Food Chemistry 111 (2008) 408–414

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

The novel trypsin Y from Atlantic cod (*Gadus morhua*) – isolation, purification and characterisation

Helga Margrét Pálsdóttir [a], Ágústa Gudmundsdóttir [b,*]

[a] *The Environment and Food Agency of Iceland, Sudurlandsbraut 24, Reykjavík, IS 108, Iceland*
[b] *Department of Food Science and Nutrition, Science Institute, University of Iceland, Laeknagardi, Vatnsmýrarvegi 16, Reykjavík, IS 101, Iceland*

ARTICLE INFO

Article history:
Received 16 December 2007
Received in revised form 28 January 2008
Accepted 1 April 2008

Keywords:
Atlantic cod
Trypsin Y
Group III
Purification
Cold-adaptation

ABSTRACT

This report describes the isolation and partial characterization of the novel group III trypsin Y from the pyloric caeca of Atlantic cod. Other Atlantic cod trypsins have been used as food processing aids with good results. Trypsin Y was purified by *p*-aminobenzamidine affinity chromatography and characterized by SDS–PAGE and western blot analysis, as well as by activity measurements towards synthetic substrates. Identification of trypsin Y was done with polyclonal antibodies raised towards the recombinant form of the enzyme and by MALDI-TOF mass spectrometry. Trypsin Y is the only group III trypsin isolated from its native source and characterized by biochemical methods. In accordance with the r-trypsin Y, the native enzyme shows dual substrate specificity, i.e. towards trypsin and chymotrypsin specific substrates. This, along with the high cold-adapted character of trypsin Y, may be valuable for its use as a processing aid for sensitive products such as seafood.

© 2008 Elsevier Ltd. All rights reserved.

1. Introduction

The commercial use of trypsins and other serine proteases isolated from the pyloric caeca of Atlantic cod (*Gadus morhua*) has recently been described (Gudmundsdóttir & Bjarnason, 2007). In a search for serine proteases with novel characteristics, the cDNA encoding trypsin Y was isolated and characterized from a cod cDNA library (Spilliaert & Gudmundsdottir, 1999). Amino acid sequence identities have been used for the classification of trypsins into three main groups, termed I, II and III (Roach, Wang, Gan, & Hood, 1997). The traditionally classified cold-adapted fish trypsins (Asgeirsson & Cekan, 2006; Asgeirsson, Fox, & Bjarnason, 1989; Smalås, Schröder Leiros, Os & Willassen, 2000) belong to group I along with most other known trypsins (Roach, 2002), such as trypsins from bovine sources (*Bos taurus*) (Stroud, Kay, & Dickerson, 1974) and rat (*Rattus norvegicus*) (MacDonald, Stary, & Swift, 1982). The recently classified group III trypsins (Roach, 2002; Spilliaert & Gudmundsdottir, 1999) include members such as Atlantic cod trypsin Y, as well as seven analogues from other cold-adapted fish species (Gudmundsdottir & Palsdottir, 2005). The cDNA sequences encoding the eight group III trypsins are known (Spilliaert & Gudmundsdottir, 1999). However, previous to this report, none of the enzymes have been isolated from their natural sources and characterized. In general, enzymes from cold-adapted organisms have higher catalytic efficiencies and lower thermal stabilities than their mesophilic analogues (Asgeirsson & Cekan, 2006; Asgeirsson et al., 1989; D'Amico, Marx, Gerday & Feller, 2003; Shoichet, Baase, Kuroki, & Matthews, 1995).

The r-trypsin Y from Atlantic cod was successfully expressed in a *Pichia pastoris* expression system (Palsdottir & Gudmundsdottir, 2004). The r-trypsin Y polypeptide was found to have interesting and unique characteristics relative to cod trypsin I, the most common and best characterized cod trypsin. The r-trypsin, Y, is a cold-adapted enzyme with dual substrate specificity, i.e. towards both trypsin and chymotrypsin specific substrates. Also, the enzyme appears to be active at lower environmental temperatures than cod trypsin I and other cold-adapted enzymes (Kishimura, Klomklao, Benjakul, & Chun, 2008; Palsdottir & Gudmundsdottir, 2004).

Trypsin I was isolated in relatively high amounts from the pyloric caeca of Atlantic cod (Asgeirsson et al., 1989; Gudmundsdóttir & Bjarnason, 2007). However, the native cod trypsin Y has proven difficult to isolate and purify. Quantitative RT-PCR analysis demonstrated that trypsin Y mRNA is expressed at a low level compared to cod trypsin I or at a ratio of 1:1340 mRNA copies (Pálsdóttir & Gudmundsdóttir, 2007). The low copy number of trypsin Y mRNA may be the main reason for the difficulties involved in the isolation and purification of the native enzyme from the Atlantic cod. Various other factors, such as autolytic degradation and sensitivity to inactivation by heat and handling, may also be the reason for the low recovery of trypsin Y, as shown for the cold-adapted cod trypsin I (Gudmundsdóttir & Bjarnason, 2007).

The main goal of the research presented here was to isolate and purify the native form of trypsin Y and compare its characteristics

* Corresponding author. Tel.: +354 525 4800; fax: +354 525 4886.
E-mail address: ag@raunvis.hi.is (Á. Gudmundsdóttir).

0308-8146/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2008.04.005

to those of the recombinant form of the enzyme (Palsdottir & Gudmundsdottir, 2004). The aim was also to estimate the feasibility of using the Atlantic cod pyloric caeca as the potential source of trypsin Y with the commercial application of the enzyme in mind.

