A thesis submitted in partial fulfillment of the requirements of the degree of

MASTER OF FOOD SCIENCE

Faculty of Food Science and Nutrition, School of Health Sciences

University of Iceland

Activity of Atlantic cod trypsin towards cytokines and other proteins.

Una Björk Jóhannsdóttir

2009

Supervisors: Professor Ágústa Guðmundsdóttir PhD and Doctor Bjarki Stefánsson

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Abstract

Previous research demonstrated that cod trypsin is the most active among 15 comparable proteases, including bovine trypsin, towards cytokines involved in immune responses and inflammation. The research presented here is a part of a larger project aiming at increasing the knowledge between the efficacy of cod trypsin towards cytokines and other pathological proteins in cell-culture studies, kinetic analysis of cod trypsin and clinical data on the wound-healing efficacy cod trypsin. In this part of the project the ability of cod trypsin and bovine trypsin to cleave isolated cytokines and other proteins in their native conformation was compared. The degradation peptides were separated by reversed phase chromatography and the extent of degradation was determined based on the resulting chromatograms. The results show that cod trypsin outperforms other bovine trypsin in cleaving pathological proteins (ICAM-1) and other proteins in their native form. The extent of degradation of the five substrates tested was greater with cod trypsin compared to bovine trypsin except for BSA where it was about the same. This is particularly intriguing since one of the substrates (ICAM-1) plays an important role in immune response, inflammation and pathogen invasion. Kinetic analysis of cod trypsin was performed by measuring the activity of trypsin I (most abundant isoform of cod trypsin) towards artificial (X-X-Arg-pNA) substrates with different amino acids in site P_2 and P_3. The data indicates that the catalytic efficiency (k_{cat}/K_m) of trypsin I was higher towards substrates containing amino acids with large side chains in site P_2 and P_3.

Ágrip

Fyrri rannsóknir sýndu að þorskatrypsín er virkast 15 samanburðarensíma gagnvart cytokínum er gegna hlutverki í ónæmissvörun og bólgumyndun. Þrjú náttúruleg afbrigði trypsíns (trypsín I, II og III) hafa fundist í þorski og er trypsín I í mestu magni. Auk þess hefur þetta trypsín afbrigði hæstu hvötunargetuna (k_{cat}/K_M) en hún er um 20 sinnum hærri en hjá sambærilegum trypsínum úr spendýrum. Verkefnið er hluti stærri rannsóknaáætlunar sem miðar að auknum skilningi á milli áhrifa þorskatrypsíns á frumur í rækt, hraðafræðilegra ensímrannsókna og klínískra rannsókna þar sem trypsín gaf góðan árangur við græðingu sára. Samanburður var gerður á getu þorskatrypsíns og nautatrypsíns til að rjúfa cytókín og einföld hnattlaga prótein í náttúrulegri myndbyggingu, niðurbrotspeptíð voru greind með HPLC tækni og hraði niðurbrotsins áætlaður á HPLC línuriti. Niðurstöðurnar sýna að þorskatrypsín er mun virkara en nautatrypsín í að brjóta niður sjúkdómstengd prótein (ICAM-1) og önnur prótein í sinni náttúrulegu myndbyggingu. Umfang niðurbrotsins á þeim fimm hvarfefnum sem prófuð voru var mun meira með þorskatrypsíni í samanburði við nautatrypsín, nema fyrir BSA þar sem það var álíka. Þetta er athyglisvert í ljósi þess að eitt hvarfefnanna (ICAM-1) gegnir mikilvægu hlutverki þegar kemur að ónæmisviðbragði, bólgu og innrás sýkla. Hraðafræðimælingar þar sem notuð voru X-X-Arg-pNA hvarfefni voru framkvæmdar á hreinsuðu trypsín I. Þær mælingar sýndu að amínósýrur í sætum P_2 og P_3 á hvarfefninu höfðu mikil áhrif á hraðafastana k_{cat}, K_m og k_{cat}/K_m, þar sem hvötunargeta trypsín I virtist meiri gagnvart hvarfefnum með stórar amínósýrur.

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Table of Content

Table of Figures

Figure of Tables.

Table of Abbreviations.

A	Absorbance
Ala	Alanine
Arg	Arginine
Asp	Aspartic acid
BSA	Bovine Serum albumin
Bz-	Benzoyl-
CBZ-	Carbo-benzyloxy-
cDNA	Complementary DNA
C-terminal	Carboxyl terminus of a protein (COOH)
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
FPLC	Fast Protein Liquid Chromatography
Gly	Glycine
His	Histidine
HPLC	High Performance Liquid Chromatography
kDa	Kilo Dalton
Lau	Leucine
Lys	Lysine
mA	Milli Amper
MeOsuc-	Methoxy-*N*-succinyl-
Met	Methionine
MonoQ	Quaternary ammonium strong anion exchange Mono beads
N-terminal	Amino terminus of a protein (NH_2)
PAGE	Polyacrylamide gel electrophoresis
Phe	Phenylalanine
*p*NA	Para nitroanilide
Pro	Proline
rpm	Rounds per minute
SDS	Sodium dodecyl sulphate
Ser	Serine
Val	Valine
TFA	Trifluoroacetic acid
Tos-	Tosyl
Tris	Tris [hydroxymethyl] aminomethane
Z-	Benzyloxycarbonyl

1. Introduction

Previous research demonstrated that cod trypsin is the most active among 15 comparable proteases available, including bovine trypsin, towards cytokines involved in immune responses and inflammation. The research presented here is a part of a larger project aiming at increasing the knowledge between the efficacy of cod trypsin towards cytokines and other pathological proteins in cell-culture studies, kinetic analysis of cod trypsin and clinical data on the wound-healing efficacy cod trypsin.

This thesis is about cod trypsin and its ability to cleave three amino acids substrates, cytokine and other proteins. It is focused on comparing the ability of cod trypsin and bovine trypsin to cleave the isolated cytokine ICAM-1 and other proteins in their native conformation. The purity of bovine trypsin and cod trypsin was analyzed using SDS-PAGE analysis, MonoQ chromatography and Western blot analysis. The ability of bovine trypsin and cod trypsin in cleaving seven different proteins was tested. The proteins were lysozyme, myoglobin, lactoferrin, bovine serum albumin (BSA), ICAM-1, α-casein dephosphorylated and β-casein. The degradation peptides were analyzed by HPLC and the rate of degradation analyzed by HPLC and the rate of reaction estimated by the integral of the degradation peaks. Kinetic analysis of MonoQ purified cod trypsin I in cleaving seven three amino acid *para*-nitroanilide (*p*NA) substrates was tested. The substrates were CBZ-Gly-Pro-Arg-*p*NA, Bz-Phe-Val-Arg-*p*NA, Tos-Gly-Pro-Lys-*p*NA, H-D-Val-Leu-Arg-*p*NA, Z-Val-Gly-Arg-*p*NA, D-Phe-Pro-Arg-*p*NA and MeoSuc-Ala-Ala-Arg-*p*NA. The steady-state kinetic constants k_{cat} and K_m were determined using the Lineweaver-Burk plot, Hanes-Woolf plot, Direct-linear plot and by doing non-linear least-squares fit to the Michaelis Menten equation to find out catalytic efficiency (k_{cat}/ K_m) for each substrate.

2. Background

2.1. **Atlantic cod**

For Iceland and other countries in the Northern hemisphere the Atlantic cod is an economically important fish species. The cod is a poikilothermic marine fish species that lives at 600 m depth in the ocean were temperature is between 0-10 °C. Usually the cod lives at 200-400 meter depth around Iceland were the temperature is about 2-4°C (Schopka, 1996). Digestive enzymes play an important role in nutrition and development of the cod because the cod is a predator and feeds on small fish and crabs (Marteinsdóttir, 2005). The pyloric caecum serves the role of a digestive organ in the Atlantic cod and therefore produces the digestive enzymes. The pyloric caeca is rich in digestive enzymes such as serine proteases (Ásgeirsson B, 1989). Many endopeptidases have been isolated from the cod pyloric caeca and characterized such as trypsin, chymotrypsin and elastase (Ásgeirsson B, 1989, Ásgeirsson B, 1991, Ásgeirsson B, 1993). Trypsin, chymotrypsin and elastase from the Atlantic cod all have typical characteristics of cold adapted (psychrophilic) enzymes.

2.2. **Trypsin**

Trypsin is a serine peptidase of the S1 family found in the digestive system of many vertebrates, where it breaks down proteins. All peptidases of the S1 family are endopeptidases and have a catalytic triad consisting of His 57, Asp 102 and Ser195 (Halfon, 1998). Among other enzymes of the S1 family are chymotrypsin and elastase. Trypsin was first named and described as the proteolytic activity in pancreatic secretions by Kühne in 1876 (Kühne, 1876). Trypsin cleaves peptide bonds C-terminal to Arg or Lys residues (Nortop, 1931). Trypsin can be classified into 3 basic groups, termed I, II and III, based on their amino acid sequence identities (Guðmundsdóttir, 2005). All these groups have been found in the Atlantic cod.
Peptidases are classified into seven classes based on their catalytic types. These are the serine, threonine- cysteine-, aspartic-, metallo- and the newly described glutamic acid peptidases (Pálsdóttir, 2006). Serine peptidase were the first enzymes to be studied extensively (Neurath, 1985). They play important roles in digestion and other biological functions in all animals including insects, fish and mammals (Keller, 1958, Neurath, 1984). The catalytic triad plays the main role during catalysis by serine peptidases. The catalytic mechanisms of serine peptidases were they hydrolyze ester and amide bonds proceeds by an identical acyl transfer mechanism shown in Figure 1.

