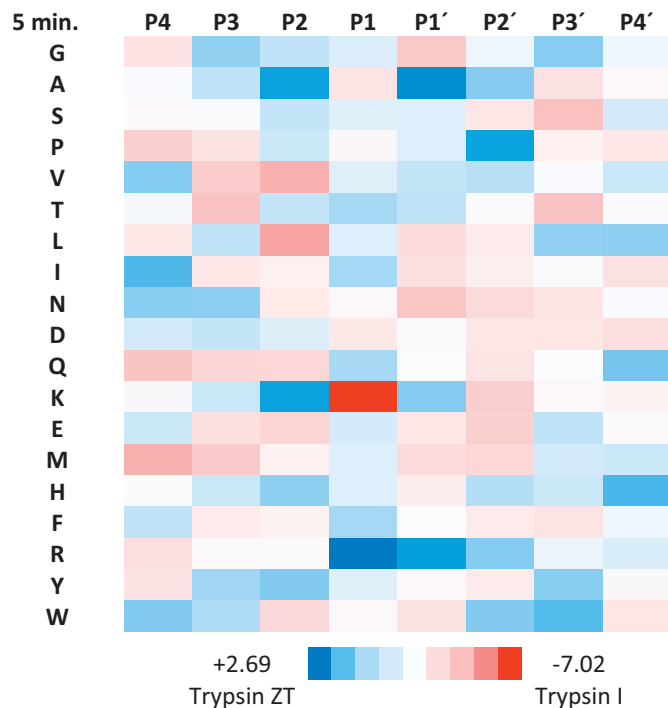
**Table 1**

The effect of inhibitors on trypsin ZT activity. The table shows the type and concentration of inhibitor used. The remaining activity (%) after 30 min incubation of trypsin ZT at 25 °C is shown.

Inhibitor	Concentration	Trypsin ZT residual activity [%]
PMSF	1 mM	15
TLCK	1 mM	26
TPCK	1 mM	67
EDTA	50 mM	9
2-mercaptoethanol	100 mM	68
Soybean trypsin inhibitor	2 µg/mL	56



**Fig. 7.** iceLogos based on the pattern of cleavage events for trypsin ZT and trypsin I after 5 min incubation with the MSP-MS peptide library. Motif analysis of time-dependent substrate specificity profiles for trypsin ZT and trypsin I are shown as percent difference in occurrences for an amino acid (relative to the available amino acids in the library), plotted at each position P4 to P4'. Amino acids most often detected at each P position are shown above the horizontal axis, and the amino acids least frequently detected are shown below the axis. Statistically significant residues ( $P < 0.05$ ) are colored by physicochemical property (black: hydrophobic; blue: basic; red: acidic). Residues in pink are absolutely disfavored (never observed in a cleavage). Purple indicates amidic amino acids (N) and residues in green are amino acids capable of forming H-bonds. Letter height corresponds to the percent difference value. Figure is made with iceLogo software, iceLogo representations.



**Fig. 8.** Heat map representation based on standard scores (Z-score) ratio at the P4-P4' positions in multiplex substrate profiling substrates comparing the substrate specificity of trypsin ZT and trypsin I after incubation for 5 min. Amino acid residues in a substrate undergoing cleavage were designated P1, P2, P3, P4 in the N-terminal direction from the cleaved bond. The residues in C-terminal direction are designated P1', P2', P3', P4'. The P positions are provided in the columns and the rows show amino acids in the different P positions. Trypsin ZT favored residues are blue (Z score, >0), and trypsin I favored residues are red (Z score, <0).

substrate profiling by mass spectrometry (MSP-MS)). Cleavage of any one of the available peptide bonds within the peptides was detected by liquid chromatography-tandem mass spectrometry (LC-MS/MS) sequencing [26].

Based on the results, motif analysis was conducted. Representations using iceLogo [28] that take into account both the cleaved and uncleaved positions in the peptide library were made. This was done to visualize the fold enrichment and de-enrichment of amino acids flanking each cleavage site (cleavage occurs between the P1 and P1' positions) (Fig. 7 and Supplementary file 4). As expected for tryptic enzymes, cod trypsin ZT and cod trypsin I both shared an enrichment of Arg and Lys at the P1 position. Differences between the two enzymes were detected. Unlike cod trypsin I, Glu at P3 was detected at a low frequency and Gln was preferred at P4' by cod trypsin ZT. After 15 min and 60 min incubation, cod trypsin I disfavored Arg at P4' in contrast to cod trypsin ZT. Both cod trypsin ZT and cod trypsin I exhibited a preference for Ser at P1' and bulky hydrophobic amino acid residues at P3 (Tyr), P2 (Phe) and P4' (Phe) after 5 min and 15 min incubation. Furthermore, Asp was detected at a low frequency at P2 and as has been observed for other trypsins [39], there was low tolerance for Pro at P1' for both enzymes. In line with the finding that cod trypsin ZT did not show activity towards a substrate (Suc-Ala-Ala-Pro-Phe-pNA) designed for chymotrypsin (data not shown), amino acid residues specific for chymotrypsin (F, Y and W) are disfavored by cod trypsin ZT and cod trypsin I at position P1 within the substrate (Fig. 7).