2. Materials and methods

2.1. Production and purification of recombinant trypsin Y

The recombinant form of Atlantic cod trypsin Y (r-trypsin Y), containing the entire prepro sequence of the molecule, was produced in the yeast *P. pastoris* by fermentation, as previously described (Palsdottir & Gudmundsdottir, 2004). The trypsin Y cDNA construct was ligated in frame with a polyhistidine *myc* DNA sequence contained in a pPICZα A expression vector. This resulted in expression of the r-trypsin Y polypeptide fused to a polyhistidine *myc* tag at its C-terminal end. After expression of the r-trypsin Y polypeptide, a two-step purification procedure was used for the isolation of r-trypsin Y. Purification of the recombinant protein was first performed on a Q-Sepharose anion-exchange column (Sigma, MO, USA). The *P. pastoris* cell supernatant was diluted 10-fold in 10 mM glycine buffer at pH 9.7 prior to its loading onto the column. The r-trypsin Y polypeptide was eluted from the ion-exchange column with a 10 mM glycine buffer at pH 9.7 containing 0.4 M NaCl. Further purification of this sample was done on a trypsin-specific *p*-aminobenzamidine affinity column (Sigma). The sample containing the trypsins was diluted 20-fold with an equilibration buffer (50 mM Tris-HCl buffer, 5 mM $CaCl_2$, pH 8.0) prior to its loading onto the column. The r-trypsin, Y, was eluted from the *p*-aminobenzamidine column with 0.4 M NaCl contained in the equilibration buffer.

2.2. SDS–PAGE gel electrophoresis

The molecular mass and the purity of the r-trypsin Y polypeptide was analysed by SDS–PAGE gel electrophoresis, carried out using a SE 250 Mighty Small II 10 × 8 cm electrophoresis unit from Hoefer (CA, USA). The stacking and resolving gels contained 5% and 12% acrylamide, respectively. Proteins separated by electrophoresis under reducing conditions were denatured with 2× treatment buffer (10% β-mercaptoethanol, 20% glycerol and 4% SDS in 0.125 M Tris-HCl, pH 6.8) for 5 min at 100 °C. The same 2× treatment buffer, without β-mercaptoethanol, was used for the proteins separated by electrophoresis under non-reducing conditions. The electrophoresis was performed in a Tris-glycine buffer (25 mM Tris, 192 mM glycine) at pH 8.5, containing 0.1% SDS.

2.3. Western blot analysis and protein concentration measurements

Western blot analysis and coomassie brilliant blue staining were used to analyse the r-trypsin Y protein bands on SDS–PAGE gels. A pageblue protein staining solution from Fermentas (St. Leon-Rot, Germany) was used for the gel staining. The pageblue protein staining solution is a ready-to-use solution containing coomassie brilliant blue G-250 dye. The pageblue dye was used according to the manufacturers' staining protocol for mini gels (Fermentas). Mouse monoclonal anti-myc antibodies (Abcam, Cambridge, UK) were used in western blot analysis for detection of the r-trypsin Y polypeptide containing the polyhistidine *myc* tag. A semi dry electroblotter from Bio-Rad (CA, USA) was used to transfer the proteins from the SDS–PAGE gels onto a Hybond-P membrane (GE Healthcare, UK) according to the manufacturerś instructions. The transfer was performed at 15 V for 30 min at room temperature in Tris-glycine buffer, at pH 8.5, containing 2% methanol. The blotted membranes were blocked with 5% skimmed milk powder (MBF, Selfoss, Iceland) in TBS-tween buffer (20 mM Tris base, 137 mM NaCl, pH 7.6, 0.1% Tween 20) at 4 °C overnight. This was followed by rotation on a rotating platform for 1 h at room temperature. The membranes were washed three times with TBS-Tween buffer (1 × 15 min and 2 × 5 min). They were then incubated at room temperature for 1 h or longer with the mouse monoclonal anti-myc antibody, diluted 1:2000 in TBS-Tween buffer. A wash was performed with the TBS-Tween buffer (1 × 15 min and 2 × 5 min) prior to incubation with the secondary sheep anti-mouse IgG antibodies conjugated with horseradish peroxidase (GE Healthcare) diluted 1:1500. The secondary incubation was also performed at room temperature for at least one hour. Detection was carried out using the ECL detection reagents from GE Healthcare according to the manufacturers instructions after washing the membranes with TBS-Tween buffer (1 × 15 min and 4 × 5 min). A protein molecular weight marker (Fermentas) in the size range of 14.4–116 kDa, was used for the coomassie brilliant blue SDS–PAGE gels. A prestained protein molecular weight marker (Fermentas), in the size range of 20–120 kDa, was used on the gels for the western blot analysis.

The protein concentration of the r-trypsin Y polypeptide was determined by the Bio-Rad protein assay kit according to the manufacturerś protocol. The method is based on the Bradford dye-binding procedure (Bradford, 1976).

2.4. Antibody production

Polyclonal antibodies, raised towards the recombinant Atlantic cod trypsin Y, were produced in mouse ascitic fluid according to the method of Overkamp, Mohammed-Ali, Cartledge & Landon (1988). Four Balb/C2 mice, aged 3 months, were injected intraperitoneally (i.p.) with two different r-trypsin Y preparations. Two mice were injected with a purified form of r-trypsin Y and two mice were injected with a reduced form of the purified r-trypsin Y, i.e. the enzyme isolated from an SDS–PAGE gel run under reducing conditions. The protocol used for preparation of the reduced form of r-trypsin Y was according to the method described by Harlow and Lane (1998). In short, proteins in the samples were separated on SDS–PAGE gels run under reducing conditions and the gel was stained with pageblue coomassie dye. The r-trypsin Y protein bands were excised from the gel and emulsified by passing the gel solution repeatedly through a 1 ml syringe, and finally through a 21 G needle, before injection.