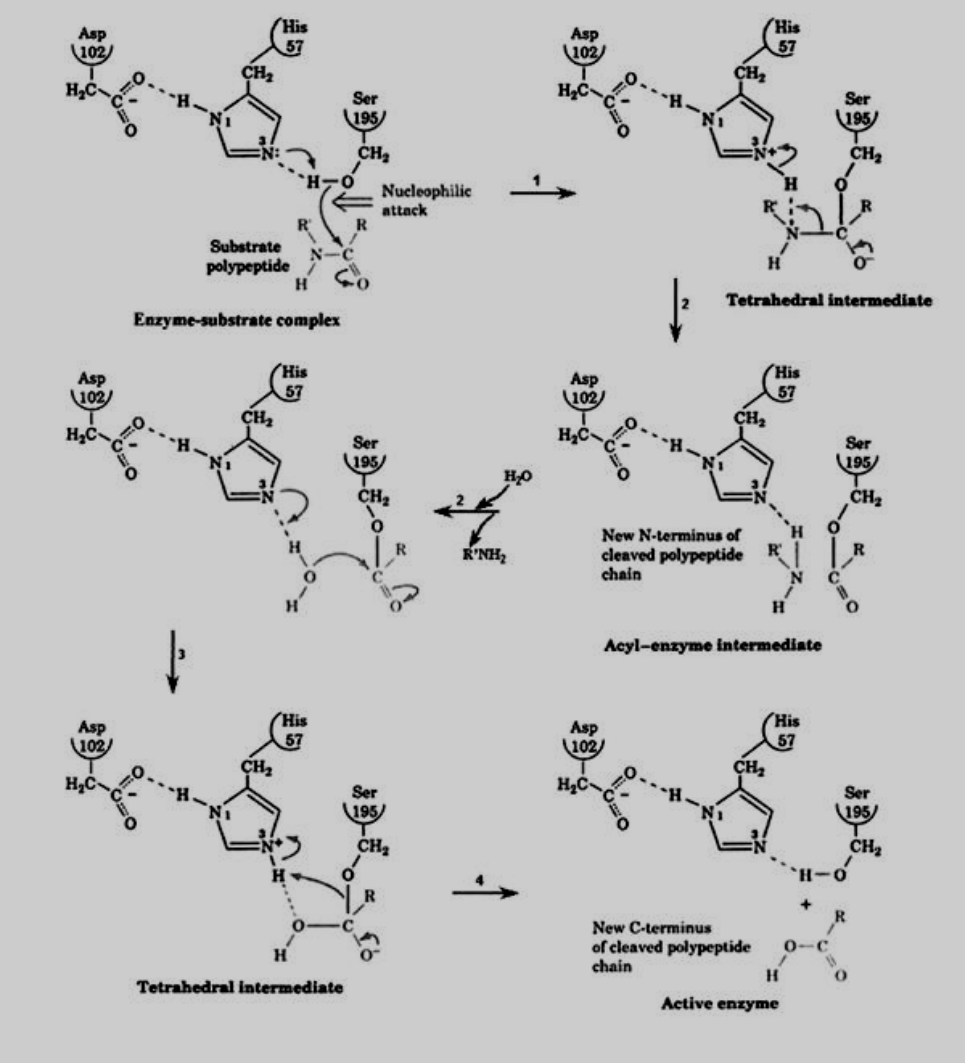

Figure 1. The serine peptidase catalytic mechanism.

Ser195 acts as a nucleophile and attacks the carbonyl carbon atom of the scissile substrate peptide bond, generating a tetrahedral intermediate. The bond between N and C in the peptide bond is broken resulting in an acyl-enzyme intermediate. One half of the peptide diffuses away. Water comes into the reaction as a nucleophile, attacking the substrate carbonyl carbon and another tetrahedral intermediate is generated. The electron deficient carbonyl carbon re-forms and the C-terminal of the peptide is ejected. The original enzyme is regenerated (Voet, 2004).

The tertiary structure of a number of peptidases in the S1 family has been solved (Birktoft, 1972, Huber, 1974, Stroud, 1974). The S1 family has a strongly conserved tertiary structure and it can clearly be seen in trypsin. Although the primary structure of trypsins is not the same between groups, their folds are very similar (Sprang, 1987). The tertiary structure of trypsin consists of two β-barrel domains arranged at right angles to each other (Smalas, 1994). An α-helix and two loops, the N-terminal loop and the inter domain loop connects the two β-barrels (Schrøder Leiros HK, 2000). The catalytic triad is located between the two domains were His57 and Asp102 residues are located in one domain and Ser 195 and the substrate

specificity pocket (seen in Figure 2) are located in the other domain (Schrøder Leiros HK, 2000). The substrate binds in that site. Asp189 residue is at the bottom of the specificity pocket and is responsible for cleaving peptide bonds with positively charged amino acid residues (Lys and Arg) (Carik, 1985). The amino acids residues in the substrate that trypsin hydrolyses are named from the N terminus to the C terminus, $P_{n,}$….P_2,P_1,P_{-1},P_{-2},….P_{-n} and trypsin cleaves the chain between P_1 and P_{-1} and Lys or Arg are in the P_1 – site, their respective enzyme binding site is $S_{n,}$….S_2,S_1,S_{-1},S_{-2},….S_{-n}. The amino acids in position Gly216 and Gly226 have a dramatic effect on the specificity and catalytic efficiency of trypsin (Perona, 1997). Gly216 is in the S_3-site in the specificity pocket and forms two hydrogen bonds with the amino acid in the P_3-site of the substrate which is required to achieve full catalytic potency (Hedstrom, 1992, Hedstrom, 1994b). The amino acids in P_2 and P_3 do contribute to catalytic efficiency (Corey, 1992, Schellenberger, 1994). Asp189 is in position S_1 and forms hydrogen bonds with Lys or Arg residues in position P_1.

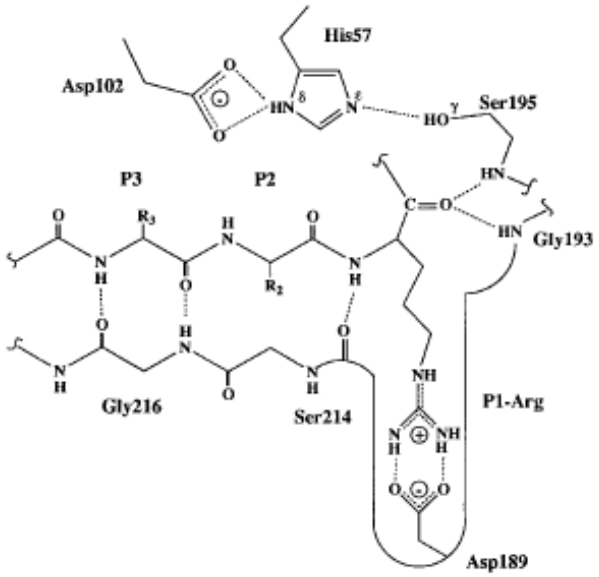

Figure 2. The chemical mechanism of trypsin catalysis.

The catalytic residues His57, Asp102 and Ser195 interact with a peptide substrate binding to the P_1 site. Five enzyme-substrate hydrogen bonds at position P_1 and P_3 are shown in addition to hydrogen bonds among the members of the catalytic amino acids (Perona, 1997).

When Asp189 in rat trypsin was replaced with a Ser residue (as in chymotrypsin) no trypsin like activity could be detected and no significant chymotrypsin activity was observed. This indicates that even though the amino acid residues in the substrate binding pocket play an important role in specificity of the enzyme, the amino acids distant from the substrate binding site are also crucial determinants of specificity (Carik, 1985, Hedstrom, 1992, Perona, 1995).

2.3. **Cold-adapted enzymes**

In general, trypsins from the Atlantic cod and other fish adapted to cold environment as the Atlantic Ocean are different from their mammalian analogues in that they have higher catalytic efficiencies, especially at low temperatures (Ásgeirsson B, 1989, Gerday C, 2000, Schrøder Leiros, 2000). Cold adapted enzymes are more sensitive to inactivation by heat, low pH, and autolysis than their mesophilic analogues (Ásgeirsson B, 1989, Simpson, 1989). Many cold adapted proteolytic enzymes, such as trypsin I from Atlantic cod and euphaulysin from Antarctic krill have been shown to be more susceptible to autolysis than their mesophilic counterparts (Kristjánsdóttir, 1999, Helgadottir, 2002). That may be connected to their increased catalytic efficiency and decreased thermal and acid stability compared to their mesophilic analogues (Shoichet, 1995, Miyazaki, 2000, Smalas, 2000, D'Amico, 2003, Feller, 2003).

The mechanisms of cold adaptation of enzymes are more complex than previously anticipated and it seems that enzymes adapted to cold in different ways. (Gerday C, 2000, Schrøder Leiros HK, 2000, Smalas, 2000, Feller, 2003) High molecular flexibility compared to their mesophilic analogues appears to be a common feature of cold-adapted enzymes (Závodszky, 1998). Flexible loops in the structure of cold adapted enzymes seem to play an important role in substrate specificity as well as in the activity (Perona, 1997). Interactions between structural regions of cold-adapted enzymes, in particular between domains, seem to be weaker than between those in mesophilic analogues. This weaker interaction seems to give the enzyme more flexibility and may decrease thermal stability of cold-adapted enzymes in many cases (Schrøder Leiros HK, 2000). Numerous authors (Gerday C, 2000, Smalas, 2000) have pointed out other determinants for cold adaptation for trypsin and other cold-active enzymes relative to their mesophilic analogues. These enzymes seems to have lower number of hydrogen bounds, less densely packed structure, increased surface hydrophobicity, a higher number of Met residues, and a different fold of the “autolysis loop”. Most cold-adapted enzymes seem to contain a higher number of Met residues compared to their mesophilic homologues (Guðmundsdóttir, 1993, Guðmundsdóttir, 1996). Met residues can increase the flexibility of the enzyme backbone and therefore reduce the overall protein stability (Guðmundsdóttir, 1996). Trypsin I from the Atlantic cod has reduced proline content at specific points in their structures (Guðmundsdóttir, 1993). Many cold adapted enzymes have reduced Pro residues in their structure while other enzymes have an increased number of Gly residues (Guðmundsdóttir, 1993, Feller, 1997a, Feller, 1997b, Russell, 1998, Smalas, 2000).

