Elucidation of different cold-adapted Atlantic cod (*Gadus morhua*) trypsin X isoenzymes

Bjarki Stefansson[1], Gunnar B. Sandholt[2], Ágústa Gudmundsdottir[1,2]*

[1] Zymetech, Fiskislod 39, 101 Reykjavík, Iceland.

Bjarki Stefansson, e-mail: bjarkis@zymetech.com

[2]Faculty of Food Science and Nutrition, Health Sciences Division, University of Iceland.

Gunnar B. Sandholt, e-mail: gbs1@hi.is

*Address correspondence to: Ágústa Gudmundsdottir, University of Iceland, Fiskislod 39, 101 Reykjavik, Iceland. Tel. +354-551-1168; e-mail: ag@zymetech.com

Abstract

Trypsins from Atlantic cod (*Gadus morhua*), consisting of several isoenzymes, are highly active cold-adapted serine proteases. These trypsins are isolated for biomedical use in an eco-friendly manner from underutilized seafood by-products. Our group has explored the biochemical properties of trypsins and their high potential in biomedicine. For broader utilization of cod trypsins, further characterization of biochemical properties of the individual cod trypsin isoenzymes is of importance. For that purpose, a benzamidine purified trypsin isolate from Atlantic cod was analyzed. Anion exchange chromatography revealed eight peaks containing proteins around 24 kDa with tryptic activity. Based on mass spectrometric analysis, one isoenzyme gave the best match to cod trypsin I and six isoenzymes gave the best match to cod trypsin X. Amino terminal sequencing of two of these six trypsin isoenzymes showed identity to cod trypsin X. Three sequence variants of trypsin X were identified by cDNA analysis demonstrating that various forms of this enzyme exist.

One trypsin X isoenzyme was selected for further characterization based on abundance and stability. Stepwise increase in catalytic efficiency (k_{cat}/K_m) of this trypsin X isoenzyme was obtained with substrates containing one to three amino acid residues. The study demonstrates that the catalytic efficiency of this trypsin X isoenzyme is comparable to that of cod trypsin I, the most abundant and highly active isoenzyme in the benzamidine cod trypsin isolate. Differences in pH stability and sensitivity to inhibitors of the trypsin X isoenzyme compared to cod trypsin I were detected that may be important for practical use.

Keywords: Biomedicine; trypsin; cold-adapted; Atlantic cod; kinetics; trypsin X.

1. Introduction

Trypsins from Atlantic cod (*Gadus morhua*) are isolated for biomedical use in an eco-friendly manner from underutilized by-products of the seafood industry in Iceland[1, 2]. Trypsins are serine proteases that specifically cleave peptide bonds on the carboxyl side of lysine and arginine amino acid residues. Different native Atlantic cod trypsin isoenzymes have been isolated and purified[3-6]. Two Atlantic cod trypsins termed I and X differ by eight amino acid residues as deduced from their cDNA sequences[5, 6]. Of the different cod trypsin isoenzymes trypsin I is most abundant and is the only thoroughly characterized cod trypsin whereas trypsin X has not yet been well characterized [3, 4]. For unknown reasons, cod trypsin I has proven to be difficult to produce recombinantly in bacteria [7] and yeast (unpublished results).

Trypsins from the Atlantic cod and other fish adapted to cold environments have higher catalytic efficiencies, especially at low and medium temperatures, compared to their mammalian analogues [1, 2, 4, 8, 9]. These properties of the cold-adapted enzymes have resulted in their commercial use as for example in the biomedical and food industries[1, 2, 10-15]. The high catalytic efficiency of Atlantic cod trypsin is especially useful in the processing of biological materials. Protein digestion at low temperatures is desirable in order to minimize undesirable chemical reactions as well as bacterial contamination [1]. Also, the use of cold adapted cod trypsins allows minimal dose of enzyme for protein digestion. Furthermore, the enzymes can be inactivated at lower temperatures than mesophilic analogues that can be an additional benefit[16].

The main question to be answered by the research undertaken is whether cod trypsin isoenzymes with new characteristics are present in the cod trypsin isolate under study. This report presents thorough characterization of a cod trypsin isoenzyme that shows identity to

cod trypsin X. The findings from this study may expand the biomedical use of cod trypsins and facilitate successful recombinant cod trypsin production.

2. Materials and Methods

2.1. **Antibodies and Chemicals**

Dimethyl sulfoxide (DMSO), Succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide (Suc-AAPF-pNA), Benzoyl-L-Arg-p-nitroanilide (BAPNA), EDTA, Bis-Tris, glycine, sodium dodecyl sulphate (SDS), dithiothreitol (DTT), benzamidine, β-mercaptoethanol, phenyl methyl sulphonyl fluoride (PMSF), soybean trypsin inhibitor, tosyl-lysyl-chloromethyl ketone (TLCK), Bowman-Birk trypsin inhibitor, 2-Amino-2-(hydroxymethyl)-1,3-propanediol (TRIS), DEAE-Sepharose Fast Flow and Benzyloxycarbonyl-Gly-Pro-Arg p-nitroanilide acetate salt (CBZ-GPR-pNA) were from Sigma Aldrich, St. Louis, MO, USA. Calcium chloride dihydrate, sodium chloride, sodium hydroxide, methanol, glycerol, acetic acid and hydrochloric acid were from Merck, Darmstadt. Succinyl-L-Ala-L-Ala-L-Pro-L-Ala-*p*-nitroanilide (Suc-AAPA-pNA), Succinyl-L-Ala-L-Ala-L-Pro-L-Leu-*p*-nitroanilide (Suc-AAPL-pNA) and Benzyloxycarbonyl-L-Phe-L-Arg-*p*-nitroanilide (CBZ-FR-pNA) were from Bachem AG, Bubendorf, Switzerland. Bio-Rad protein assay was from Bio-Rad laboratories, Hercules, California, USA. PageRuler Prestained Protein Ladder SDS from Fermentas (now Thermo Fisher Scientific, Rockford, IL). NucleoSpin Plasmid kit from Macherey-Nagel (US).

Benzamidine purified cod trypsin was purified as described before [4] and obtained from Zymetech (Reykjavik, Iceland).

2.2. **MonoQ-HPLC anion exchange chromatography**

The cod trypsin isoenzymes were separated 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. For isolation of trypsin 1 to 3 the column was equilibrated with 20 mM Tris, 5 mM ethanolamine, 10 mM $CaCl_2$, pH 9.0, and the enzymes were eluted with a linear 0-220 mM NaCl gradient in 80 column volumes at a flow rate of 1 mL/min. The sample pH was adjusted to pH 9.1 with 1 M NaOH before application. For isolation of trypsin 4 to 8 the column was equilibrated with 20 mM Tris, 10 mM $CaCl_2$, pH 8.0, and the enzymes were eluted with a linear 0-150 mM NaCl gradient in 40 column volumes, linear 150-620 mM NaCl in 6 column volumes, 620-850 mM NaCl in 10 column volumes at a flow rate of 1 mL/min. The sample pH was adjusted to pH 8.1 with 1 M NaOH before application.

For characterization of cod trypsin X variant 7 (CTX-V7) the pyloric caeca filtrate [4] was run through a DEAE-Sepharose Fast Flow column before purification on a benzamidine column. The DEAE column (2.5 cm x 10 cm) was equilibrated in 100 mM Tris, 5 mM $CaCl_2$, pH 8.2 at a flow rate of 3 mL/min. The filtrate was applied to the column and a fraction containing CBZ-Gly-Pro-Arg activity collected using a 100 mM Tris, 5 mM $CaCl_2$, 500 mM NaCl, pH 8.2 buffer. All the steps were conducted at 4°C. The DEAE eluate was applied to a benzamidine column and proteins eluted as previously described [4]. The DEAE/benzamidine purified cod trypsin was applied to a MonoQ HR 5/5 ion exchange column equilibrated with 50 mM Tris, 5 mM $CaCl_2$, pH 8.2, and CTX-V7 was eluted with a linear 0-200 mM NaCl gradient in 20 column volumes at a flow rate of 1 mL/min. The fraction containing CTX-V7 was frozen in liquid nitrogen and stored below -20°C until used. All the MonoQ purification steps were conducted at room temperature.

2.3. **Electrophoresis**

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was done according to Laemmli as previously described[3]. Silver staining was performed as previously described[17].