On day "0", the mice received 4 μg and 2.5 μg of the non-reduced and the reduced r-trypsin Y, respectively, emulsified with a Complete Freud's Adjuvant (CFA) (Sigma) in a total volume of 200 μl. The injection was repeated on day 14, when the mice received 13.6 μg of the non-reduced r-trypsin Y in a total volume of 250 μl in CFA and 12 μg of the reduced r-trypsin Y in the volume of 200 μl. The mice were also injected with 500 μl of pristine (Sigma) i.p. On day 23, the mice injected with the reduced r-trypsin Y were sacrificed and the ascitic fluid was collected. The other two mice were injected for the last time with 30.5 μg of the non-reduced r-trypsin Y in a total volume of 300 μl emulsified with an incomplete Freud's adjuvant (Sigma). The mice were then sacrificed on day 34 and the ascitic fluid was collected.

2.5. Specificity of the r-trypsin Y polyclonal antibodies

The specificity of the r-trypsin Y antibodies was tested in western blot analysis on the r-trypsin Y sample used for the antibody production. The western blot techniques used were as described above. The r-trypsin Y antibodies were diluted in the ratio 1:4000–5000 in TBS-Tween buffer. Sheep anti-mouse Ig antibodies conjugated with horseradish peroxidase (GE Healthcare), diluted 1:1500 in TBS-Tween buffer, were used as a secondary antibody. The *P. pastoris* cell supernatant, containing the pPICZα A plasmid

without the trypsinogen Y insert, was used as a negative control in the western blot analysis. The native Atlantic cod trypsin I was used as a standard to examine a possible cross-reaction with the r-trypsin Y antibodies. A negative control experiment was also performed using a serum from non-immunized mice as the primary antibodies in western blot analysis, in the same dilution as the r-trypsin Y antibodies.

2.6. Extraction of serine peptidases from Atlantic cod

Atlantic cod (*G. morhua*) samples were obtained from the Marine Research Institute at Stadur (Grindavík, Iceland). The pyloric caeca was excised from a freshly caught three-month old cod (approximately 5 g) and placed in a cooled serine peptidase extraction buffer (10 mM Tris-HCl, 5 mM $CaCl_2$, 30% glycerol, pH 8.0). Ten ml of the extraction buffer were used per 1 g of the pyloric caeca tissue. The sample was placed on ice for 2 h during transport to the laboratory where it was placed on a magnetic stirrer at 4 °C overnight. The sample was then spun twice at 10,000 rpm and 4 °C for 30 min. The supernatant was used for the isolation of the native trypsin Y polypeptide.

2.7. Isolation and purification by p-aminobenzamidine affinity chromatography

Trypsins were isolated from the other serine peptidases in the cod pyloric caeca supernatant on a trypsin specific *p*-aminobenzamidine affinity column (Sigma). The supernatant was loaded onto the column after equilibration with the serine peptidase extraction buffer. The elution of cod trypsin Y was performed with the serine peptidase extraction buffer, containing 0.4 M NaCl. The tightly bound cod trypsin I was eluted from the resin with 25 mM acetic acid, 20 mM $CaCl_2$, pH 3.2 in 30% glycerol. Due to the sensitivity of cod trypsin I to acidic conditions (Asgeirsson et al., 1989), the elution fractions were mixed with 20 mM Tris-HCl, pH 8.5, 30% glycerol buffer, to increase the pH of the samples immediately upon their release from the column. The ratio was one proportion of buffer to four proportions of sample.

2.8. Purification on a MonoQ-HPLC anion exchange chromatograph

The *p*-aminobenzamidine purified sample was run through a MonoQ 5/5 ion-exchange column (Sigma) in an HPLC chromatograph (Äkta Purifier, GE Healthcare) for further isolation and separation of the native trypsin Y from the other trypsin forms. Prior to loading onto the column, the sample was dialysed against 20 mM Tris, pH 9.3, 5 mM ethanolamine, 10 mM $CaCl_2$ (equilibration buffer), through cellulose dialysis tubing (Sigma), retaining proteins of a molecular weight of 12 kDa or higher. The sample was then condensed approximately 50-fold by using centrifugal filter devices from Millipore (MA, USA). The condensed sample was loaded onto a MonoQ HR 5/5 ion-exchange column equilibrated with equilibration buffer and the proteins were eluted form the column using a linear salt gradient, ranging from 0–1 M NaCl. One ml fractions were collected from the MonoQ column into tubes containing 30% glycerol. The absorbance at 280 nm was monitored during the entire run by the software incorporated with the HPLC system.

2.9. Identity analysis of native trypsin Y

Western blot analysis was performed on the *p*-aminobenzamidine and MonoQ purified trypsin Y fractions. In order to distinguish between the two native forms of trypsins I and Y, the r-trypsin Y antibodies and the native trypsin I antibodies were used for detection on the western blots. The cod trypsin I antibodies were diluted 1:1000 in TBS-Tween buffer. Goat polyclonal antibodies, raised to rabbit IgG conjugated with horseradish peroxidase (Abcam), were used as the secondary antibodies after dilution (1:3000) in TBS-Tween buffer.

Identification of the trypsin Y polypeptide was also performed by MALDI-TOF mass spectrometry. The purified native and recombinant trypsin Y samples were run separately on an SDS–PAGE gel. The band on each gel corresponding to the molecular mass of trypsin Y was excised from the gel and subjected to in-gel trypsin digestion. MALDI-TOF mass spectrometry was performed on the peptide fragments at the Aberdeen Proteome Facility, University of Aberdeen, UK. The mass spectra of the peptide fragments were obtained on an Applied Biosystems Voyager-DE STR MALDI-TOF mass spectrometer. The molecular masses of the peptide fragments were compared to a theoretical trypsin digest of the known trypsin Y amino acid sequence (Spilliaert and Gudmundsdottir, 1999).