This entire feature seems to give the enzymes more flexibility and decreased thermal stability and that seems to give higher catalytic efficiency within cold-adapted enzymes compared to their mesophilic analogues.

2.4. Trypsin from the Atlantic cod.

Trypsins are produced in the pyloric caecum in the Atlantic cod. Numerous trypsin isozymes are produced in the Atlantic cod (Ásgeirsson B, 1989, Guðmundsdóttir, 1993, Helgadottir, 2002). As described above most trypsins can be classified into three basic groups, numbered I, II and III based on their amino acid sequence. The largest of these is group I which includes members like Atlantic cod trypsin I and Atlantic salmon trypsin I (see Figure 3). All the three groups have the same catalytic triad residues His57, Asp102 and Ser 195, they also share the same Asp 189 in the bottom of their substrate-binding pocket and the Tyr172 residue which is considered to be a key residue in determining substrate specificity for trypsin (Hedstrom, 1994b).

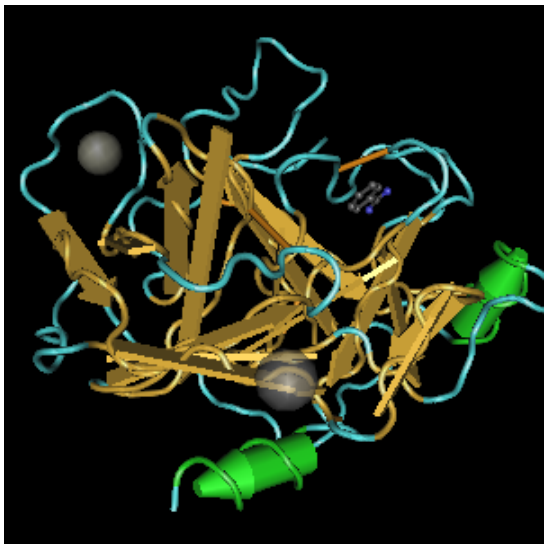

Figure 3. Trypsin from the Atlantic cod (PDB ID: 2EEK).

Several of the cod trypsin isozymes have been isolated from their native source and trypsin I is the most predominant one. Trypsin I has the highest catalytic efficiency, which is nearly 20 times higher than mesophilic bovine trypsin and trypsin I is by far the best characterized form of the trypsin isozymes (Ásgeirsson B, 1989, Jónsdóttir, 2004). The high catalytic efficiency and low thermal stability of Atlantic cod trypsin I makes it a very interesting enzyme to study with respect to its structure and function. A cDNA clone corresponding to trypsin I was isolated earlier from an Atlantic cod pyloric caeca cDNA library and sequenced (X76886) (Guðmundsdóttir, 1993). Two additional Atlantic cod trypsin clones, termed trypsin X

(Guðmundsdóttir, 1993) and trypsin Y (Spilliaert, 1999, Pálsdóttir, 2007) have been identified. The precursor form of the recombinant Atlantic cod trypsin I has been produced in an *E. coli* expression system and an active recombinant trypsin I was generated by cleavage of the purified fusion protein (Jónsdóttir, 2004).

2.5. **ractical Applications of trypsin**

The igh catalytic efficiency of Atlantic cod trypsin is especially useful in the processing of fresh food in which protein digestion at low temperatures is required (Bjarnason, 1993, Bjarnason, 2000). The food industry requires high safety and with cold-adapted enzymes the processing can be done at low temperatures and that minimizes undesirable chemical reactions as well as bacterial contamination, which may indeed be elevated at higher temperatures (Guðmundsdóttir, 2005). Because of the high catalytic efficiency of cold adapted trypsin the dose of the enzyme can by minimized and they can easily be inactivated by relatively low heat. The trypsin from the Atlantic cod is already used in all-natural seafood flavoring on the international market (Guðmundsdóttir, 2005).

Proteolytic enzymes, such as trypsin and chymotrypsin from the pancreas of cattle or pigs, have been used in clinical trials and as therapeutic agents for humans and animals for over a hundred years (Morris, 1891, Guðmundsdóttir, 2005). In general, the proteolytic enzymes have been used therapeutically in four areas; 1) as oral agents for special gastrointestinal disorders, 2) as local agents to debride or solubilize proteinaceous materials which either foster or cause disease, 3) as anti-inflammatory agents, and 4) as thrombolytic agents in the treatment of thromboembolic disorders (Sherry, 1960). Mammalian trypsins and chymotrypsins at high concentrations have been used for wound healing (Spittler, 1954) and as an anti-inflammatory agents (Martin, 1955) both in human and animals (Lecht, 1968, Leipner, 2000). The medical application of trypsin from the Atlantic cod includes the use of the native trypsin for inflammation, fungal diseases, acne, wound healing and other dermatologic indications, as well for general and dental hygiene (Bjarnason, 2000). In this study the ability of cod trypsin to cleave three amino acids substrates, cytokine and other proteins is tested. It is focused on comparing the ability of cod trypsin and bovine trypsin to cleave isolated cytokine and other proteins in their native conformation.

3. Materials and Methods

3.1. **Chemicals.**

Bovine trypsin was obtained from Sigma Aldrich (catalog number T9201) and cod trypsin was obtained from Zymetech. Lysozyme (Sigma Aldrich, Product no. L7651), myoglobin (Sigma Aldrich, Product no. M0630), lactoferrin (Sigma Aldrich, Product no. L4765), bovine serum albumin (BSA) (Sigma Aldrich, Product no. A3059), ICAM-1 (R&D Systems, Inc., Catalog number ADP4), α-casein dephosphorylated (Sigma Aldrich, Product no. C8032) and β-casein (Sigma Aldrich Product, no. C6905). N-CBZ-Gly-Pro-Arg-pNA (Sigma), Bz-Phe-Val-Arg-*p*NA (Bachem Americas, Inc), Tos-Gly-Pro-Lys-*p*NA (Bachem Americas, Inc), H-D-Val-Leu-Arg-*p*NA (Bachem Americas, Inc), Z-Val-Gly-Arg-*p*NA (MP Biomedicals), D-Phe-Pro-Arg-*p*NA (MP Biomedicals) and MeoSuc-Ala-Ala-Arg-*p*NA (MP Biomedicals).

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

HPLC chromatography (Äkta Purifier, GE Healthcare) using the MonoQ 5/5 ion exchange column was used to characterize the samples used for the studies and for purification of trypsin I. Before loading the column, the column was prepared with 20 mM Tris, 10 mM $CaCl_2$ and 5 mM ethanolamin pH 9,1 (Buffer A). The sample (1 mL) was applied to the MonoQ 5/5 ion exchange column at a flow rate of 1.0 mL/min. After washing the column with buffer A, the trypsin was eluted using a linear salt gradient, ranging from 0-30 % of 20 mM Tris, 10 mM $CaCl_2$, 5 mM ethanolamin and 1 M NaCl pH 9,1 (buffer B) and then 100 % buffer B. The absorbance at 280 nm was analyzed during the entire run by software equipped with the HPLC system.

3.3. **SDS-PAGE gel electrophoresis**

The size and purity of the cod trypsin and bovine trypsin was analyzed by SDS-PAGE gel electrophoresis carried out using a SE 250 Mighty Small II 10 x 8 cm electrophoresis equipment from Hoefer (CA, USA). The acrylamide concentrations of the stacking and the resolving gels were 5 % and 12 %, respectively. Samples were mixed in 6x SDS sample buffer containing DTT and boiled for 10 min. SDS-PAGE was done according to Laemmli (Laemmli, 1970).

3.4. **Protein staining**

Coomassie Brilliant Blue protein stains were used to visualize the trypsin protein bands on the SDS-PAGE gels. Commassie Brilliant Blue R-250 (Sigma) dye was used. The gel was dyed with 0,1% (w/v) Commassie Brilliant Blue R-250 50% (v/v) methanol and 10% (v/v) acetic acid. After electrophoresis, the gel was immersed in at least 5 volumes of staining solution and placed on a slowly rotating platform for a minimum of 4 hours at room temperature. The gel was then distained in a 15% (v/v) methanol, 10% (v/v) acetic acid solution on a slowly rotating platform for 4-8 hours. Protein Molecular Weight Marker (Fermentas) in the size range of 11-130 was used.

3.5. **Protein concentration measurements**

3.5.1. **A280.**

Absorbance at 280 nm was used to measure protein concentration of purified trypsin I. Absorbance at 280 nm was measured in an Ultrospec 4000 UV/visible spectrophotometer and using the SWIFT II applications software (Pharmacia Biotech). To find out trypsin I concentration we used evaluation from Pace et al. (Pace, 1995);

$$A = \varepsilon * b * c$$

ε (280) for Trypsin I is 40.630 $M^{-1}cm^{-1}$ and b is 1 cm. A is absorbance at 280 nm and c is concentration in M/L. c is then multiple with 24200 mg/mmól witch is Trypsin I molecular mass (Guðmundsdóttir, 1993) to find out Trypsin I concentration in mg/mL.

3.5.2. **Protein measurement using Coomassie Brilliant Blue.**

Protein standard solutions were made from 2,0 mg/mL BSA solution, containing 40-400 µg/mL in 7 test tubes. The volume in the test tube was adjusted to 250 µL with dH_2O. 2,75 mL of protein reagent (Coomassie Brilliant Blue G-250) (Z.Zaman, 1979) was added to the test tube and the contents mixed well. 250 µL of protein sample added to a test tube and fill up with 2,75 mL of protein reagent. The absorbance at 620 nm was measured after 5-10 min in 3 mL cuvettes against a reagent blank prepared from 250 µL dH_2O and 2,75 mL of protein reagent. The weight of protein was plotted against the corresponding absorbance resulting in a standard curve used to determine the protein in unknown sample.