To compare differences in global substrate specificity between cod trypsin ZT and cod trypsin I, a heat map representation based on standard scores (Z-score) ratio at the P4-P4' positions in the substrate was generated where the substrate specificity of cod trypsin ZT was compared to cod trypsin I (Fig. 8 and Supplementary file 5). Blue color (Z-score, >0) reflects a preference for the amino acid residue in a certain P position in favor of cod trypsin ZT compared to cod trypsin I. The analysis revealed significant physicochemical differences in substrate specificity between cod trypsin ZT and cod trypsin I. For example, cod trypsin ZT showed preference for Arg at P1 compared to cod trypsin I whereas cod trypsin I showed preference for Lys at P1 compared to cod trypsin

ZT (Fig. 8 and Supplementary file 5). Overall, cod trypsin ZT showed an increased preference for amino acids with basic side chains (Lys, Arg and His) at the different subsites compared to cod trypsin I. In addition, cod trypsin ZT displayed higher preference for Gly at P3 and P3', Ala at P2 and P1', Pro at P2', Gln at P4' and Trp at P4 over cod trypsin I.

#### 4. Discussion

Biochemical characterization of cod trypsin ZT in the present study revealed that this group III trypsin shows multiple differences compared to its previously characterized isoenzyme, cod trypsin I. The temperature stability of cod trypsin ZT was found to be lower than that for cod trypsin I with a  $T_{50}$  value of 49.8 °C compared to a  $T_{50}$  value for cod trypsin I of 50.8 °C (Fig. 5). Also, cod trypsin ZT is less stable at lower pH values (below pH 7, Fig. 4) than cod trypsin I [14]. In general, proteins are highly charged at extreme pH that can lead to their partial denaturation due to electrostatic repulsions [20]. The lower stability of cod trypsin ZT can be of importance for certain practical purposes as the activity of cod trypsin ZT can be better restricted than cod trypsin I [5,14]. In addition, cod trypsin ZT has narrower maximal activity range at different pH than cod trypsin I (Fig. 3). This characteristic can also be utilized for more stringent control of maximal tryptic activity. It is well known that stability is an important parameter which in part determines the practical feasibility of applying enzymes in industrial processes [40]. Various stabilizing liquid formulations have been developed for cod trypsin in order to facilitate its application in biomedicine (unpublished results). Stability studies involving chemical modifications of trypsin have also been undertaken with some success [3,5,41].

Structural variances between the two cod trypsin isoenzymes, ZT and I, are indicated by their difference in pH and temperature stability. This may not come as a surprise as cod trypsin ZT and cod trypsin I share <50% amino acid sequence identity. Structural difference can also be noted from the fact that cod trypsin ZT shows maximal activity at a lower temperature (50 °C) than cod trypsin I (55 °C) (Fig. 6). For comparison, the optimal temperature of trypsins from cold climate fish, such as Pacific cod (*G. macrocephalus*) and saffron cod (*Eleginus gracilis*) is generally around 50 °C [4]. As expected, the optimal temperature of temperate climate fish is slightly higher or about 60 °C [4].

The phylogenetic relationship of trypsin isoenzymes with identity (>62%) to cod trypsin ZT-4 is seen in Fig. 2. All the sequences belong to fish that live at different climate regions. As expected, the group III cod trypsin isoenzymes cluster together on the same branch within the phylogenetic tree. Notably, the sequences showing the closest phylogenetic relationship to the cod trypsin ZT sequences are found in olive flounder (*Paralichthys olivaceus*) that lives in subtropical climate regions. This is followed by a number of sequences from fish living at temperate, subtropical or tropical climate regions. Thus, it seems clear that the group III trypsins are not only found in fish living under extreme cold conditions as previously suggested [15,42].

Cod trypsin ZT was found to be a true serine protease based on its inhibition by PMSF (Table 1). In line with those findings, cod trypsin ZT has an overall preference for arginine and lysine amino acid residues, in favor of arginine, at the cleavage site (P1) based on MSP-MS analysis (Fig. 7 and Supplementary file 4). Furthermore, cod trypsin ZT belongs to the trypsin family as shown by its susceptibility to inhibition by TLCK and Soybean inhibitor (Table 1). The sensitivity of proteases to inhibitors is of importance for their potential therapeutic application [43]. Interestingly, despite the fact that cod trypsin ZT is partly inhibited by a chymotrypsin inhibitor (TPCK) it showed no activity towards a chymotrypsin substrate (data not shown) unlike the previously identified native- and recombinant group III trypsin, cod trypsin Y [11,44]. The difference observed in the sensitivity of trypsin ZT to inhibitors relative to trypsin I might be important for effective digestion because of inhibitors present in feed. This has been proposed for human trypsin isoenzyme (mesotrypsin) that might serve a specific function in digestion because of low sensitivity to inhibitors [45].