Trypsin, chymotrypsin and elastase activities of the purified fractions were measured towards the following synthetic substrates: *N*-CBZ-Gly-Pro-Arg-*p*NA (Sigma) for trypsin activity, suc-Ala-Ala-Pro-Phe-*p*NA (Sigma) for chymotrypsin activity and suc-Ala-Ala-Ala-*p*NA (Sigma) for elastase activity. The substrate solutions used were 25 mM dissolved in dimethylsulfoxide (Sigma). The activation assays contained 50 μl of the supernatant, 100 μl 0.1 M Tris buffer, pH 9.0, and 10 μl of 25 mM synthetic substrate. The reactions were monitored at room temperature at 410 nm in a Spectra Max 250 Microtiter plate reader (Molecular Devices, CA, USA).

The protein concentration of the purified fractions was measured for the specific activity determinations, using the same method as that used for the r-trypsin Y polypeptide (Palsdottir & Gudmundsdottir, 2004).

3. Results

3.1. Production of recombinant Atlantic cod trypsin Y antibodies

The r-trypsin Y polypeptide used for antibody production was expressed in the yeast *P. pastoris* and purified by Q-Sepharose anion-exchange and *p*-aminobenzamidine affinity chromatography. The purified r-trypsin Y polypeptide is seen in Fig. 1 where part A shows a Coomassie stained SDS–PAGE gel and part B shows a western blot, using anti-myc antibodies for detection. The HisMyc fusion part adds about 2.5 kDa to the 27.4 kDa precursor form of the trypsin Y polypeptide. This gives rise to the 30 kDa r-trypsin

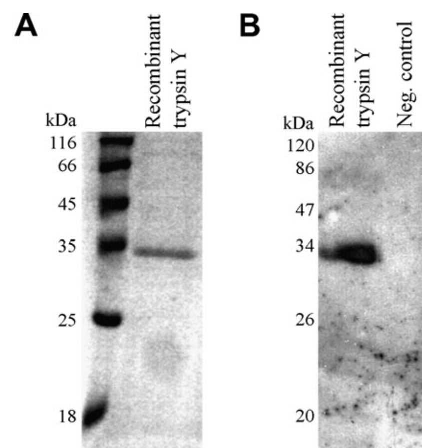

Fig. 1. Analysis of the r-trypsin Y polypeptide used for generation of the r-trypsin Y antibodies. (A) A Coomassie-stained SDS–PAGE gel. (B) Western blot analysis using anti-myc antibodies. The approximately 30 kDa r-trypsin Y band is seen in both parts (A and B). *Pichia pastoris* cells containing the pPICZα A plasmids without the trypsinogen Y insert were used as a negative control on the gel in part B.

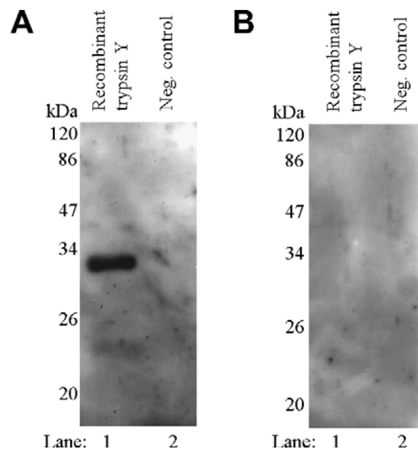

Fig. 2. Western blot analysis, showing the specificity of the r-trypsin Y antibodies. (A) r-trypsin Y antibodies used for detection of the r-trypsin Y polypeptide. (B) Mouse serum used as primary antibodies for detection of the r-trypsin Y polypeptide. The approximately 30 kDa r-trypsin Y band was seen in part A, but no band was detected in part B. *Pichia pastoris* cells containing the pPICZα A plasmid without the trypsinogen Y insert were used as a negative control. No bands were detected in the negative controls (parts A and B), indicating high specificity of the antibodies.

Y (trypsin Y-HisMyc fusion protein) band seen in Fig. 1 (parts A and B). The negative control, consisting of *P. pastoris* cells containing the pPICZα A plasmid without the trypsinogen Y insert, showed no protein bands when blotted with the anti-myc antibodies in western blot analysis (Fig. 1B).

3.2. Specificity of the r-trypsin Y antibodies towards the r-trypsin Y polypeptide

Fig. 2A (lane 1) shows a western blot, demonstrating a protein band of 30 kDa, corresponding to the r-trypsin Y polypeptide. No protein bands were detected in the negative control (pPICZα A expression vector without a trypsinogen Y insert) (lane 2), indicating a strong specificity of the r-trypsin Y antibodies. The r-trypsin Y antibodies used for detection in Fig. 2A were generated towards the non-reduced form of the trypsin Y polypeptide. Antibodies generated towards the reduced form of r-trypsin Y were also shown to give a clear r-trypsin Y band in western blot analysis (results not shown). However, high background contamination was observed on the blot. The serum from non-immunized mice was used as a negative control in the specificity analysis of the r-trypsin Y antibodies. The results are seen on the western blot in Fig. 2B. Notably, no protein bands were detected by the mouse serum in the r-trypsin Y sample (lane 1) or in the control sample (lane 2).