3.6. **Protein degradation**

The ability of bovine trypsin and cod trypsin in cleaving lysozyme, myoglobin, lactoferrin, bovine serum albumin (BSA), ICAM-1, α-casein dephosphorylated and β-casein was tested. The degradation reaction was performed in a waterbath at 25°C for all the proteins and as well at 4° and 37°C for lysozyme and lactoferrin. The length of the reaction at 4°C was 72 hours, at 25°C 0, 3, 6 and 24/25 hours but for myoglobin it was 48 hours and at 37°C the length was 3 and 6 hours for lysozyme and 2 hours for lactoferrin. The amount of enzyme used was 2,0 µg for cod trypsin and 2,4 µg for bovine trypsin and amount of proteins used was 50 µg, in 100 mM Tris and 5 mM $CaCl_2$ pH 8,0 in a volume of 150 µL. After the reaction 450 µL 0.1 % Trifluoroacetic Acid (TFA) was used to stop the reaction.

3.7. **Reversed phase HPLC chromatography**

The resulting peptides in the digest were separated using HPLC chromatography (Äkta Purifier, GE Healthcare) using the reversed phase C2/C18 ST 4.6/100 column from Pharmacia Biotech. For all the samples tested, the column was prepared with buffer A. The samples were eluted from the column with 0-70% gradient (buffer A: 0.1 % TFA in dH_2O; buffer B: 0.1 % TFA, 80 % acetonitrile in dH_2O) with a flow rate of 1 mL/min. The absorbance at 215 nm was monitored during the entire run by software equipped with the HPLC system.

3.8. **OPA**

The OPA (ortho-phtaldialdehyde) method was tested to measure the extent of degradation of the proteins tested. The OPA reagent was prepared essentially as described by Goodno et al. (Goodno, 1981). The OPA solution was made by combining the following reagents and diluting to a final volume of 50 mL water: 25 mL of 100 mM sodium tetraborate, 2,5 mL of 20% (w/w) SDS, 40 mg of OPA (dissolved in 1 mL of methanol) and 100 µL of β-mercaptoethanol. This reagent was prepared daily. To assay proteolysis of the resulting peptides from the digest with trypsin, the digest was added directly to 1 mL of OPA reagent in a 1,5 mL quartz cuvette, the solution was mixed and incubated for 15 min at room temperature and the absorbance at 340 nm measured in an Ultrospec 4000 UV/visible spectrophotometer and using SWIFT II applications software (Pharmacia Biotech).

3.9. **Mass spectrometry**

The peptides from the tryptic digests were spotted on a MALDI-TOF analysis plate using the dried droplet method. Peptide calibration standards, a-cyano-4-hydroxy cinnaminic acid (HCCA), 2,5-dihydroxy benzoic acid (DHB) and sinapinic acid were from Bruker Daltonik (Bremen, Germany). MALDI-TOF analysis on tryptic digests was performed on a Bruker autoflex III instrument. The instrument was operated in the reflection delayed extraction mode. In silico digest of protein sequences was performed using ProteinProspector (http://prospector.ucsf.edu/).

3.10. **Activity measurement**

In the comparison of the ability of bovine trypsin and cod trypsin to cleave simple globular proteins in their native conformation the activity in the samples was measured using Benzoyl-Arg-pNA (BAPNA) (Sigma) was used as substrate. The stock concentration of BAPNA was 25 mM dissolved in dimethylsulfoxide (Me_2SO) (DMSO). For the kinetic analysis of cod trypsin I following substrates were used: N-CBZ-Gly-Pro-Arg-pNA, Bz-Phe-Val-Arg-*p*NA, Tos-Gly-Pro-Lys-*p*NA, H-D-Val-Leu-Arg-*p*NA, Z-Val-Gly-Arg-*p*NA, D-Phe-Pro-Arg-*p*NA and MeoSuc-Ala-Ala-Arg-*p*NA. The substrates were dissolved at 12,5 mM or 50 mM concentration in DMSO and used at a final concentration of 0,5-0,005 mM. The assay buffer contained 100 mM Tris, 5 mM $CaCl_2$ at pH 8,0. Assay buffer (940 µL) and substrate stock solution (10 µL) were measured into a 1,5 mL cuvette. Then stirred thoroughly and placed in a waterbath at 25°C for 3 - 30 minutes. At the end of the incubation time the cuvette is dried on the outside with a paper towel and placed in the cuvette holder. 50 µL of the enzyme solution was add to the substrate solution and mixed thoroughly using cuvette stirrer before measurement. The first two seconds of each assay were ignored when fitting a straight line to the absorbance slope. Each sample was measured 4 times and the mean was taken of the latest three. The k Factor was 2,27. The reactions were monitored at room temperature at 410 nm in an Ultrospec 4000 UV/visible spectrophotometer and SWIFT II applications software (Pharmacia Biotec).

3.11. **Determination of kinetic constants.**

The Lineweaver-Burk plot finds out the Michaelis-Menten constant K_m and the limiting velocity (V_{max}) from a set of measurements of velocity at different substrate concentrations. If

$1/V_o$ is plotted against 1/[S], a straight line is obtained where the slope is equal to K_m/V_{max} and the y-intercept is equal to $-1/V_{max}$.

The Hanes-Woolf plot finds the Michaelis-Menten constant K_m and the limiting velocity V_{max} if [S]/Vo are plotted against [S], a straight line is obtained where the slope is equal to $1/V_{max}$ and the y-intercept is equal to K_m/V_{max}.

Direct linear plot, pairs of (v,s) values are obtained in the usual way. Then a v value is plotted onto a V_{max} vertical axis and the corresponding negative s values are plotted onto a K_m horizontal axis. The two points are joined and the line is extrapolated into V_{max}, K_m parameter space. The process is repeated for each pair of (v,s) values. Hence n lines are obtained for n points. With data that fit perfectly, all the lines intersect at the coordinates of the best fit K_m and V_{max}, if not a number [n(n-1)/2] of different intersections are obtained. (Eisenthal R, 1992).

Michaelis-Menten constant K_m and the limiting velocity V_{max} were also determined by fitting the data to the Michaelis-Menten equation using Excel see (http://www-biol.praisley.ac.uk/Kinetics/Chapter_2/chapter2_2_1.html).

4. Results and discussion

4.1. Characterization of samples used for comparison of the ability of bovine trypsin and cod trypsin in cleaving simple globular proteins in their native conformation.

The samples used for this part of the project were bovine trypsin and cod trypsin. The cod trypsin is benzamidine purified and therefore different isozymes of trypsins are in this fraction. Our studies indicate that it contains at least 3 different isoforms of trypsin where trypsin I is the most abundant isoform. The purity of bovine trypsin and cod trypsin was analyzed using SDS-PAGE analysis (Figure 4). In both samples proteins were detected migrating just above the 24 kDa protein marker, which matches to the expected size of full length bovine trypsin and cod trypsin isoforms. Multiple faint protein bands were detected at and below the 17 kDa marker in both samples. These are most likely autolytic degradation products of trypsin. No bands were detected above the 33 kDa protein marker. Based on these findings, the bovine trypsin and cod trypsin samples used for the studies are thought to be highly pure. Further studies on the cod trypsin sample, using Mono Q chromatography and Western blot analysis, support this conclusion.

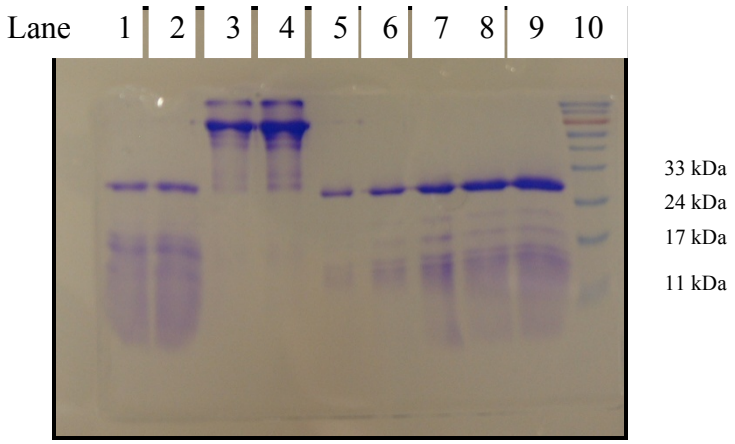

Figure 4. SDS-Page analysis.

Lane 1 and 2 contain 8 µg and 10.7 µg of cod trypsin respectively. Lane 3 and 4 contain 10 µg and 20 µg of bovine serum albumin (BSA) respectively. Lane 5 to 9 contains 1.5 µg, 3 µg, 6 µg, 8.9 µg and 12 µg of bovine trypsin respectively. Protein markers are in lane 10, size from top: 130 kDa, 100 kDa, 72 kDa (red), 55 kDa, 40 kDa, 33 kDa, 24 kDa, 17 kDa and 11 kDa.

The tryptic activity in the bovine trypsin and cod trypsin samples was measured using BAPNA (Benzoyl-arginine-p-nitroanilide) as substrate (Table 1). The protein concentration in

these samples was determined using the method of Zaman and Verwilghen (Z.Zaman, 1979) (Table 1).

Table 1. Activity using BAPNA and protein concentration in bovine trypsin and cod trypsin samples.

Sample	*Activity (U/mL)*	*Protein concentration (mg/mL)*
Cod trypsin	2.38	0.267
Bovine trypsin	2.28	0.296

These measurements were used in the comparison of the ability of bovine trypsin and cod trypsin in cleaving simple globular proteins in their native conformation. The degradation reactions using the globular proteins were conducted using equal amounts of bovine trypsin and cod trypsin based on their activity against BAPNA. BAPNA is a simple tryptic substrate (contains only one amino acid) and is therefore thought to show the smallest difference in catalytic activity between bovine trypsin and cod trypsin. This means, based on the measurements in table 1, that the protein concentration of bovine trypsin is higher compared to cod trypsin in the degradation reactions.