Two dimensional electrophoresis was performed at Proteome Factory AG, Germany. 200 µl of sample was mixed with 60 mg thio urea, 168 mg urea and 20 µl 1.4 M DTT. The mix was concentrated to a volume of 40 µl with ultrafiltration through a Centricon microconcentrator (VWR) with a molecular weight cutoff of 3 kDa. The sample was washed twice with 9 M urea, 25 mM Tris, 50 mM KCl and protease inhibitor mix. The two dimensional gel electrophoresis separation was performed based on the technique according to Klose and Kobalz[18]. 15 µg protein was applied to vertical rod gels (9 M urea, 4% acrylamide, 0.3% piperazine diacrylamide (PDA), 5% glycerol, 0.06% TEMED and 2% carrier ampholytes (pH 2-11), 0.02% ammonium persulfate (APS) for isoelectric focusing at 8820 Vh in the first dimension. After focusing, the IEF gels were incubated in equilibration buffer, containing 125 mM trisphosphate (pH 6.8), 40% glycerol, 65 mM DTT and 3% SDS for 10 minutes and subsequently frozen at -80°C. The second dimension SDS-PAGE gel (20 cm x 30 cm x 0.1 cm) was prepared, containing 375 mM Tris-HCl buffer (pH 8.8), 12% acrylamide, 0.2% bisacrylamide, 0.1% SDS and 0.03% TEMED. After thawing, the equilibrated IEF gels were immediately applied to SDS-PAGE gels. Electrophoresis was performed using 140mA for 5.5 hours until the front reached the end of the gel. After the separation the gel was stained with FireSilver (Proteom Factory, PS 2001).

2.4. **Mass spectrometry**

MALDI-TOF analysis was performed as previously described[3]. *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).

2.5. **Construction of cDNA Library and cloning of cod trypsinogens**

RNeasy MIDI kit from Qiagen was used to isolate mRNA from Atlantic cod pyloric caeca. The mRNA was reverted to cDNA using RevertAidTM First Strand cDNA Synthesis

Kit from Fermentas (now Thermo Fisher Scientific, Rockford, IL). PCR amplification was done using a 2-step PCR program with Phusion polymerase (high-fidelity DNA polymerase, Finnzyme) according to manufacturer instructions. Primers were designed based on sequences found by searching the codgene database (http://ri.imb.nrc.ca/codgene) with trypsinogen I (ACO90397) and trypsinogen X (Q91041) using the Basic Local Alignment Search Tool (BLAST). PCR amplification of trypsinogen I was performed using primers 5' ATG AAG TCT CTT ATC TTC GTT CTG CTC CTC 3' as forward and 5' ATA ATT TGC CAT GGT ATC GCG GA 3' as backward. Trypsinogen X forward primer of 5' ATG AAG TCT CTT ATC TTC GTT CTG CTC CTC 3' and 5' ATA ACT TGC CAT GGT ATC GCG AAC C 3' as backward. The PCR fragments were ligated into the pJET1.2/blunt end cloning vector using the CloneJET PCR Cloning kit (Fermentas). The vector ligation mixture was transformed into 100 μl DH5α highly competent cells. Several colonies from each transformation were picked and inoculated in 5 ml of LB containing 100 μl of ampicillin overnight for plasmid purification. Miniprep was done using NucleoSpin Plasmid kit from Macherey-Nagel. Multiple plasmids containing inserts, verified using PCR and digestion with *Bgl*II, were sent to Cogenics (Essex, UK) for sequencing. The resulting DNA sequences were translated to amino acid sequences.

2.6. **Sequence alignment**

Sequence alignments were performed using ClustalW method (default settings at http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_clustalw.html). PostScript output from aligned sequences was generated using ESPript (http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi)[19].

2.7. **Enzyme assays**

Measurements of trypsin activity were made using a 25 mM stock solution of CBZ-GPR-pNA (in DMSO) as the substrate and 100 mM Tris, 5 mM $CaCl_2$, pH 8.2 buffer. Final substrate concentration in the cuvette was 500 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-1 cm-1 [20]. The contents of the cuvette (930 μl of buffer and 20 μl substrate were mixed thoroughly with a plastic stirrer and allowed to equilibrate for at least 3 minutes in a 25°C water bath before 50 μl of enzyme sample containing 0.25-1.05 U/ml were added. More concentrated samples were diluted to fall within this range. The enzymatic assays were performed using the Ultraspec 4000 spectrophotometer.

2.8. **Protein determination**

Protein concentration was determined using the Bio-Rad protein assay which is based on the Bradford dye-binding method [21]. Serum albumin/globulin was used as a protein standard (P8119, Sigma Aldrich, St. Louis, MO). Absorbance was measured on an Ultraspec 4000 spectrophotometer linked to a computer with SWIFT II software (Pharmacia Biotech).

2.9. **Determination of pH and temperature dependence and stability**

To determine the relative activity of CTX-V7 at different pH and the effect of pH on the stability of CTX-V7 and cod trypsin I 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). The buffer solutions contained 5 mM $CaCl_2$ for CTX-V7 and 10 mM $CaCl_2$ for cod trypsin I. For pH dependence determination, activity measurements were conducted using the different buffers using the method described above (Materials and Methods 2.7). The effect of pH on the stability of CTX-V7 and cod trypsin I was determined by measuring residual

CBZ-GPR-pNA activity after incubation for one hour and 24 hours at 25°C in the different buffer solutions described above.

To determine the relative activity of CTX-V7 at different temperatures the activity (Materials and Methods 2.7) was measured in a buffer (50 mM Tris, 5 mM CaCl2, pH 8.2) in a preheated thermostated cuvette at the assay temperature before addition of the enzyme. The effect of temperature on the stability of CTX-V7 was determined by measuring residual CBZ-GPR-pNA activity (Materials and Methods 2.7) after incubation for 10 min. at different temperatures in 100 mM Tris, 5 mM CaCl2, pH 8.2. The pH of the buffers was corrected to pH 8.2 at each temperature that was tested.

Cod trypsin I was purified as described before[3].

2.10. **Inhibition studies**

The serine protease inhibitors PMSF, TLCK, soybean trypsin inhibitor and Bowman-Birk trypsin inhibitor were used for inhibition studies. CTX-V7 was incubated for 30 minutes with the inhibitors and residual CBZ-GPR-pNA activity measured.

2.11. **Amino terminal sequencing**

Cod trypsin X variant 5 (CTX-V5) and CTX-V7 were isolated from DEAE/benzamidine purified cod trypsin using the MonoQ purification procedure for CTX-V7 as described above. Fractions containing CTX-V5 and CTX-V7 were immediately inhibited by adding TLCK to a final concentration of 2 mM. The fractions were frozen in liquid nitrogen and freeze dried in a SpeedVac SCS 100 before being subjected to amino terminal sequencing in an Applied Biosystem Procise protein sequencer coupled to a PTH amino acid analyzer in the Biomedical Research Facility at the University of Virginia (USA).