3.3. Isolation and identification of the native Atlantic cod trypsin Y

Trypsins were isolated from the Atlantic cod pyloric caeca and purified on a trypsin-specific *p*-aminobenzamidine column. Fig. 3 (parts A and B) shows western blots of the purified fractions, where

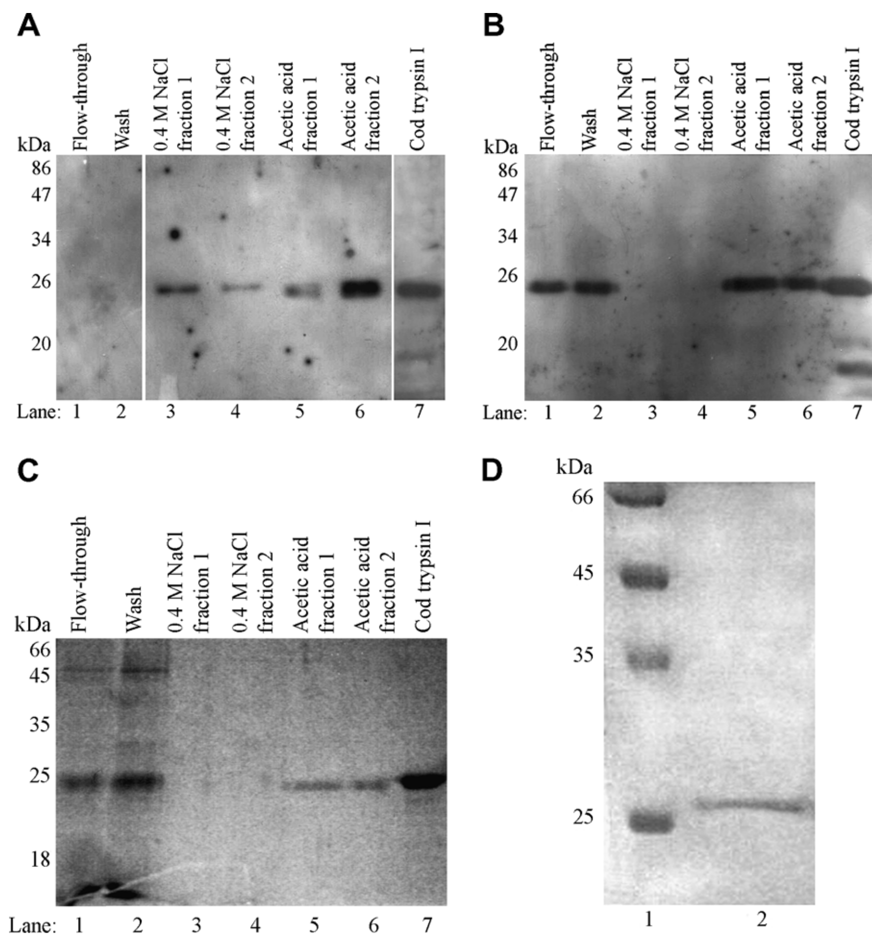

Fig. 3. Analysis of *p*-aminobenzamidine purified native trypsin fractions. (A) Western blot analysis using r-trypsin Y antibodies for detection. (B) Western blot analysis using trypsin I antibodies for detection. (C) A coomassie blue-stained SDS–PAGE gel. (D) A coomassie blue-stained SDS–PAGE gel showing the purified trypsin Y polypeptide not detected in part (C) lanes 3 and 4. The sample (lanes 3 and 4 part, C) was condensed 50-fold and loaded onto the gel (D) for visualization, in-gel trypsin digestion and identification of the peptide fragments by MALDI-TOF mass spectrometry analysis. Native Atlantic cod trypsin I was used as a control sample (parts A, B and C).

the r-trypsin Y antibodies (Fig. 3A) and the native trypsin I antibodies (Fig. 3B) were used for detection. The fractions are also seen on the Coomassie stained SDS–PAGE gel in Fig. 3C. The calculated molecular mass of the putative active form of trypsin Y is about 25.2 kDa or 1.7 kDa higher than that of trypsin I (23.5 kDa). A protein band of about 25 kDa, corresponding to the mass of the putative native trypsin Y polypeptide, was detected by the r-trypsin Y antibodies in the fractions eluted from the *p*-aminobenzamidine column with 0.4 M NaCl (Fig. 3A, lanes 3 and 4). Protein bands were also detected in the fractions eluted with acetic acid (Fig. 3A, lanes 5 and 6) and in the native Atlantic cod trypsin I control sample (Fig. 3A, lane 7). However, the protein bands eluted with the acetic acid, as well as the Atlantic cod trypsin I control sample, appear to be slightly smaller than the protein band eluted with 0.4 M NaCl, as noted by comparing lanes 4 and 5 in Fig. 3A. Although the r-trypsin Y antibodies cross-reacted with the trypsin I polypeptide (Fig. 3A, lane 7), the trypsin I antibodies did not cross-react with the putative trypsin Y polypeptide. As seen, no bands were detected in the 0.4 M NaCl elution fraction on the western blot using the trypsin I antibodies (Fig. 3B, lanes 3 and 4). These only reacted with the approximately 23.5 kDa protein band in the acetic acid elution fraction (Fig. 3B, lanes 5 and 6), as well as in the flow-through and wash fractions (Fig. 3B, lanes 1 and 2). No protein bands corresponding to the 25 kDa putative trypsin Y or the 23.5 kDa trypsin I were detected in the 0.4 M NaCl elution fraction on the Coomassie-stained SDS–PAGE gel seen in Fig. 3C (lanes 3 and 4). This elution fraction, presumed to contain the trypsin Y polypeptide (Fig. 3 C, lanes 3 and 4), was condensed 50-fold and run again on a Coomassie-stained SDS–PAGE gel (Fig. 3D). A faint protein band of about 25 kDa, corresponding to the molecular mass of the trypsin Y polypeptide, was observed on the gel. The presence of trypsin Y in this band was confirmed by MALDI-TOF mass spectrometry. Notably, a protein band of about 23.5 kDa was detected in the acetic acid elution fraction (Fig. 3C, lane 5 and 6) and in the lane containing the native trypsin I control sample (Fig. 3C, lane 7).