4.2. Comparison of the ability of bovine trypsin and cod trypsin in cleaving simple globular proteins in their native conformation at 25°C.

The ability of bovine trypsin and cod trypsin in cleaving seven different proteins was tested. The proteins were lysozyme, myoglobin, lactoferrin, bovine serum albumin (BSA), ICAM-1, α-casein dephosphorylated and β-casein. The degradation of α-casein dephosphorylated and β-casein with bovine trypsin and with cod trypsin was too fast for comparison, so the results using these proteins are not reported on. This is not surprising since casein is known to have an open structure that is more easily digested. Furthermore, TNF-α and TNF-β were purchased to test as substrates but running the undigested proteins (not treated with trypsin) on reversed phase column resulted in multiple peaks on the chromatogram. This indicates that the protein preparations contained multiple different proteins or the proteins were degraded or both. Therefore these proteins were not tested as substrates for cod trypsin and bovine trypsin. Of the five protein substrates reported on, four of the proteins are extracellular or membrane proteins (lysozyme, lactoferrin, BSA and ICAM-1). These proteins are considered excellent model substrates, when comes to testing the ability of cod trypsin cleaving native proteins. This is because their structure is adapted to the extracellular environment where cod trypsin is thought to act when used for example for therapeutic purposes.

Here the degradation reaction was performed in a waterbath at 25°C but the length of the reaction and amount of enzyme used was dependent on the protein used as substrate. After the reaction the resulting peptides in the digest were separated using reversed phase chromatography column. For all the samples tested, the column was prepared as described under Materials and Methods.

By running undigested samples of the protein substrates their elution point was determined. To compare the extent of degradation by bovine trypsin or cod trypsin, the percentage of the area of the peak for the undigested protein of the total area of all the peaks eluting from the column was calculated.

Both cod trypsin and bovine trypsin eluted around the 37 mL elution point. Since the proteins tested as substrates eluted at another point the peaks for cod trypsin and bovine trypsin were typically included in the total area of peaks. Since the protein amount of bovine trypsin used was higher than of cod trypsin the calculated amount of degradation was slightly inaccurate in favor of bovine trypsin. Despite the fact, the overall conclusion is that the activity of cod trypsin against all the proteins reported on is greater compared to bovine trypsin.

The OPA (ortho-phtaldialdehyde) method was tested to measure the extent of degradation of the proteins tested (F.C. Church, 1985). This method is based on the specific reaction between primary amino groups and OPA. The amount of the resulting molecule is then quantified spectrophotometrically at 340 nm. In this way the formation of new amino ends during protein degradation can be followed. After testing, the sensitivity and consistency of the assay was deemed not good enough for our purposes and therefore it was not used.

4.2.1. **Degradation of lysozyme.**

The extent of degradation of lysozyme by bovine trypsin or cod trypsin at different time points is shown in Table 2. Representative chromatograms of the degradation by cod trypsin (in red) and by bovine trypsin (in blue) are shown in Figure 6 and Figure 7

Table 2. Degradation of lysozyme.

Degradation of lysozyme by bovine trypsin and cod trypsin analyzed using reversed phase chromatography. The length of the reaction (in hours), the percentage of undigested lysozyme of the total area of all the peaks and the standard deviation based on n number of experiments is shown.

Enzyme	*Reaction time (hours)*	*Undigested protein (%) (average)*	*STDEV*	*(n)*
Cod trypsin	3	77.84	-	1
Bovine trypsin	3	86.68	-	1
Cod trypsin	6	59.07	4.5	3
Bovine trypsin	6	82.09	9.85	3
Cod trypsin	25	18.46	5.51	3
Bovine trypsin	25	81	3.02	3

Already, in 6 hours there is a significant and statistical difference (according to a t-test: paired two sample for means) in the percentage of undigested protein left after digestion with cod trypsin or bovine trypsin. Therefore, this shows that cod trypsin is more efficient in degrading lysozyme compared to bovine trypsin. Longer digestion (25 hours) further substantiates this observation. Digestion of lysozyme with bovine trypsin for 25 hours did not increase compared to a 6 hour digestion with cod trypsin. On the other hand, there was a substantial increase in the digestion of lysozyme with cod trypsin when comparing digestion for 6 hours and 25 hours. In conclusion, the ability of cod trypsin in degrading lysozyme is much greater compared to bovine trypsin.

Close inspection of the chromatograms of the digests of lysozyme by cod trypsin or bovine trypsin (Figure 6 and Figure 7) reveals that the fragmentation pattern is basically the same. This indicates that cod trypsin is not cleaving lysozyme at different sites within lysozyme compared to bovine trypsin. Therefore, the increased ability of cod trypsin in digesting lysozyme seems not to be caused by novel cleavage sites within lysozyme compared to bovine trypsin. As a result, cod trypsin seems to cleave lysozyme at the same sites but at a faster rate.

Structure of lysozyme.

Hen egg white lysozyme is composed of 129 amino acids. Structural features are five helices, in addition to five beta-strands, which are organized in two antiparallel sheets (Figure 5).

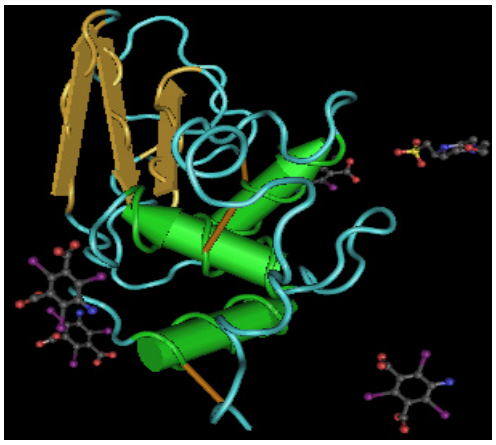

Figure 5. Structure of hen egg white lysozyme (PDB ID: 3E3D).

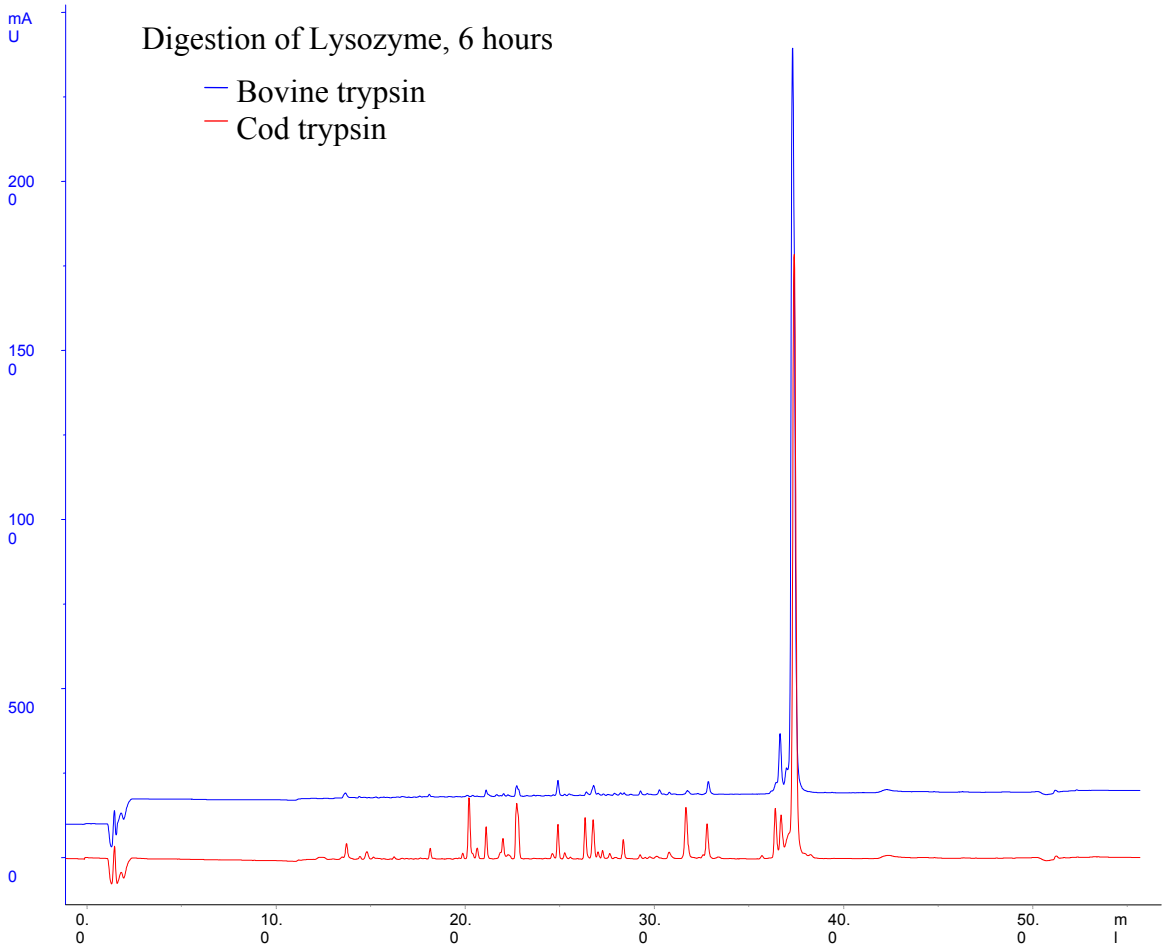

Figure 6. Degradation of lysozyme, 6 hours at 25°C.

50 µg of lysozyme with 2.0 µg of cod trypsin (red line) or with 2.4 µg bovine trypsin (blue line) after 6 hours at 25°C analyzed using reversed phase chromatography. Y axis indicates absorbance at 215 nm. X axis indicates the volume of eluent in mL.