2.12. **Determination of K_m, k_{cat} and k_{cat}/K_m for CTX-V7**

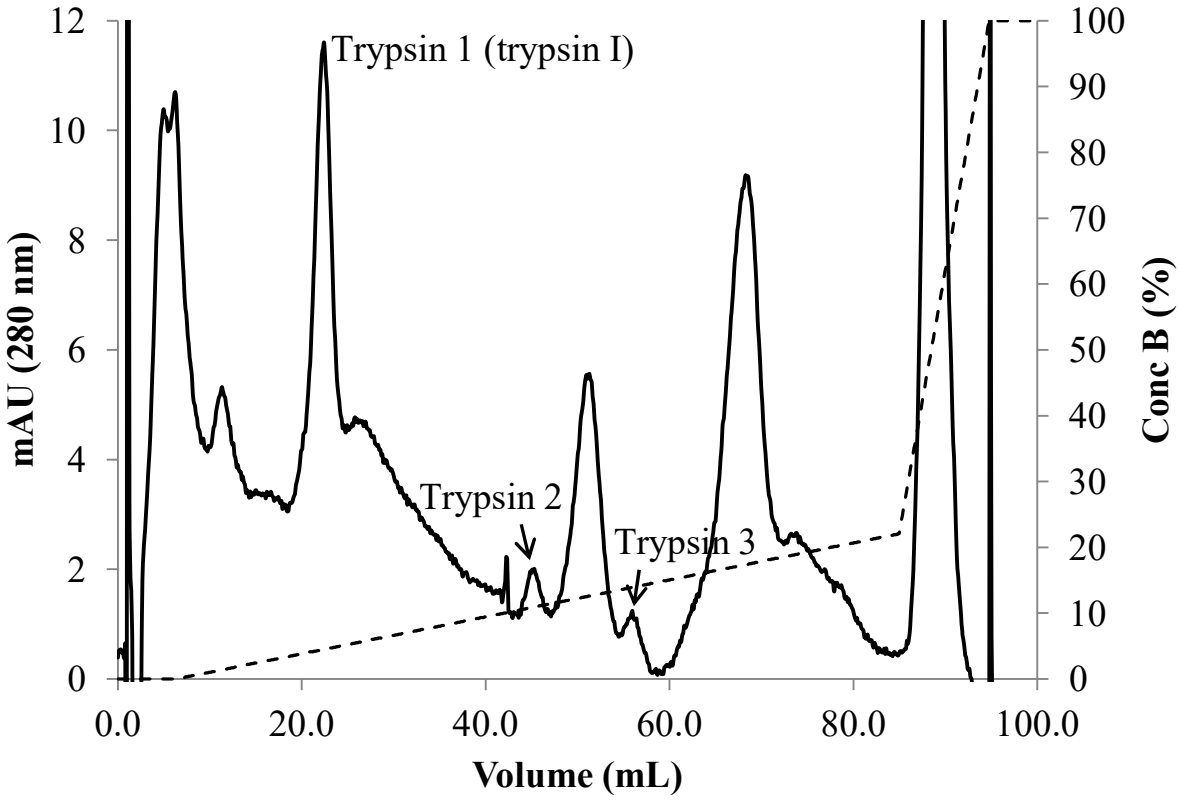
Measurements of CTX-V7 activity towards different chromogenic substrates to determine K_m, k_{cat} and k_{cat}/K_m were performed as described above (see 2.7) except the final substrate concentration was varied. Substrate stock solutions were made by dissolving the substrate in DMSO and further dilutions of substrate stock solutions were prepared with DMSO in order to maintain the concentration of DMSO the same in all assay measurements. The K_m was determined at 5-8 substrate levels at a final concentration of 1.5-15 mM for BAPNA, 0.005-0.125 mM for CBZ-FR-pNA and 0.01-0.5 mM for CBZ-GPR-pNA. Kinetic parameters (K_m and V_{max}) were determined from the best fit line of inverse substrate concentration versus inverse activity (Lineweaver–Burk plots) using Excel (Microsoft Corp, Redmond, WA, USA). Errors were determined using the LINEST function in Excel. k_{cat} was calculated from V_{max} where the concentration of the enzyme was determined as described in section 2.8. The molecular weight of CTX-V7 was 25 kDa based on SDS-PAGE analysis.

3. Results

3.1. **Characterization of cod trypsin isolate**

A benzamidine purified cod trypsin isolate (see Materials and Methods 2.1) was analyzed with anionic exchange chromatography using a MonoQ column. The resulting chromatograms can be seen in Figure 1A and Figure 1B. Fractions from the peaks were subjected to SDS-PAGE. Silver staining of the gels revealed that the peaks labeled trypsin 1 to trypsin 8 contained proteins migrating at or just below bovine trypsin (24 kDa), which was used as a protein marker (Figure 1C).

A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Trypsin 2
Trypsin 3
mAU (280 nm)
Conc B (%)
Volume (mL)
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12
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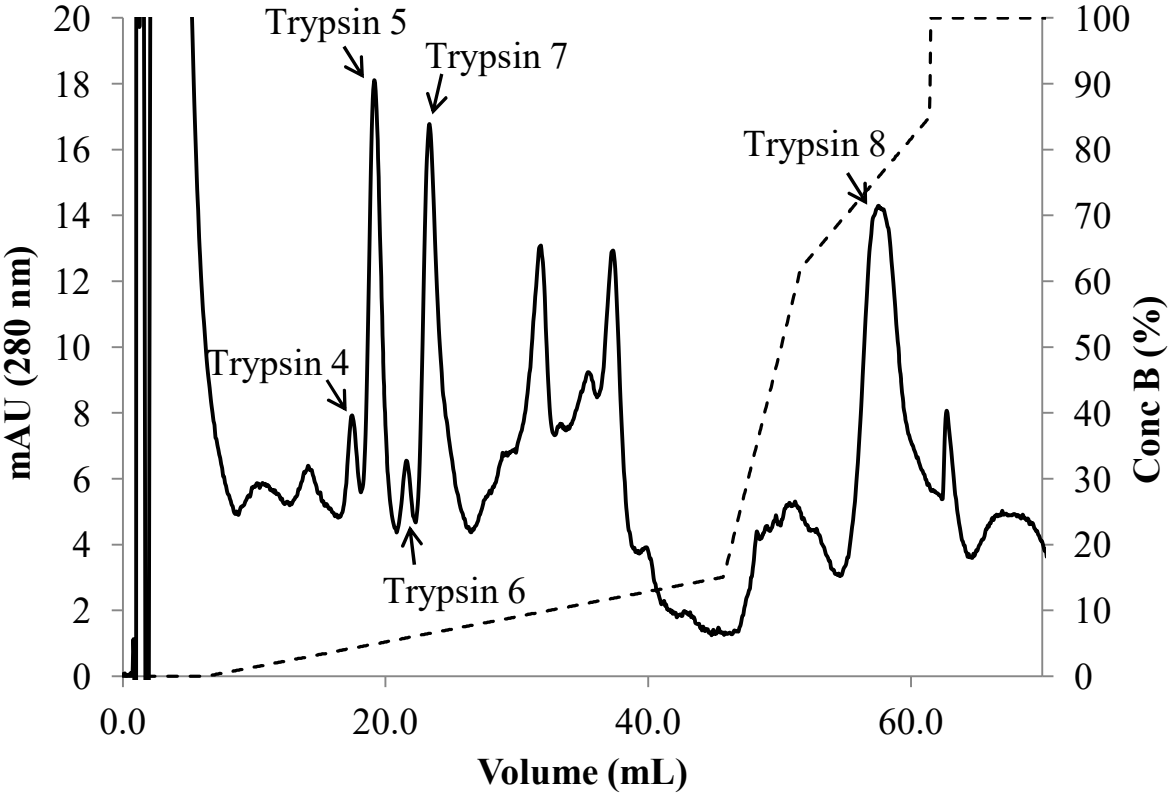
B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Trypsin 7
Trypsin 8
Trypsin 4
Trypsin 6
mAU (280 nm)
Conc B (%)
Volume (mL)
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C

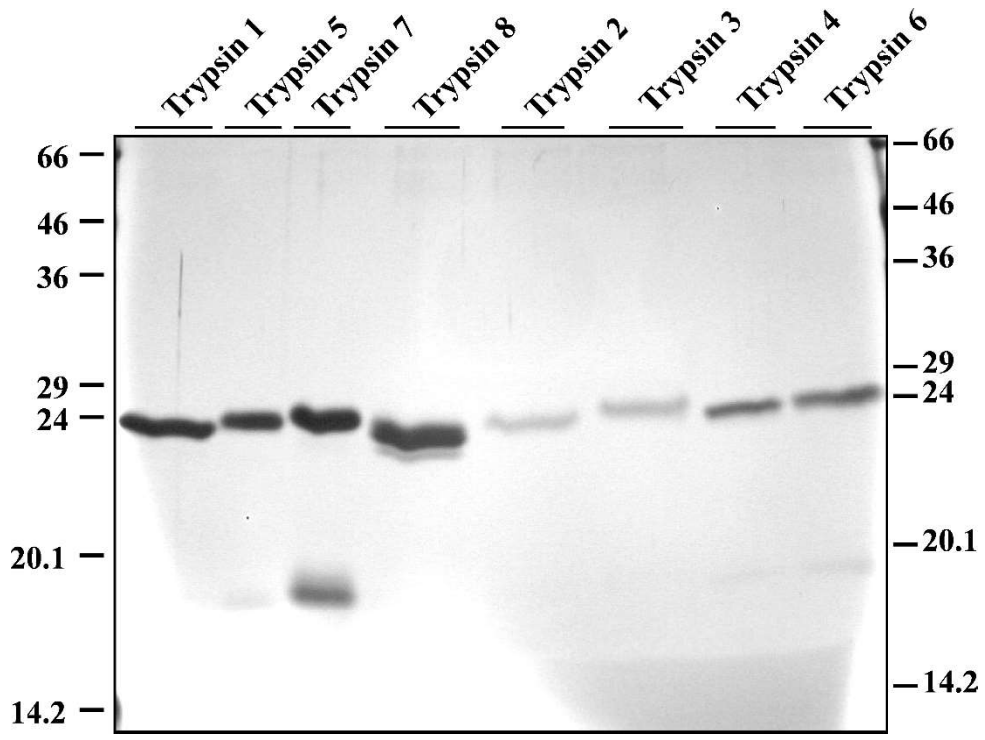

Figure 1. Anionic exchange chromatography of benzamidine purified cod trypsin.