3.4. Activity measurements

Activity measurements toward synthetic substrates specific for trypsin (*N*-CBZ-Gly-Pro-Arg-*p*NA), chymotrypsin (suc-Ala-Ala-Pro-Phe-*p*NA) and elastase (suc-Ala-Ala-Ala-*p*NA) were performed on the purified fractions from the *p*-aminobenzamidine column. As expected, the highest trypsin activity was found in the acetic acid elution fraction (Fig. 4A) where the most abundant trypsin I normally comes off the column. Trypsin activity, although lower than in the acetic acid elution fraction, was also found in the 0.4 M NaCl elution fraction containing the native trypsin Y polypeptide. Trypsin activity was also detected in the flow-through and the wash fractions (Fig. 4A). Chymotrypsin activity was found in the 0.4 M NaCl elution fraction but no such activity was detected in the acetic acid elution fraction (Fig. 4B). High chymotrypsin activity was also found in the flow-through and the wash fractions (Fig. 4B). However, no bands corresponding to trypsin Y were detected in these fractions on western blots using the r-trypsin Y antibodies for detection (Fig. 3A, lanes 1 and 2). No elastase activity was seen in any of the fractions eluted from the *p*-aminobenzamidine column.

Table 1 shows the trypsin activity, as measured towards the synthetic substrate *N*-CBZ-Gly-Pro-Arg-*p*NA. It also shows the protein concentrations and the specific activities of the various elution fractions from the *p*-aminobenzamidine column. The highest specific activity was seen in the acetic acid elution fraction (54.4 U/mg), a fraction normally containing the trypsin I polypeptide in abundance. The specific activity of the 0.4 M NaCl elution fraction, containing the putative trypsin Y, was about 8.3 U/mg.

Further purification and identification of trypsin Y was performed on a condensed fraction of the *p*-aminobenzamidine purified sample. Prior to loading onto the MonoQ column, the presence of the putative 25 kDa trypsin Y band in the sample was confirmed by western blot analysis, using the r-trypsin Y antibodies for detection. However, no protein bands were detected on western blots or SDS–PAGE gels in the samples collected from the MonoQ column.

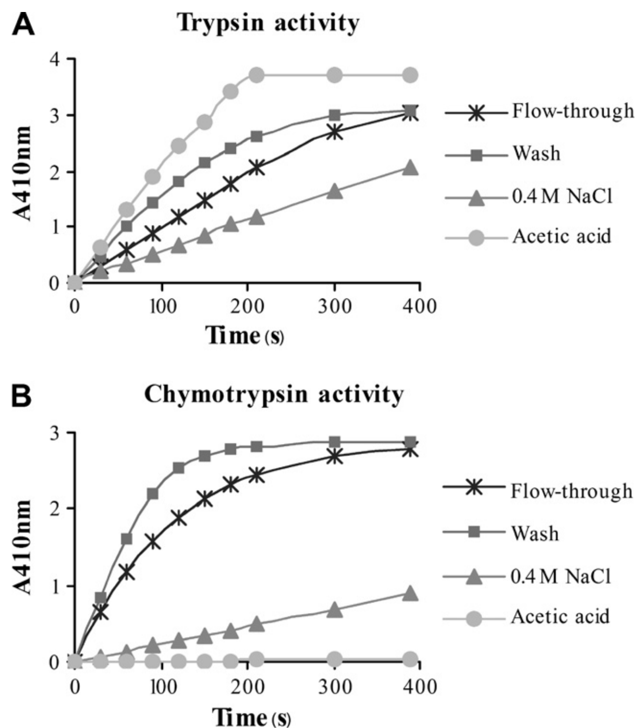

Fig. 4. Enzymatic activity measurements of the *p*-aminobenzamidine-purified trypsin samples. (A) Trypsin activity was measured towards the synthetic substrate *N*-CBZ-Gly-Pro-Arg-*p*NA. (B) Chymotrypsin activity was measured towards the synthetic substrate suc-Ala-Ala-Pro-Phe-*p*NA.

Table 1
Specific activity of purified trypsin fractions from the *p*-aminobenzamidine affinity column

	Activity (U/ml)	Protein (mg/ml)	Specific activity (U/mg)
Flow-through	0.95	0.25	3.80
Wash	1.58	0.28	5.68
0.4 M NaCl	0.45	0.054	8.25
Acetic acid	2.07	0.038	54.4

The specific activity was calculated from the protein concentration and enzymatic activity measured towards the synthetic substrate *N*-CBZ-Gly-Pro-Arg-*p*NA.

4. Discussion

This is the first report describing the isolation, partial characterization and identification of a native group III trypsin. Enzymatic activity measurements toward synthetic substrates do not distinguish between the activity contribution of individual enzyme isoforms or types, such as trypsins Y and I. Therefore, in order to facilitate isolation and detection of the putative native trypsin Y polypeptide, polyclonal r-trypsin Y antibodies were produced in mice.