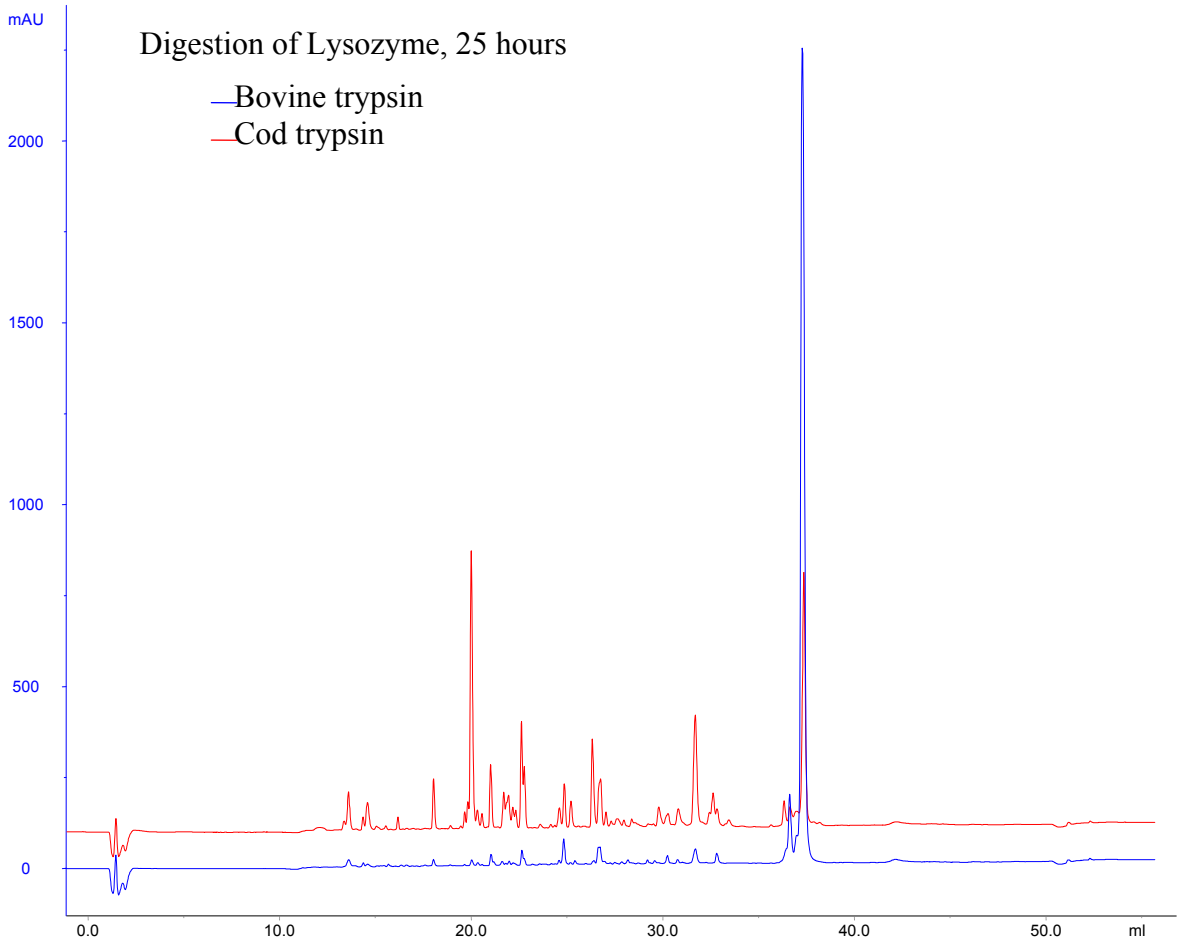

Figure 7. Degradation of lysozyme, 25 hours at 25°C.

50 µg of lysozyme with 2.0 µg of cod trypsin (red line) or with 2.4 µg bovine trypsin (blue line) after 25 hours at 25°C analyzed using reversed phase chromatography. Y axis indicates absorbance at 215 nm. X axis indicates the volume of eluent in mL.

4.2.2. **Degradation of lactoferrin.**

The extent of degradation of lactoferrin by bovine trypsin or cod trypsin at different time points is shown in Table 3. Representative chromatograms of the degradation by cod trypsin (in red) and by bovine trypsin (in blue) are shown in Figure 9 and Figure 10.

Table 3. Degradation of lactoferrin 25°C.

Degradation of lactoferrin by bovine trypsin and cod trypsin analyzed using reversed phase chromatography. The length of the reaction (in hours), the percentage of undigested lactoferrin of the total area of all the peaks and the standard deviation based on n number of experiments is shown.

Enzyme	*Reaction time (hours)*	*Undigested protein (%) (average)*	*STDEV*	*(n)*
Cod trypsin	3	57,35		1
Bovine trypsin	3	76,35		1
Cod trypsin	6	22,52	3,52	3
Bovine trypsin	6	74,61	2,89	3
Cod trypsin	24	0,00	0	3
Bovine trypsin	24	64,52	1,11	3

In only 3 hours there is a difference in the percentage of undigested protein of total protein after digestion with cod trypsin or bovine trypsin. The length of digestion was extended to 6 hours and a substantial increase in the amount of digested lactoferrin after proteolysis with cod trypsin was detected. Conversely, there was almost no change in the percentage of undigested lactoferrin after treatment with bovine trypsin. Digestion for 24 hours further underscores how dramatic the difference is between the ability of cod trypsin and bovine trypsin in degrading lactoferrin. The peak for undigested lactoferrin has disappeared in the cod trypsin lysate whereas 64% of undigested lactoferrin remains after proteolysis with bovine trypsin (see Figure 9 at 40 mL elution point). These results clearly show that cod trypsin is far superior in degrading lactoferrin than bovine trypsin. This is especially true considering that cod trypsin has probably digested all of the full length lactoferrin at an earlier time point than 24 hours.

The chromatograms of the digests of lactoferrin by cod trypsin or bovine trypsin (Figure 9 and Figure 10) reveals that the fragmentation pattern is essentially the same. Therefore, as was true for the digestion of lysozyme, cod trypsin seems to cleave lactoferrin at the same sites as bovine trypsin but at a faster rate.

Structure of lactoferrin.

Lactoferrin is a glycoprotein with a molecular weight of about 80 kDa, which shows high affinity for iron (Adlerova, L. 2008). Lactoferrin was classified as a member of the transferrin family, due to its 60% sequence identity with serum transferrin. Lactoferrin is comprised of a single polypeptide chain containing 703 amino acids folded into two globular lobes (Figure 8). These lobes, also called C – (carboxy) and N – (amino) terminal regions, are connected with a α-helix. Each lobe consists of two domains known as C1, C2, N1, and N2. The domains create one iron binding site on each lobe.

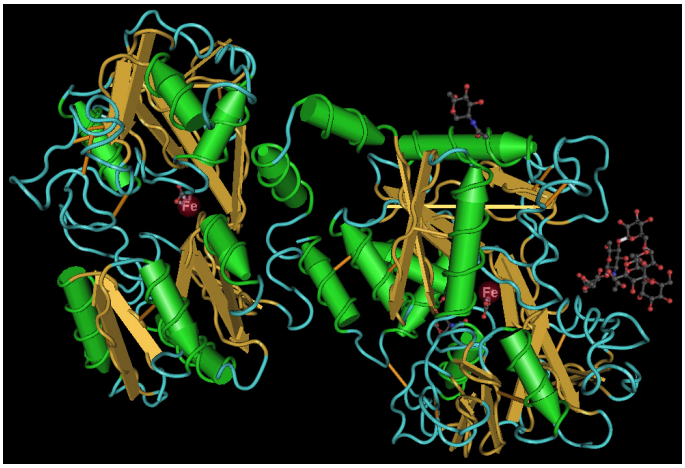

Figure 8. Structure of Bovine Lactoferrin (PDB ID: 1BLF).

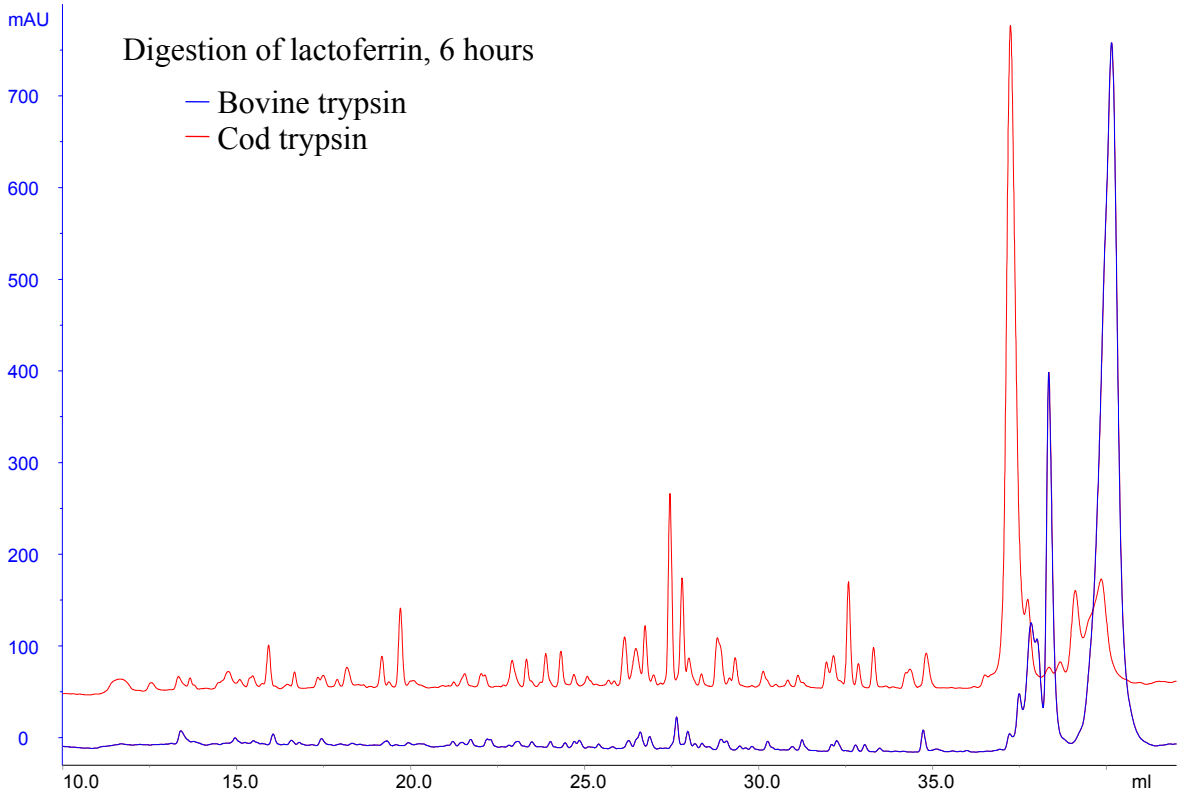

Figure 9. Degradation of lactoferrin, 6 hours at 25°C.