A and B. Chromatograms from a MonoQ separation of cod trypsin showing absorbance at 280 nm vs. elution volume. Cod trypsin was loaded on a MonoQ column and proteins eluted with a salt gradient (dotted line). In panel A the column was equilibrated with 20 mM Tris, 5 mM ethanolamine, 10 mM CaCl2, pH 9.0 and elution performed with a linear 0-220 mM NaCl gradient in 80 column volumes at a flow rate of 1 mL/min (see Materials and Methods 2.2). In panel B the column was equilibrated with 20 mM Tris, 10 mM CaCl2, pH 8.0, and elution performed with a linear 0-150 mM NaCl gradient in 40 column volumes, linear 150-620 mM NaCl in 6 column volumes and 620-850 mM NaCl in 10 column volumes at a flow rate of 1 mL/min (see Materials and Methods 2.2). C. Proteins in fractions from peaks labelled trypsin 1-8 were resolved by SDS-PAGE and the gel was silver stained (see Materials and Methods 2.3). The bars and numbers on the left and right show the migration and molecular weight in kDa of standard proteins (Dalton Mark VII-L) separated on the gel.

All the fractions containing trypsin 1 to trypsin 8 showed activity towards the tryptic substrate CBZ-Gly-Pro-Arg-pNA. Fractions from other peaks contained proteins migrating below 17.4 kDa (data not shown) that were considered to be degradation products and therefore not subjected to further analysis. The proteins migrating at 24 kDa (Figure 1C) were

cut out of the gel and after tryptic digestion the proteins were subjected to MALDI-TOF analysis for identification. By performing a Mascot search using the mass spectrometry data, trypsin 1 was identified as trypsin I (P16049) (Mowse score 124 returned by MASCOT software). Trypsin X (Q91041.2) was the best match to trypsin 2-7 with a Mowse score of 75 (trypsin 2), 82 (trypsin 3), 124 (trypsin 4), 145 (trypsin 5), 124 (trypsin 6), and 145 (trypsin 7). None of the masses from the mass spectra obtained by MALDI-TOF analysis on trypsin 2 to trypsin 7 have a match to trypsin I except masses that are common to both trypsin I and trypsin X. On the other hand, masses unique to trypsin X compared to trypsin I are three in trypsin 2, two in trypsin 3, four in trypsin 4 and trypsin 6 and five in trypsin 5 and trypsin 7 (Supplemental Tables 1 to 7). A Mascot search based on the mass spectra obtained with trypsin 8 did not return a match to any trypsin like protein.

The benzamidine purified cod trypsin was analyzed using two dimensional gel electrophoresis (Figure 2).

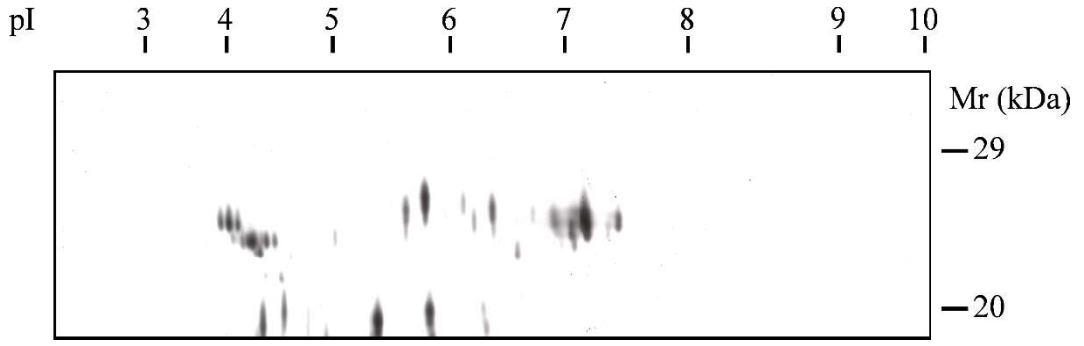

Figure 2. Two dimensional gel electrophoresis of benzamidine purified cod trypsin.

Benzamidine purified cod trypsin was analyzed by separating proteins by isoelectric focusing in the first dimension and by SDS-PAGE in the second dimension. Bars and numbers show the isoelectric point (pI) values and molecular weight in kilodaltons (kDa) of standard proteins.

The stained two dimensional gel (Figure 2) revealed more than eight protein spots migrating around 25 kDa (24 – 27.5 kDa) within a pI range of 3.8-7.5. The rest of the proteins migrated at or below 21 kDa.

Two of the most abundant trypsin 2-7 isoenzymes from the anion exchange chromatography were subjected to amino terminal sequencing. The isoenzymes, trypsin 5 and trypsin 7, were termed cod trypsin X variant 5 (CTX-V5) and cod trypsin X variant 7 (CTX-V7) respectively. As seen in Figure 1C the fraction labelled trypsin 7 contained two protein bands based on SDS-PAGE analysis. One band migrating along with the 24 kDa marker and another band migrating below the 20.1 kDa marker. Different preparation of the trypsin 7 fraction (e.g. sample preparation, keeping the sample cold when possible and inhibition with TLCK) showed a single band migrating at 24 kDa using SDS-PAGE analysis (data not shown). This is indicates that the band migrating below 20.1 kDa is a degradation product of CTX-V7. The N-terminal sequence ^{16}IVGGYExTRHSQAHQVSLNS was detected in CTX-V5 and CTX-V7 (Figure 3). In addition, two sequences were detected in CTX-V5. The amino acid sequences obtained by the amino terminal sequencing of CTX-V5 and CTX-V7 matched completely to the amino acid sequence of trypsin X (Q91041.2) (Figure 3). However, these sequences did not give a full match to the deduced amino acid sequence of trypsinogen I (Figure 3). A revised sequence of trypsinogen I (ACO90397.1) is depicted in Figure 3 instead of an older trypsinogen I sequence P16049[3].

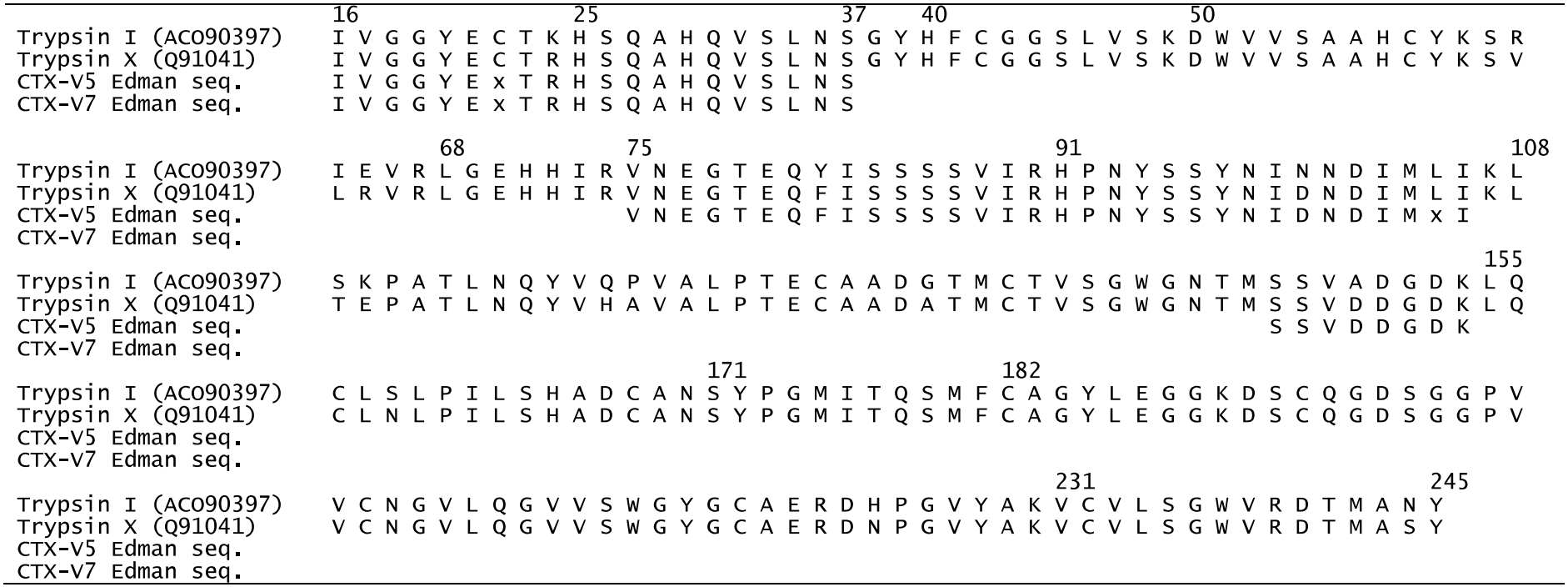

Figure 3. Amino terminal sequencing of cod trypsin isoenzymes.