Recent studies showed that trypsin Y mRNA is expressed in very low amounts relative to that of trypsin I in the pyloric caeca of Atlantic cod (Pálsdóttir & Gudmundsdóttir, 2007). In spite of that, polyclonal antibodies raised towards the r-trypsin Y polypeptide facilitated isolation and purification of the native trypsin Y poly-

peptide from its native source. Unexpectedly, the r-trypsin Y antibodies were found to cross-react with the trypsin I polypeptide, despite only about 45% amino acid sequence identity between the trypsin I and Y polypeptides. On the other hand, polyclonal antibodies raised towards the native trypsin I polypeptide did not cross-react with the native or the r-trypsin Y polypeptide. Thus, the use of both antibodies promoted purification of trypsin Y from the other cod trypsins.

Identity confirmation of the native trypsin Y is based on MALDI-TOF mass spectrometry of the polypeptide (Fig. 3 D) subjected to in-gel trypsin digestion, as well as on the following criteria. The native enzyme was eluted from the trypsin-specific *p*-aminobenzamidine column under the same conditions as the r-trypsin Y or at 0.4 M NaCl (Fig. 3A, lanes 3 and 4) while trypsin I was eluted with acetic acid (Fig. 3B, lanes 5 and 6). Also, detection with the r-trypsin Y antibodies showed a protein band of 25 kDa corresponding to the native trypsin Y polypeptide (Fig. 3A, lands 3 and 4) in the 0.4 M NaCl elution fraction. A band on a coomassie-stained SDS–PAGE gel, corresponding to the trypsin Y polypeptide, was also detected in a condensed sample from the 0.4 M NaCl fraction (Fig. 3D). However, no band corresponding to trypsin Y was detected by the trypsin I antibodies in Fig. 3B (lanes 3 and 4). Furthermore, the calculated molecular mass of the active form of trypsin Y is about 1.7 kDa higher than that of trypsin I. Notably, a slight difference in mass between the putative trypsin Y and trypsin I polypeptides can be noted by comparing lanes 4 and 5 (Fig. 3A). Also, the fraction containing the trypsin Y polypeptide shows both trypsin and chymotrypsin activities (Fig. 4A and B) in agreement with the recombinant form of the enzyme (Palsdottir & Gudmundsdottir, 2004). No chymotrypsin activity was detected in the trypsin I sample. Nonetheless, the flow-through and wash fractions showed high chymotrypsin activity that does not appear to stem from trypsin Y, as the polypeptide was not detected in these fractions by the r-trypsin Y antibodies (Fig. 3A, lanes 1 and 2). High chymotrypsin activity is to be expected in the flow-through and wash fractions as the initial pyloric caeca supernatant contains numerous proteolytic enzymes, including chymotrypsin that do not bind to the *p*-aminobenzamidine column. The specific activities of trypsin I and trypsin Y towards the synthetic substrate *N*-CBZ-Gly-Pro-Arg-*p*NA were shown to be 54.4 U/mg and 8.3 U/mg, respectively (Table 1). Thus, trypsin I shows about 6.5-fold higher activity than does trypsin Y towards this substrate.

Trypsin Y appears to have lower affinity for *p*-aminobenzamidine than trypsin I, indicating a different substrate binding site. In addition, both the recombinant and the native trypsin Y have dual substrate specificity. These results, along with data from amino acid sequence alignments, show that trypsin Y is indeed different from the traditionally classified cold-adapted trypsins of group I (Gudmundsdottir, A., Gudmundsdottir, E., Oskarsson, Bjarnason, Eakin & Craik, 1993; Brandsdal, Heimstad, Sylte & Smalas, 1999; Leiros, Willassen & Smalas, 1999; Schrøder Leiros, Willassen, & Smalås, 2000; Toyota, Kuninaga, Sekizaki, Itoh, Tanizawa & James, 2002). The r-trypsin Y polypeptide was shown to be active at lower environmental temperatures than are other known trypsins and to be completely inactivated at 30 °C. However, this characteristic has not yet been analysed for the native trypsin Y polypeptide. Despite many differences and indications of different substrate-binding sites in trypsins Y and I (Palsdottir & Gudmundsdottir, 2004), trypsin Y has greater amino acid sequence similarities to the traditionally classified trypsins than to any other known groups of proteins (Spilliaert & Gudmundsdottir, 1999).

Presently, it is not understood why the Atlantic cod and many other vertebrates produce more than one form of trypsin. The different trypsin forms may facilitate survival under variable environmental and physical conditions (Roach, 2002). Research on winter flounder (*Pleuronectes americanus*) (Murray, Perez-Casanova, Gallant, Johnson, & Douglas, 2004) and Atlantic salmon (*Salmo salar*) (Rungruansak-Torrissen, Pringle, Moss, & Houlihan, 1998) has shown that environmental temperatures, as well as the developmental stage, affect the expression of individual trypsin types in these organisms.

5. Conclusion

The trypsin Y polypeptide isolated, purified and identified in this study has dual substrate specificity, a relatively high activity at low temperatures and is completely inactivated at 30 °C. These characteristics may offer an advantage of using trypsin Y, over other enzymes, as a processing aid for sensitive products such as seafood. As demonstrated in this study, the native trypsin Y is present in low amounts in the cod pyloric caeca. Therefore, the r-trypsin Y is more likely to be the source for commercial production of the enzyme. Future research will focus on further characterization of the native trypsin Y polypeptide and its potential applications in the food industry.

Acknowledgements

This work was supported by grants from the University of Iceland Research Fund and the Research Fund of Iceland. We thank Professor Jón Bragi Bjarnason for his advice during preparation of the manuscript and Dr. Bjarnheidur Gudmundsdóttir for her contribution to the production of the r-trypsin Y polyclonal antibodies.

References

Asgeirsson, B., & Cekan, P. (2006). Microscopic rate-constants for substrate binding and acylation in cold-adaptation of trypsin I from Atlantic cod. *Febs Letters, 580*, 4639–4644.