Digestion of 50 µg of lactoferrin with 2.0 µg of cod trypsin (red line) or with 2.4 µg bovine trypsin (blue line) after 6 hours at 25°C analyzed using reversed phase chromatography. Y axis indicates absorbance at 215 nm. X axis indicates the volume of eluent in mL.

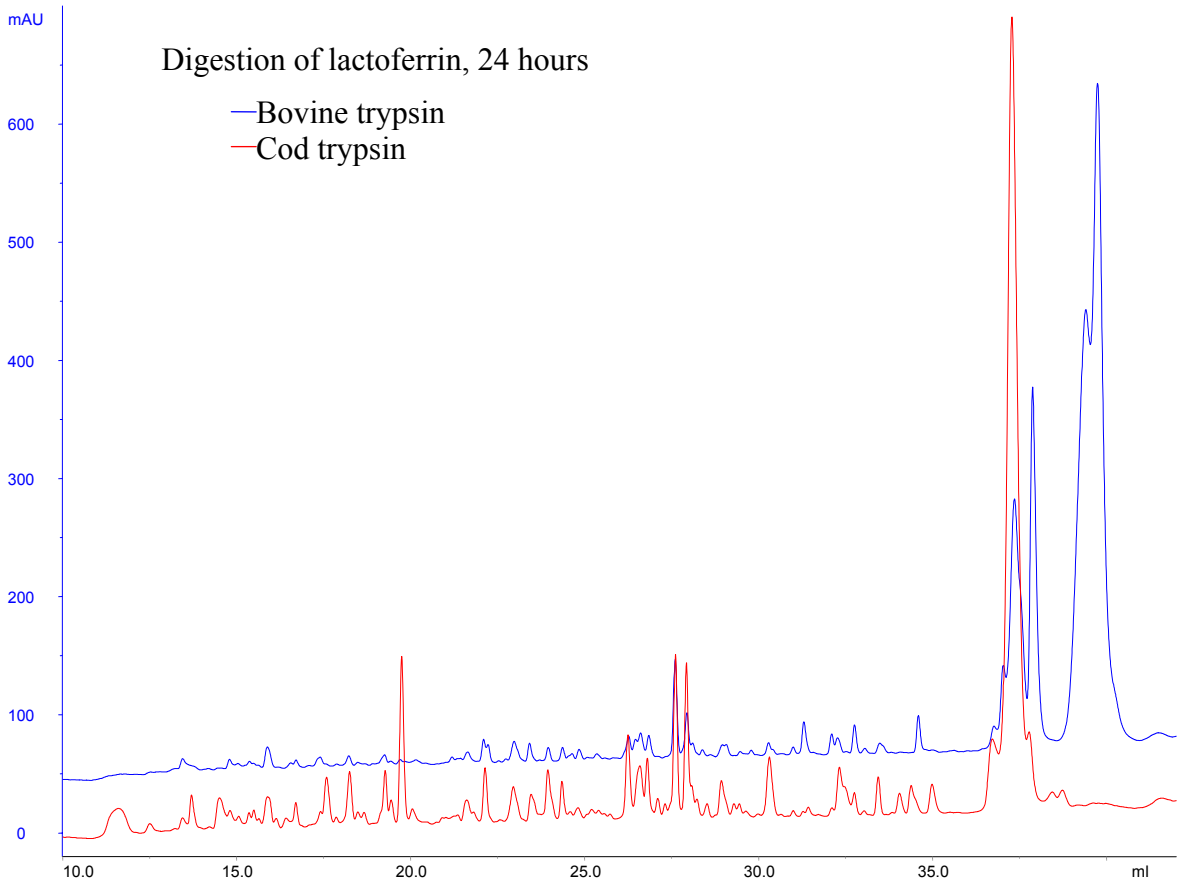

Figure 10. Degradation of lactoferrin, 24 hours at 25°C.

Digestion of 50 µg of lactoferrin with 2.0 µg of cod trypsin (red line) or with 2.4 µg bovine trypsin (blue line) after 24 hours at 25°C analyzed using reversed phase chromatography. Y axis indicates absorbance at 215 nm. X axis indicates the volume of eluent in mL.

4.2.3. **Degradation of myoglobin.**

The extent of degradation of myoglobin by bovine trypsin or cod trypsin at different time points is shown in Table 4. Representative chromatograms of the degradation by cod trypsin (in red) and by bovine trypsin (in blue) are shown in Figure 12 and Figure 13.

Table 4. Degradation of myoglobin 25°C.

Degradation of myoglobin by bovine trypsin and cod trypsin analyzed using reversed phase chromatography. The length of the reaction (in hours), the percentage of undigested myoglobin of the total area of all the peaks and the standard deviation based on n number of experiments is shown.

Enzyme	*Reaction time (hours)*	*Undigested protein (%) (average)*	*STDEV*	*(n)*
Cod trypsin	24	45.64	-	1
Bovine trypsin	24	64.15	-	1
Cod trypsin	48	36.61	3.56	3
Bovine trypsin	48	55.60	5.59	3

Myoglobin is well known to be difficult to cleave. Therefore the reaction time was longer for myoglobin (see Table 4) compared to the digestion of other proteins.

Digestion of myoglobin for 24 hours with cod trypsin is more extensive compared digestion with bovine trypsin (see Table 4 and Figure 12). Incubation for 48 hours does not increase this difference but these measurements were repeated 2 times and a statistical difference was observed between the degradation of myoglobin with cod trypsin and bovine trypsin. Thus, it can be concluded that cod trypsin is more efficient in degrading myoglobin than bovine trypsin but the difference is not as striking as with lysozyme and lactoferrin as substrates.

As with lysozyme and lactoferrin, the chromatograms of the digests of myoglobin by cod trypsin or bovine trypsin (Figure 12 and Figure 13) reveal that the fragmentation pattern is very similar. This indicates that cod trypsin seems to cleave myoglobin at the same sites as bovine trypsin but at a faster rate.

Structure of myoglobin.

Myoglobin is a cytoplasmic hemoprotein which consists of a single polypeptide chain of 154 amino acids (G.A. Ordway, 2004). Myoglobin backbone is a polypeptide chain that consists of eight α–helices (Figure 11). The polypeptide chain is folded and cradles a heme prosthetic group.

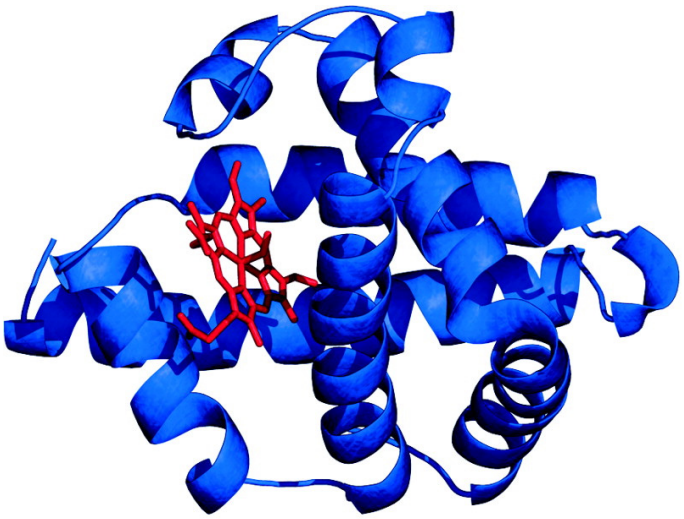

Figure 11. Structure of myoglobin (taken from (G.A. Ordway, 2004)).