CTX-V5 and CTX-V7 were purified as described in Materials and Methods 2.2. After addition of TLCK the samples were freeze dried and subjected to amino terminal sequencing. The sequences of trypsinogen I (ACO90397) and trypsinogen X (Q91041), excluding the signal peptide and propeptide, are shown on top. The sequences obtained from the amino terminal analysis of CTX-V5 and CTX-V7 are shown below. The label x within the sequences denotes a residue that could not be determined in the amino terminal analysis. Numbering is given relative to the standard chymotrypsinogen-A sequence [22].

3.2. **Cloning of cod trypsin isoenzymes**

Based on the results from the MALDI-TOF and amino terminal sequencing analysis on the benzamidine purified cod trypsins, cDNA library from Atlantic cod pyloric caeca was constructed to see if novel trypsinogen X isoenzymes could be identified by cloning.

Polymerase chain reaction (PCR) using primers based on the trypsinogen X (X76887) nucleotide sequence previously identified was performed[5]. The resulting DNA fragments were ligated into cloning vectors and transformed into bacteria (see Material and Methods 2.5). Several clones were isolated and the DNA insert in the cloning vector sequenced. Sequencing analysis on all the translated amino acid sequences revealed three different clones, named trypsinogen X-1 (GenBank accession number KU565326), trypsinogen X-2 (GenBank accession number KU565327) and trypsinogen X-3 (GenBank accession number KU565328). The three clones had an amino acid sequence identity of 97.1%, 96.3% and 96.7% respectively to trypsinogen X (Q91041.2) that was previously identified[5]. Sequence alignment of the three amino acid sequences of trypsinogen X like clones isolated in this study and the deduced amino acid sequence of previously identified trypsinogen X (Q91041.2) reveals amino acid differences at nine sites (Figure 4)[5].

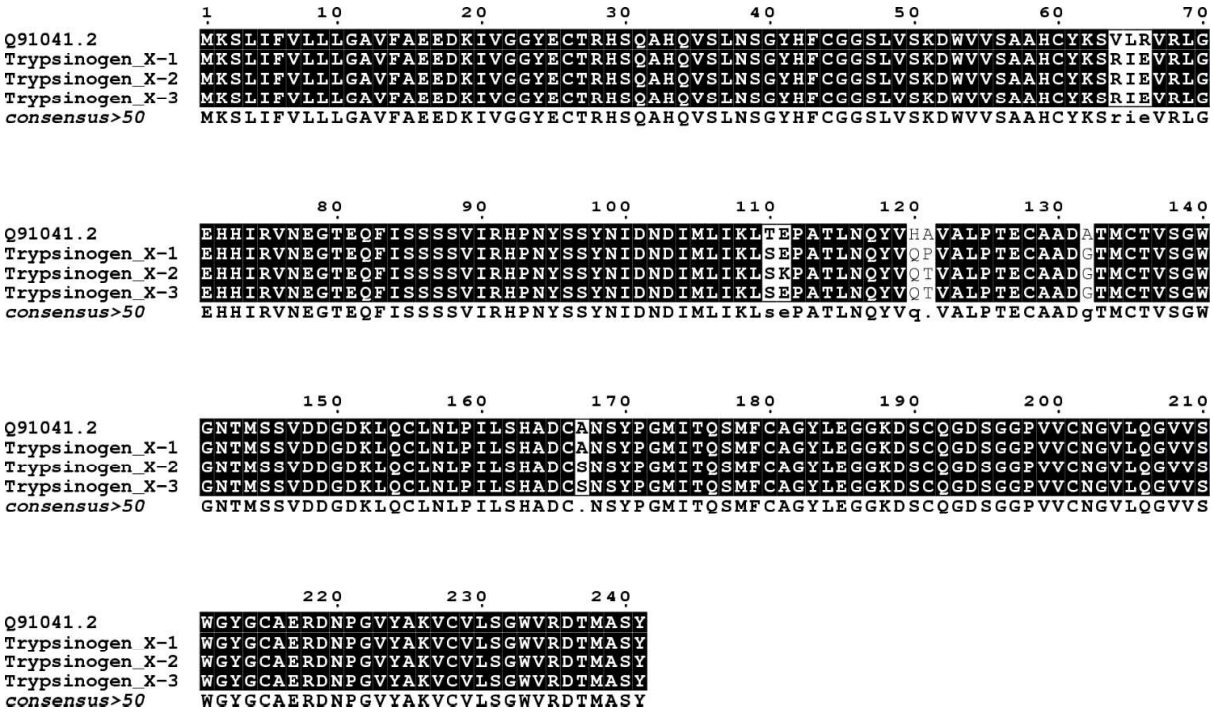

Figure 4. Protein sequences of trypsinogen X proteins and sequence alignment.

Translated amino acid sequences for trypsinogen X-1 (KU565326), trypsinogen X-2 (KU565327) and trypsinogen X-3 (KU565328) and the amino acid sequence of trypsinogen X (Q91041.2) were aligned using Clustal method (see Material and Methods 2.5). Identical residues in all four proteins are printed in reverse.

At amino acid site 121 there are three different amino acids (Ala, Pro or Tyr) found within the four translated amino acid sequences. At the other eight sites there are two different amino acids found: 64 (Val or Arg), 65 (Leu or Ile), 66 (Glu or Arg), 110 (Tyr or Ser), 111 (Glu or Arg), 120 (His or Gln), 132 (Ala or Gly), and 167 (Ala or Ser). As a control, polymerase chain reaction (PCR) using primers based on the trypsinogen I (FJ665432.1) sequence was performed. Several clones were isolated and the DNA inserts in the cloning vectors sequenced. The translated amino acid sequence for all the clones was a perfect match to the amino acid sequence of trypsinogen I, ACO90397.1 (data not shown).

Amino acid residues in the new trypsin X like cDNA sequences that differ from the amino acid sequence of trypsin X (Q91041.2) can all be found in expressed sequence tags (ESTs) from *Gadus morhua.* This was established with a search in an EST database (Database of GenBank+EMBL+DDBJ sequences from EST Divisions) using the translated

nucleotide basic local alignment search tool (tblastn) with the amino acid sequence of trypsinogen X-1. On the other hand, few amino acid residues in trypsin X (Q91041.2) could not be found in ESTs, Val-Leu-Arg 64-66, Tyr110, Ala121 and Ala132. Furthermore, two ESTs were identified (ATLCOD1ESTi08513_3 and ATLCOD1ESTi08514_3) in the codgenome.no database that seem to contain a full length cod trypsin X like sequence where ATLCOD1ESTi08514_3 has the same sequence as trypsinogen X-2 (Figure 4). The sequence of ATLCOD1ESTi08513_3 is identical to trypsinogen X-2 except that it contains Ala at amino acid site 167 instead of Ser in trypsinogen X-2.

In an attempt to match these different trypsin X sequences to trypsin 2-7 an *in silico* digestion was performed on the trypsin X sequences. Based on the in silico digestion, the size of peptides containing amino acid sequences that differentiate between the trypsin X isoenzymes have a monoisotopic mass-to-charge ratio (m/z) below 516.3140 or above 3845.7828. None of the mass spectra obtained with MALDI-TOF analysis on trypsin 2 to trypsin 7 had peaks with a mass that matched these peptides. Therefore, the MALDI-TOF analysis could not be used to determine whether trypsin 2 to trypsin 7 have a better match to trypsin X, trypsinogen X-1, trypsinogen X-2 or trypsinogen X-3.

3.3. **Characterization of CTX-V7**

One of the trypsin X isoenzymes was selected for further characterization. CTX-V5 and CTX-V7 are the most abundant trypsin X isoenzymes based on the area of the peaks for trypsin 2-7 in the chromatograms in Figure 1A and Figure 1B. Stability analysis by storing the enzymes in 100 mM Tris-HCl, 5 mM $CaCl_2$, pH 8.2 at 25°C showed that CTX-V7 is more stable than cod CTX-V5 (data not shown). Based on these findings CTX-V7 was chosen for further analysis.