Distinct differences between cod trypsin ZT and cod trypsin I in their preference for certain amino acid residues surrounding the cleavage site were observed (Fig. 8 and Supplementary file 5). Thus, cod trypsin ZT is better adapted to cleave peptides containing several positively charged residues (arginine, histidine or lysine) surrounding the P1 amino acid compared to cod trypsin I. Notably, lysine and arginine rich amino acid sequences are frequently found in surface proteins of viruses infecting humans [46–48]. For that reason, cod trypsin ZT might be of value against certain human pathogenic viruses. In support of this assumption, a recent *in vitro* study shows that cod trypsin ZT is very effective in cleaving a viral surface protein of coronavirus (unpublished results).

Interestingly, serine proteases play an important role in the natural defence of fish to pathogens such as viruses and bacteria [6,7,9]. Serine proteases with tryptic activity and a number of other proteases are found in the gut- and epidermal mucosa of fish [8,49]. Many of these proteases are likely to play a role in fish innate immunity providing defence against different pathogenic microbes. Proteases can degrade pathogens or may indirectly prevent pathogen invasion [8,9,49,50]. Trypsin ZT and other cod trypsin isoenzymes may be part of the natural resistance of Atlantic cod to pathogens [50]. The distribution and possible immunological role of trypsin ZT within the Atlantic cod is interesting and needs further attention [51].

Isoenzymes seem to have emerged in some cases by natural selection to diversify the application of a certain group of enzymes [52]. Isoenzymes can for example allow fine-tuning of metabolism to meet the particular needs of a given tissue or developmental stage (for example lactate dehydrogenase, LDH) [53]. Trypsin diversity is particularly high in fish, decapod crustaceans and insects [52,54,55]. The occurrence of multiple isoenzymes is thought to provide adaptive advantage in some insects feeding on plants containing inhibitors [10,54]. In salmon (*Salmon salar*), different trypsin isoenzymes can have an effect on physiological conditions such as fish size, growth rate, protein utilization and feed efficiency [55]. Enzyme polymorphism has also been shown to be important for adaptation to a broad food spectrum in caridean shrimp (*Crangon crangon*) [56]. The results presented here demonstrate that cod trypsin ZT and cod trypsin I show differences in stability to pH and heat, sub-site substrate specificity and sensitivity to inhibitors. It is therefore tempting to suggest that trypsin polymorphism plays an adaptive role in the Atlantic cod as it appears to do in salmon, decapod crustaceans and insects.

The difficulty in producing recombinant cod trypsins and mutants thereof limits the development of recombinant cod trypsin based products for specific therapeutic applications [11,12]. However, using naturally selected isoenzymes to make enzyme combinations for certain therapeutic use is a novel approach. The knowledge gathered on the biochemical properties of cod trypsin ZT and cod trypsin I allows for development of different combinations of cod trypsin isoenzymes aimed for biomedical applications. Future research will focus on further characterization of cod trypsin ZT including kinetic analysis and stability studies using circular dichroism (CD) and other known techniques [19–21]. Furthermore, research on the efficacy of cod trypsin ZT against human viruses as well as fish viruses will be conducted. The results will give insight into the physiological role of cod trypsin ZT and aid in its practical application.

#### 5. Conclusion

In this study, cod trypsin ZT was biochemically characterized in comparison to the well characterized cod trypsin I. Trypsin ZT is the first thoroughly studied group III trypsin reported. This isoenzyme shows differences in pH and heat stability compared to cod trypsin I. Stability is a key parameter which co-determines the practical feasibility of applying an enzyme in an industrial process. Differences were also observed between the two enzymes in their sensitivity to inhibitors and sub-site substrate specificity. The results indicate that cod trypsin ZT may be of value for inactivating viruses. Thorough understanding of

cod trypsin ZT biochemistry increases basic scientific understanding and may lead to multiple novel uses within the biotechnology sector.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijbiomac.2018.12.099>.

## Acknowledgments

We thank Charles Craik, Francesco Caiazza and Michael Winter for their work and support regarding the MSP-MS analysis.

This work was supported by the AVS R&D Fund of Ministry of Fisheries and Agriculture in Iceland [grant reference number: R15 046-15, R069-08, R11 028-11 and R14 044-14]; and Technology Development Fund (The Icelandic Centre for Research) [grant reference number: 120852-0611 and 131804-0611]. The funding source had no involvement in study design; in the collection, analysis and interpretation of data; in the writing of the report; or in the decision to submit the article for publication.

## Competing interests

RS and BS are employed at Zymetech. AG is Professor emeritus at the University of Iceland and is GS mentor. She is partially employed at Zymetech. GS is a PhD student and holds a part time research position at Zymetech. Contents in the manuscript are used in a patent No. PCT/EP2016/067531 and WO2017017012A1.

## Human and animal rights

Not applicable. This manuscript does not contain any studies with human or animal subjects.

## Contributors

GS, BS, RS and ÁG designed the experiments. GS, BS, RS and ÁG wrote, reviewed, edited and approved the manuscript. GS performed the experiments. The MSP-MS experiments were performed by Charles Craik, Francesco Caiazza and Michael Winter at the Department of Pharmaceutical Chemistry, University of California San Francisco, San Francisco, California 94158, United States.

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