Asgeirsson, B., Fox, J. W., & Bjarnason, J. B. (1989). Purification and characterization of trypsin from the poikilotherm *Gadus morhua*. *European Journal of Biochemistry, 180*, 85–94.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry, 72*, 248–254.

Brandsdal, B. O., Heimstad, E. S., Sylte, I., & Smalas, A. O. (1999). Comparative molecular dynamics of mesophilic and psychrophilic protein homologues studied by 1.2 ns simulations. *Journal of Biomolecular Structure and Dynamics, 17*, 493–506.

D'Amico, S., Marx, J. C., Gerday, C., & Feller, G. (2003). Activity-stability relationships in extremophilic enzymes. *Journal of Biological Chemistry, 278*, 7891–7896.

Gudmundsdóttir, Á., & Bjarnason, J. B. (2007). Applications of cold adapted proteases in the food industry. In R. Rastall (Ed.), *Novel enzyme technology for food applications* (pp. 205–221). Cambridge, UK: Woodhead Publishing Limited.

Gudmundsdottir, A., Gudmundsdottir, E., Oskarsson, S., Bjarnason, J. B., Eakin, A. K., & Craik, C. S. (1993). Isolation and characterization of cDNAs from Atlantic cod encoding two different forms of trypsinogen. *European Journal of Biochemistry, 217*, 1091–1097.

Gudmundsdottir, A., & Palsdottir, H. M. (2005). Atlantic cod trypsins: From basic research to practical applications. *Marine Biotechnology (NY), 7*, 77–88.

Harlow, E., & Lane, D. (1998). *Immunizations. Antibodies a Laboratory Manual*. USA: Cold Spring Harbor Laboratory.

Kishimura, H., Klomklao, S., Benjakul, S., & Chun, B.-S. (2008). Characteristics of trypsin from the pyloric ceca of walleye pollok (*Theragra chalcogramma*). *Food Chemistry, 106*, 194–199.

Leiros, H. K., Willassen, N. P., & Smalas, A. O. (1999). Residue determinants and sequence analysis of cold-adapted trypsins. *Extremophiles, 3*, 205–219.

MacDonald, R. J., Stary, S. J., & Swift, G. H. (1982). Two similar but nonallelic rat pancreatic trypsinogens. Nucleotide sequences of the cloned cDNAs. *Journal of Biological Chemistry, 257*, 9724–9732.

Murray, H. M., Perez-Casanova, J. C., Gallant, J. W., Johnson, S. C., & Douglas, S. E. (2004). Trypsinogen expression during the development of the exocrine pancreas in winter flounder (*Pleuronectes americanus*). *Comparative Biochemistry and Physiology, Part A. Molecular and Integrative Physiology, 138*, 53–59.

Overkamp, D., Mohammed-Ali, S., Cartledge, C., & Landon, J. (1988). Production of polyclonal antibodies in ascitic fluid of mice: technique and applications. *Journal of Immunoassay, 9*, 51–68.

Palsdottir, H. M., & Gudmundsdottir, Á. (2004). Recombinant trypsin Y from Atlantic cod – properties for commercial use. *Journal of Aquatic Food Production Technology, 13*, 85–100.

Pálsdóttir, H. M., & Gudmundsdóttir, Á. (2007). Development of a qRT-PCR assay to determine the relative mRNA expression of two different trypsins in Atlatic cod (*Gadus morhua*). *Comparative Biochemistry and Physiology, Part B, 146*, 26–34.

Roach, J. C. (2002). A clade of trypsins found in cold-adapted fish. *Proteins, 47*, 31–44.

Roach, J. C., Wang, K., Gan, L., & Hood, L. (1997). The molecular evolution of the vertebrate trypsinogens. *Journal of Molecular Evolution, 45*, 640–652.

Rungruansak-Torrissen, K., Pringle, G. M., Moss, R., & Houlihan, D. F. (1998). Effects of varying rearing temperatures on expression of different trypsin isozymes, feed conversion efficiency and growth in Atlantic salmon (*Salmo salar* L.). *Fish Physiology and Biochemistry, 19*, 247–255.

Schrøder Leiros, H. K., Willassen, N. P., & Smalås, A. O. (2000). Structural comparison of psychrophilic and mesophilic trypsins. Elucidating the molecular basis of cold-adaptation. *European Journal of Biochemistry, 267*, 1039–1049.

Shoichet, B. K., Baase, W. A., Kuroki, R., & Matthews, B. W. (1995). A relationship between protein stability and protein function. *Proceedings of the National Acadademy of Sciences USA, 92*, 452–456.

Smalås, A. O., Schrøder Leiros, H. K., Os, V., & Willassen, N. P. (2000). Cold adapted enzymes. *Biotechnology Annual Review, 6*, 1–57.

Spilliaert, R., & Gudmundsdottir, A. (1999). Atlantic cod trypsin Y-member of a novel trypsin group. *Marine Biotechnology, 1*, 598–607.

Stroud, R. M., Kay, L. M., & Dickerson, R. E. (1974). The structure of bovine trypsin: electron density maps of the inhibited enzyme at 5 Angstrom and at 2–7 Angstrom resolution. *Journal of Molecular Biology, 83*, 185–208.

Toyota, E., Ng, K. K., Kuninaga, S., Sekizaki, H., Itoh, K., Tanizawa, K., et al. (2002). Crystal structure and nucleotide sequence of an anionic trypsin from chum salmon (*Oncorhynchus keta*) in comparison with Atlantic salmon (*Salmo salar*) and bovine trypsin. *Journal of Molecular Biology, 324*, 391–397.