CTX-V7 was purified as described in Materials and Methods 2.2. SDS-PAGE analysis on purified TLCK treated CTX-V7 revealed a single band migrating at 25 kDa and

isoelectric focusing showed that CTX-V7 has a pI value of 5.4 (data not shown). Kinetic parameters for CTX-V7 were determined using three *para*-nitroanilide substrates of different peptide lengths (Table 1). The catalytic efficiency (k_{cat}/K_m) of CTX-V7 was lowest towards the substrate Bz-Arg-pNA (Bz-R-pNA or BAPNA) (3.5 $s^{-1}mM^{-1}$) containing only one amino acid. The catalytic efficiency increases with longer substrates, 149-fold with CBZ-FR-pNA compared to BAPNA and more than 2300-fold with CBZ-Gly-Pro-Arg-pNA compared to BAPNA. The increase in catalytic efficiency compared to BAPNA was for the most part due to a decrease in the K_m value but a stepwise increase in the k_{cat} value was observed (Table 1).

Table 1. Kinetic parameters of CTX-V7 obtained with substrates of different lengths.

Substrate	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1}mM^{-1}$)
Bz-R-pNA (BAPNA)	13.0 ± 0.2	3.9 ±0.6	3.5 ± 1.0
CBZ-FR-pNA	25.9± 0.3	0.050 ± 0.007	520 ± 130
CBZ-GPR-pNA	64.9± 1.2	0.0080 ± 0.0008	8210 ± 1050
Suc-AAPF-pNA	No activity detected		
Suc-AAPL-pNA	No activity detected		
Suc-AAPA-pNA	No activity detected		

CTX-V7 did not cleave substrates designed for chymotrypsin (Suc-Ala-Ala-Pro-Phe-pNA) and elastase (Suc-Ala-Ala-Pro-Leu-pNA or Suc-Ala-Ala-Pro-Ala-pNA).

The effect of various protease inhibitors on the CBZ-Gly-Pro-Arg-pNA activity of CTX-V7 was measured in order to verify the identity of this isoenzyme as a trypsin-like enzyme. Table 2 shows the effect of five serine protease inhibitors (PMSF, TLCK, soybean trypsin inhibitor, Bowman-Birk and benzamidine) on CTX-V7 activity after 30 minute incubation at 25°C.

Table 2. Inhibition of CTX-V7 with PMSF, TLCK, Soybean trypsin inhibitor, Bowman-Birk inhibitor and benzamidine.

Inhibitor	Concentration	% Inhibition
PMSF	1 mM	99
TLCK	1 mM	100
Soybean trypsin inhibitor	100 μg/ml	100
Bowman-Birk	100 μg/ml	100
Benzamidine	100 μg/ml	0
Benzamidine	1 mg/ml	65

All of the inhibitors except benzamidine almost fully inhibited CTX-V7 at concentrations that typically inhibit trypsins.

The pH dependence and pH stability of CTX-V7 was determined. Activity of CTX-V7 was measured at different pH (Figure 5).

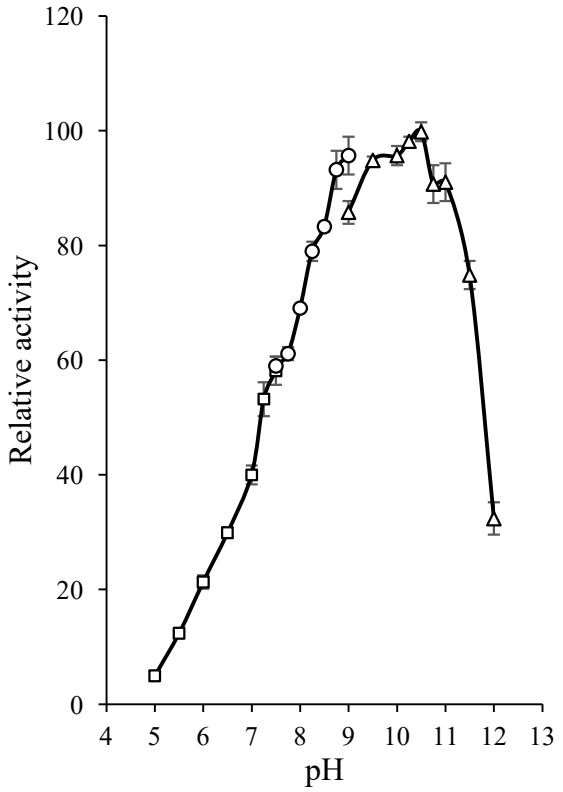

Figure 5. pH dependence of CTX-V7.

Activity of CTX-V7 was measured in buffers at different pH (see Materials and Methods 2.9). The relative activity vs. pH is shown. Boxed symbol (□) Bis-Tris buffer (pH 5.0-7.5), circular symbol (○) Tris-HCl buffer (pH 7.5-9.0) and triangle symbol (△) glycine buffer (pH 9.0-12.0).

The pH stability was determined by incubating CTX-V7 at different pH for 1 hour or 24 hours and measuring the remaining activity (Figure 6A). The pH stability of cod trypsin I was determined for comparison (Figure 6B).

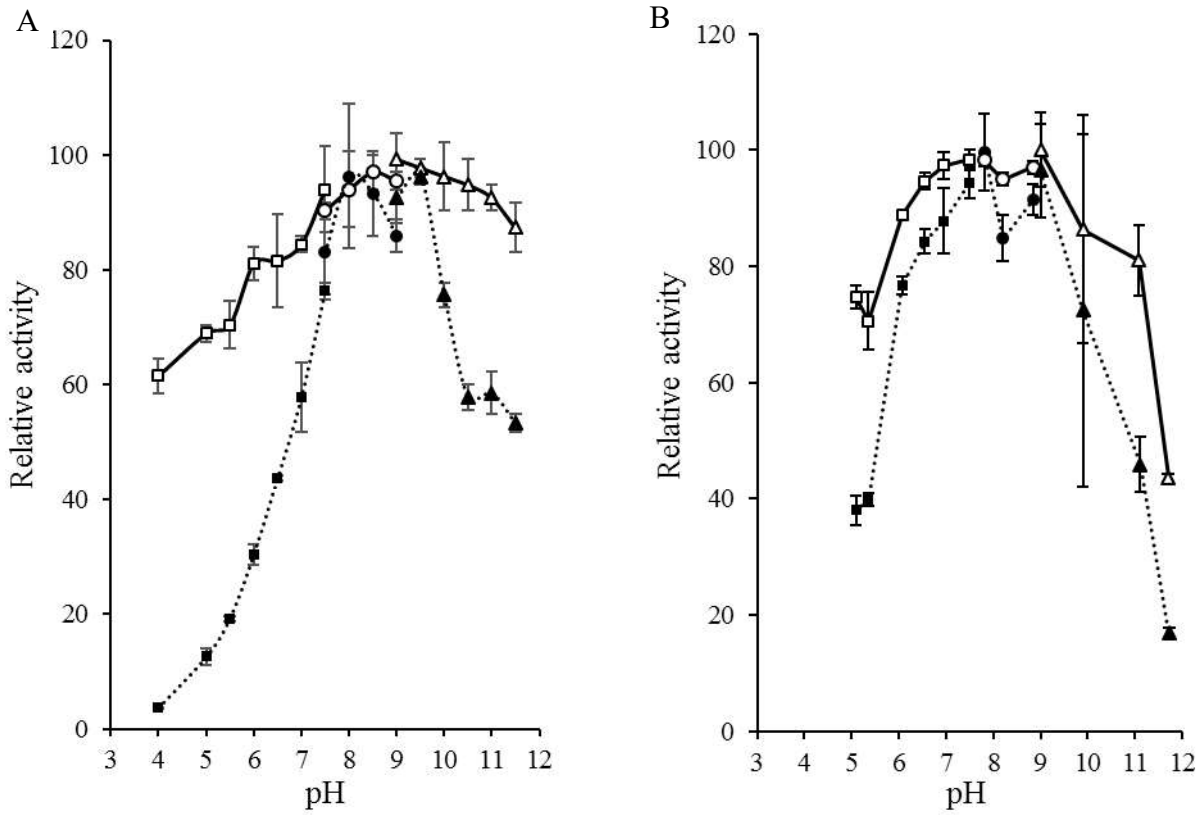

Figure 6. pH stability of CTX-V7 and cod trypsin I.

Effects of pH on activity and stability of CTX-V7 (A) and cod trypsin I (B). Samples of the enzymes were incubated at 25°C for 1 hour (solid line and open symbols) or 24 hours (dotted line and filled symbols) in buffers at different pH (see Materials and Methods 2.9). The relative activity after incubation vs. pH is plotted. Boxed symbol (□ and ■) Bis-Tris buffer (pH 4.0-7.5), circular symbol (○ and ●) Tris-HCl buffer (pH 7.5-9.0) and triangle symbol (△ and ▲) glycine buffer (pH 9.0-12.0).

Most CTX-V7 activity is retained after incubation at pH 7.5-10.5 and at pH 6.0-9.0 for cod trypsin I after 1 hour incubation. Little change in activity between 1 hour and 24

hours incubation was observed for CTX-V7 at pH 8.0-9.5 and for cod trypsin I at pH 7.5-9.0. Marked acid lability is detected for CTX-V7 and cod trypsin I as has been observed for anionic trypsins from invertebrates and lower vertebrates[16, 23-27].

Temperature dependence of CTX-V7 was determined by measuring the activity of CTX-V7 at different temperatures (Figure 7A). As well, temperature stability was determined by incubating CTX-V7 at different temperatures for 10 min. and measuring the remaining activity (Figure 7B).

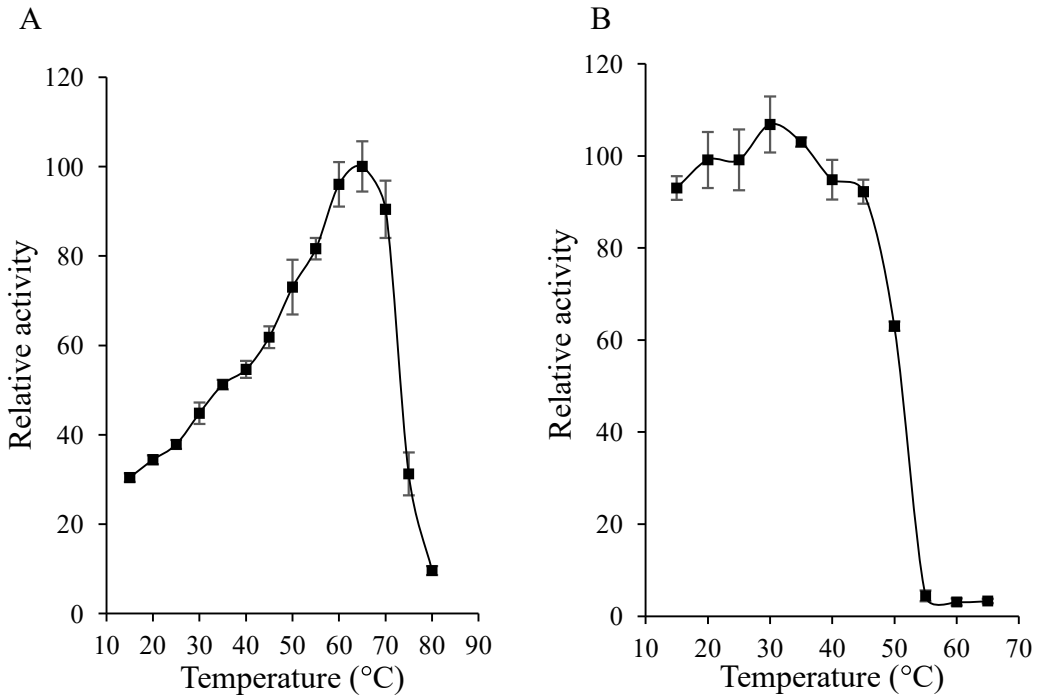

Figure 7. Temperature dependence of CBZ-GPR-pNA activity of CTX-V7 and temperature stability of CTX-V7.

A. Temperature dependence of CTX-V7. Activity of CTX-V7 was measured at different temperatures as described (see Materials and Methods 2.9). The relative activity vs. temperature is plotted. **B.** Temperature stability of CTX-V7. A sample of the enzyme was incubated at different temperatures for 10 min. and the activity measured (see Materials and Methods 2.9). The relative activity after incubation vs. temperature is plotted.

An increase in activity of CTX-V7 towards CBZ-GPR-pNA is observed as the temperature is increased up to 65°C (Figure 7A). At temperatures above 65°C a rapid decrease in activity is observed most likely due to instability and/or autolysis. The temperature dependence of trypsin I has been tested using the same substrate and activity assay methodology (data not shown). An increase in activity was observed as the temperature was increased up to 55°C and at temperatures above 55°C a rapid decrease in activity is observed.

Most of CTX-V7 activity is retained after incubation at 15-45°C for 10 min. (Figure 7B). About half of the activity of CTX-V7 is remaining after incubation at 50°C and no activity is remaining after incubation above 50°C. Results from analysis on trypsin I at similar conditions are almost identical to those for CTX-V7 (data not shown).

4. Discussion

The aim of this study was to isolate and characterize new trypsin isoenzymes from Atlantic cod. In this report, the cod trypsin isoenzyme CTX-V7 was shown to have new interesting biochemical properties different from the previously characterized cod trypsin I. These include differences in thermal- and pH stability as well as altered sensitivity to inhibitors. Interestingly, the effective concentration of proteases used for biomedical purposes depends on several factors including sensitivity to inhibitors, catalytic efficiency and stability[10]. Thus, CTX-V7 with its novel properties may be beneficial for certain biomedical applications.

4.1. **Identification of cod trypsin isoenzymes in a cod trypsin isolate using anion exchange chromatography and mass spectrometry**

After anion exchange chromatography of a benzamidine purified cod trypsin fraction, eight peaks containing proteins in the size range of bovine trypsin (24 kDa) and tryptic activity were identified (trypsin 1 - 8) (Figure 1). Elution of trypsin 8 using anion exchange

chromatography required much higher salt concentration compared to trypsin 1-7 (Figure 1A and Figure 1B). Further anion exchange chromatography analysis indicates that the trypsin 8 peak contains multiple trypsin isoenzymes with very similar pI values that are much lower compared to the pI values of trypsin 1-7 (data not shown).

Results from two dimensional gel electrophoresis analysis on the benzamidine purified cod trypsin (Figure 2) align well with the findings from the anion exchange chromatography analysis (Figure 1). The two dimensional gel electrophoresis revealed multiple protein spots migrating around 24 kDa (Figure 2). Of these spots, seven major protein spots (based on quantitation, data not shown) are found within the pI range of 5.5-7.5. Furthermore, a cluster of protein spots is found within the pI range of about 3.8 – 4.5 that might be the proteins found in the trypsin 8 peak. Further studies are needed confirm this.

In a previous study, three anionic trypsin isoenzymes termed trypsin I, II and III were isolated from Atlantic cod[4]. In another study, two complementary DNAs of trypsin isoenzymes termed trypsinogen I and trypsinogen X were isolated and sequenced[5]. Trypsin I isolated from Atlantic cod appears to derive from trypsinogen I[5] but the sequence of trypsin II and trypsin III has not yet been analyzed.

Mass spectrometry analysis on trypsin 1 from the anion exchange chromatography gave a match to cod trypsin I. Same type of analysis on trypsin 2 to trypsin 7 gave the best match to trypsin X (Q91041). Trypsin X (Q91041) is the deduced amino acid sequence of the trypsinogen X cDNA previously isolated[5]. Furthermore, amino terminal sequencing analysis on CTX-V5 (trypsin 5) and CTX-V7 (trypsin 7) demonstrated identity to trypsin X (Figure 3). Based on the data from this study it cannot be concluded whether the amino acid sequences of trypsin 2 to trypsin 7 are different. It is possible that trypsin 2 to trypsin 7 have the same amino acid sequence but are different because of post translational modifications and/or truncations (for example caused by autolysis) in the proteins. Small differences in

migration of cod trypsin 2-7 are observed on a SDS-PAGE gel (Figure 1C). It is considered more likely that these differences are caused by dissimilarities in the amino acid sequences of cod trypsin 2-7 rather than autolysis. Analysis indicated that the trypsin X isoenzyme variants are not formed by degradation such as autolysis. Storage of cod trypsin isoenzymes at 25°C followed by anion exchange chromatography analysis of samples taken at different time-points did not lead to an increase in any of the cod trypsin isoenzymes (data not shown).

4.2. **Identification of cDNAs encoding new cod trypsin X isoenzymes**

Cloning of the different trypsinogen X like transcripts in this study and the identification of different cod trypsinogen X like transcripts in DNA databases indicates that there is high sequence variability in trypsinogen X isoenzymes (Figure 4). Interestingly, unlike trypsinogen X, high sequence variability is not observed with the closely related cod trypsinogen I. Based on this study, along with the trypsinogen X cDNA previously isolated[5], five different trypsinogen X like sequences have been identified. Three trypsinogen X sequences were isolated by cloning (trypsinogen X-1, trypsinogen X-2 and trypsinogen X-3) and one more different sequence (ATLCOD1ESTi08513_3) was identified in the codgenome.no database. For that reason, trypsin 2 to trypsin 7 can be different versions of trypsin X that derive from the same or different gene but further studies are needed to confirm this. As to why this variability is found within trypsinogen X like sequences but not within trypsinogen I is not known. The presence of multiple trypsinogen genes within a genome, six for example in Senegalese sole (Solea senegalensis), has been reported in other fish species[28-32]. In Atlantic salmon three trypsins (SalTRP-I, SalTRP-IA and SalTRP-IB) with very similar sequences were identified[32]. These trypsins may represent allelic differences at the same gene locus or represent members of tetrasomic loci and/or isoloci[32]. One explanation as to why multiple trypsinogen genes are present within a genome is that

high gene dosage is needed to supply large amounts of trypsin for digestion within a short timeframe[33].

4.3. **Characterization of a new cod trypsin X isoenzyme with respect to sensitivity to inhibitors, catalytic efficiency and stability**

To our knowledge, the characterization of cod trypsin X variant 7 (CTX-V7) is the first extensive biochemical analysis of a cod trypsin X isoenzyme whereas cod trypsin I has been well characterized[3, 4, 34]. The pI value of CTX-V7 is 5.4 (data not shown) which aligns well with the calculated values of the trypsin X isoenzymes and trypsin III. The calculated pI values of the mature form (excluding the signal peptide and activation peptide) of the deduced amino acid sequence of the five trypsinogen X cDNA sequences is in the range of 5.36-5.69 and for trypsin I the value is 6.12. Based on the pI values, these enzymes can be classified as anionic trypsins. The measured pI value of cod trypsin I, II and III was 6.6, 6.2 and 5.5 respectively[4].

The substrate specificity of CTX-V7 is consistent with the substrate specificity of trypsins and trypsin-like enzymes (Table 1)[35]. Kinetic parameters of CTX-V7 obtained with CBZ-Gly-Pro-Arg-pNA are comparable to those obtained for cod trypsin I. CTX-V7 has a catalytic efficiency (k_{cat}/K_m) of 8210 $s^{-1}mM^{-1}$ at pH 8.2 compared to 7590 $s^{-1}mM^{-1}$ at pH 8.0 for cod trypsin I[3]. Furthermore, the catalytic efficiency (k_{cat}/K_m) obtained with CBZ-Gly-Pro-Arg-pNA is two-fold higher for CTX-V7 compared to bovine trypsin[3]. This is line with previous findings that show psychrophilic enzymes having higher catalytic efficiency (k_{cat}/K_m) than mesophilic counterparts[36]. As has been observed for cod trypsin I and other proteases, the catalytic efficiency (k_{cat}/K_m) of CTX-V7 increases as the substrate increases in length from one to three amino acid residues (Table 1)[3, 37-39]. CTX-V7 is stable up to 45°C at the conditions tested in this study (Figure 7B). These results are comparable to the thermal stability of other trypsins isolated from cold adapted marine organisms such as from

Antarctic fish (*Paranotothenia magellanica*)[40], Japanese Anchovy (*Engraulis japonicas*)[31], Greenland cod (*Gadus ogac*)[16] and Arctic fish capelin (*Mallotus villosus*)[25]. In comparison the thermal stability of trypsin from a mesophilic organism (bovine trypsin) is much higher[16]. This low thermal stability of cold adapted enzymes compared to other enzymes is well established[36, 41].

It has been suggested that the cold-adaptation of trypsins is due to higher degree of softness found in the outer regions of the proteins[42]. A few residues in anionic salmon trypsin were proposed to play a key role in increasing the softness of the surface of cold-adapted trypsins relative to the mesophilic ones. These key residues are Glu21, Glu64, Lys74, Arg90, Tyr97, Lys110, Tyr117, Asp150, Lys154, Asp167 and Glu221 in anionic salmon trypsin. All of these residues are found in cod trypsin I (accession number ACO90397) except for a conserved change of a residue, arginine 74 compared to a lysine in anionic salmon trypsin. The trypsin X isoenzyme amino acid sequences elucidated in this publication contain the same key residues as cod trypsin I. However, some of the trypsin X isoenzymes contain a glutamic acid at position 111 instead of a lysine at the comparable position 110 in anionic salmon trypsin. The conservation of the proposed key residues in the cod trypsin X isoenzymes and cod trypsin I indicates that cold-adaption of these enzymes is based on surface softness as has been proposed for anionic salmon trypsin[42].

Biochemical differences between CTX-V7 and cod trypsin I were observed in this study. Incubation of CTX-V7 with benzamidine at 1000 μg/ml leads to 65% reduction in activity (Table 2) whereas cod trypsin I is fully inhibited at 780 μg/ml [4]. Maximal activity for CTX-V7 is observed in the pH range of 8.5-10.5 which is higher and broader than that of cod trypsin I[4]. Interestingly, CTX-V7 demonstrates higher acid lability compared to cod trypsin I when stored at a pH below 7.5 for 1 hour and 24 hours (Figure 6). On the other hand, increased stability is observed for CTX-V7 when stored at a pH above 10 for 1 hour

and 24 hours compared to cod trypsin I (Figure 6). In addition, when measuring the activity of CTX-V7 at different pH, maximal activity is observed over a broad pH range (8.5-11) which is higher compared to cod trypsin I and other serine proteases (Figure 5)[4, 43-46]. CTX-V7 shows maximal activity at 65°C towards CBZ-GPR-pNA (Figure 7A) whereas cod trypsin I has a maximal activity at 55°C towards the same substrate. This difference can reflect a dissimilarity in structure between CTX-V7 and cod trypsin I that has an effect on flexibility, stability and/or the geometry of the catalytic site. A difference in stability would be short lived considering that the results from thermal stability analysis of CTX-V7 (Figure 7B) and cod trypsin I are very similar.

4.4. **Possible physiological importance of trypsin X isoenzymes in Atlantic cod**

The biochemical findings on CTX-V7 may reflect on the physiological importance of cod trypsin X isoenzymes in the Atlantic cod. Trypsin X isoenzymes can be important for effective digestion at varied pH in the cod digestive tract caused by different feed. As well, trypsin X isoenzymes may be critical to facilitate digestion in the presence of inhibitors found in feed. As an example of this, it has been proposed that a human trypsin isoenzyme (mesotrypsin) may play a unique role in digestion because of reduced affinity to inhibitors that allows degradation of trypsin inhibitors in the diet[47]. Further studies on the sensitivity of cod trypsin X isoenzymes to inhibitors are needed to support this notion.

4.5. **Potential practical use of cod trypsin X isoenzymes**

The number of proteases used in medicine is greatly expanding[10]. CTX-V7 has unique properties in terms of sensitivity to inhibitors and stability compared to trypsin I. Therefore, this and possibly other trypsin X isoenzymes can be considered highly relevant for biomedical applications and other practical use. Recombinant trypsin X derivatives with increased stability towards autolytic cleavage may broaden the commercial applicability of

the recombinant enzymes. The amino terminal sequencing analysis revealed a potential autolytic cleavage site within CTX-V5 at Arg74 (Figure 3). Furthermore, amino terminal analysis of other proteins collected during the anion exchange chromatography analysis revealed a potential autolytic cleavage site within cod trypsin X isoenzymes at Arg24 (data not shown). Therefore, site directed mutagenesis of trypsin X cDNA at Arg24 and/or Arg74 may enhance the stability of recombinant trypsin X.

Seven amino acid residues in the deduced amino acid sequences of trypsin I and the trypsin X isoenzymes are unique to trypsin I. These are the amino acids residues in trypsinogen I and trypsinogen X sequences, respectively: Lys28Arg, Tyr83Phe, Asn101Asp, Ala148Asp, Ser157Asn, His220Asn and Asn240Ser. All these residues are exposed on the surface based on the structure of trypsin I (PDB ID: 2EEK) except Asn240 which is missing from the structure but is most likely found on the surface as well. His220Asn is located within structural elements which are thought to be determinants of substrate specificity[48]. The amino acid residues different between cod trypsin I and trypsin X are responsible for the difference between the isoenzymes. Therefore, these residues are good targets for manipulation in the design of recombinant cod trypsin derivatives.

5. Conclusions

In this study, three new cod trypsin X isoenzyme sequences were isolated from an Atlantic cod cDNA library. Furthermore, six proteins were separated from a cod trypsin isolate that were identified as trypsin X isoenzymes. The most abundant trypsin X isoenzyme (CTX-V7) showed reduced inhibitor affinity and comparable catalytic efficiency as cod trypsin I. In addition, relative to cod trypsin I, CTX-V7 displayed increased stability at high pH but was less stable at lower pH. The findings demonstrate that cod trypsin isoenzymes with new characteristics are present in the cod trypsin isolate. These isoenzymes may be beneficial in their native or recombinant form for biomedical purposes.

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