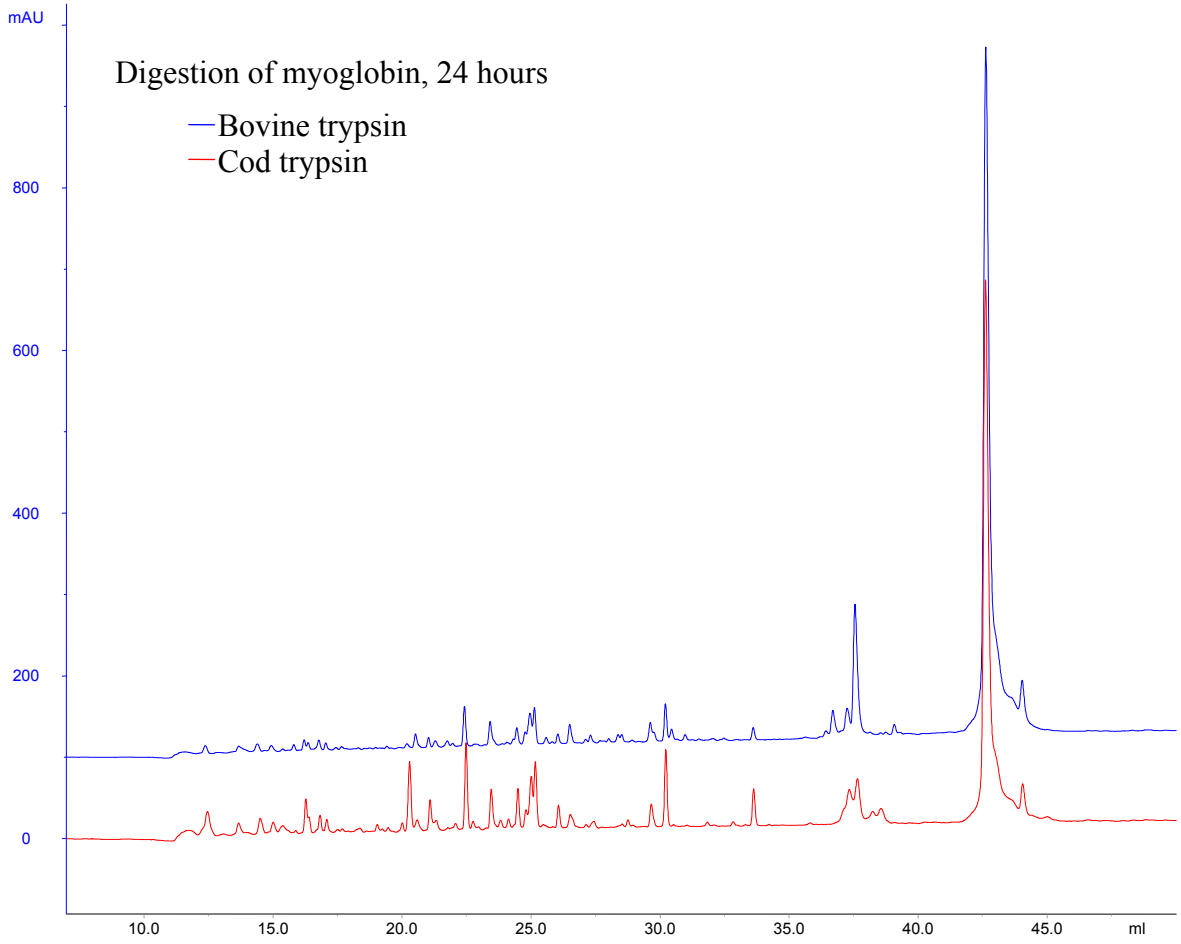

Figure 12. Degradation of myoglobin, 24 hours at 25°C.

Digestion of 50 µg of myoglobin with 2.0 µg of cod trypsin (red line) or with 2.4 µg bovine trypsin (blue line) after 24 hours at 25°C analyzed using reversed phase chromatography. Y axis indicates absorbance at 215 nm. X axis indicates the volume of eluent in mL.

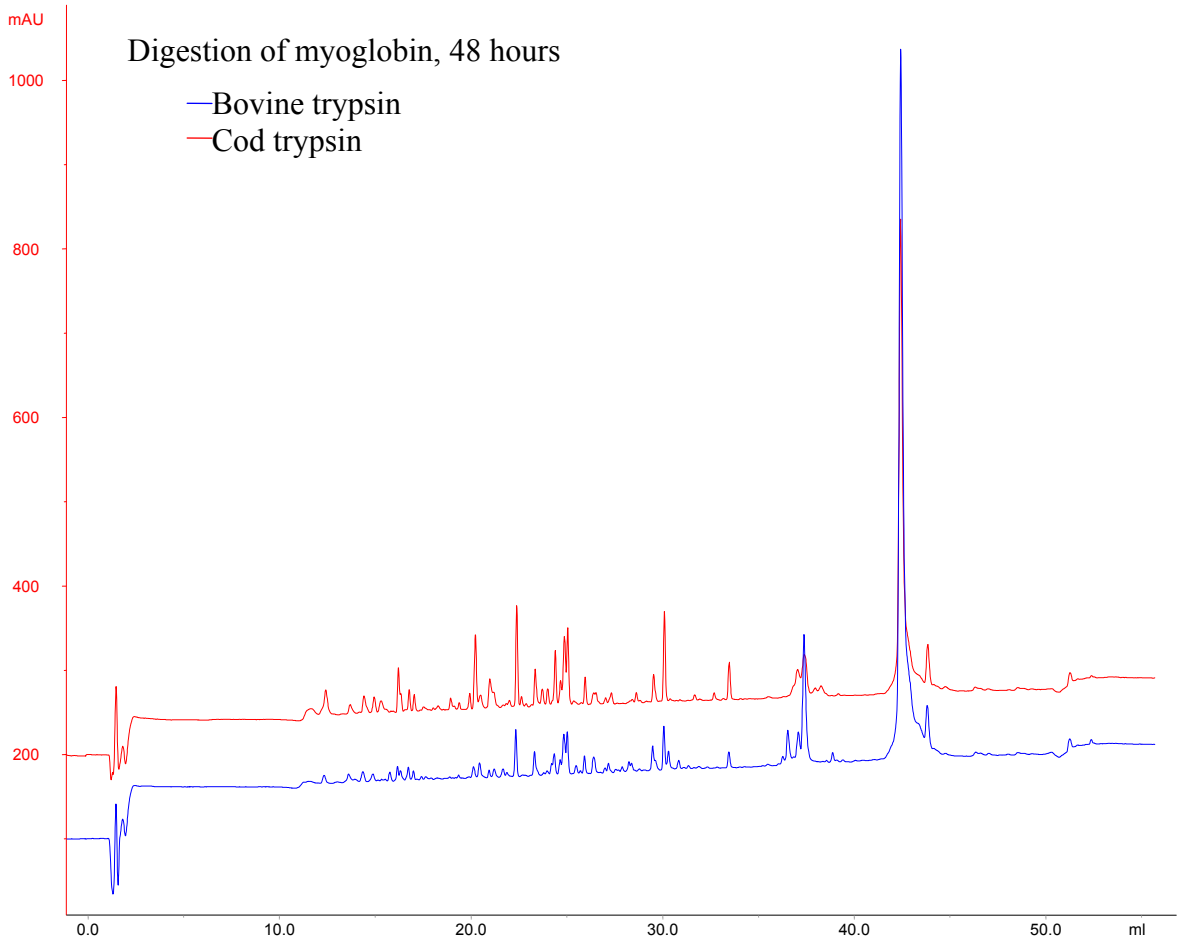

Figure 13. Degradation of myoglobin, 48 hours at 25°C.

Digestion of 50 µg of myoglobin with 2.0 µg of cod trypsin (red line) or with 2.4 µg bovine trypsin (blue line) after 48 hours at 25°C analyzed using reversed phase chromatography. Y axis indicates absorbance at 215 nm. X axis indicates the volume of eluent in mL.

4.2.4. **Degradation of bovine serum albumin (BSA).**

The extent of degradation of BSA by bovine trypsin or cod trypsin at different time points is shown in Table 5. Representative chromatograms of the degradation by cod trypsin (in red) and by bovine trypsin (in blue) are shown in Figure 15 and Figure 16.

.

Table 5. Degradation of BSA

Degradation of BSA by bovine trypsin and cod trypsin analyzed using reversed phase chromatography. The length of the reaction (in hours), the percentage of undigested BSA of the total area of all the peaks and the standard deviation based on n number of experiments is shown.

Enzyme	*Reaction time (hours)*	*Undigested protein (%) (average)*	*STDEV*	*(n)*
Cod trypsin	3	75,73		1
Bovine trypsin	3	70,05		1
Cod trypsin	6	64,19	2,99	3
Bovine trypsin	6	63,73	2,96	3
Cod trypsin	24	37,00	3,95	3
Bovine trypsin	24	48,51	6,95	3

Digestion of BSA for 3 hours was slightly more efficient with bovine trypsin compared to cod trypsin. After 6 hour and 24 hour digestion there is not a statistical significant difference between the ability of cod trypsin and bovine trypsin in degrading BSA. This is the first substrate tested where proteolysis by cod trypsin was not superior to bovine trypsin. This indicates that the difference in the rate of degradation by cod trypsin and bovine trypsin depends on the structure of the substrate. Therefore based on digestion of different substrates, some cleavage sites seem to be cleaved at a faster rate by cod trypsin while others are cleaved at a comparable rate by cod trypsin and bovine trypsin.

The chromatograms of the digests of BSA show that the degradation patterns are very similar but few differences are observed.

Structure of BSA.

BSA is 607 amino acids with a molecular weight of 69 kDa. BSA is stabilized by 17 disulphide bridges and is therefore relatively stable. The 3-dimension structure of bovine serum albumin has not been determined. However, the structure of the homologue human serum albumin, with 76% identity has been solved (Figure 14). The structure reveals that the protein is mostly composed of alpha helixes. It comprises three homologous domains that assemble to form a heart-shaped molecule.

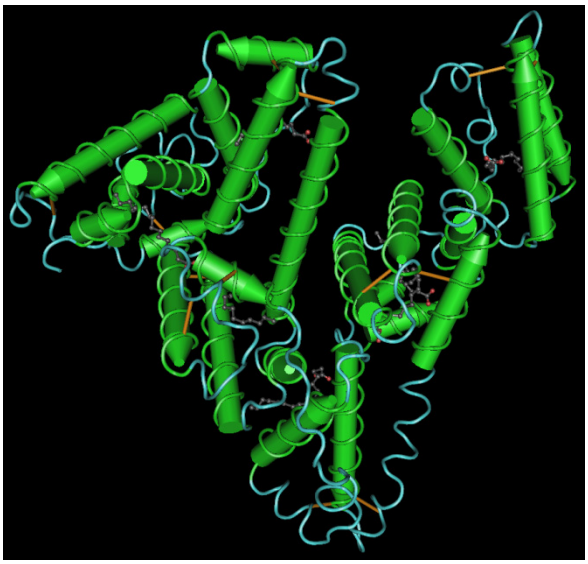

Figure 14. Structure of human serum albumin (HSA) (PDB ID: 1E7H).

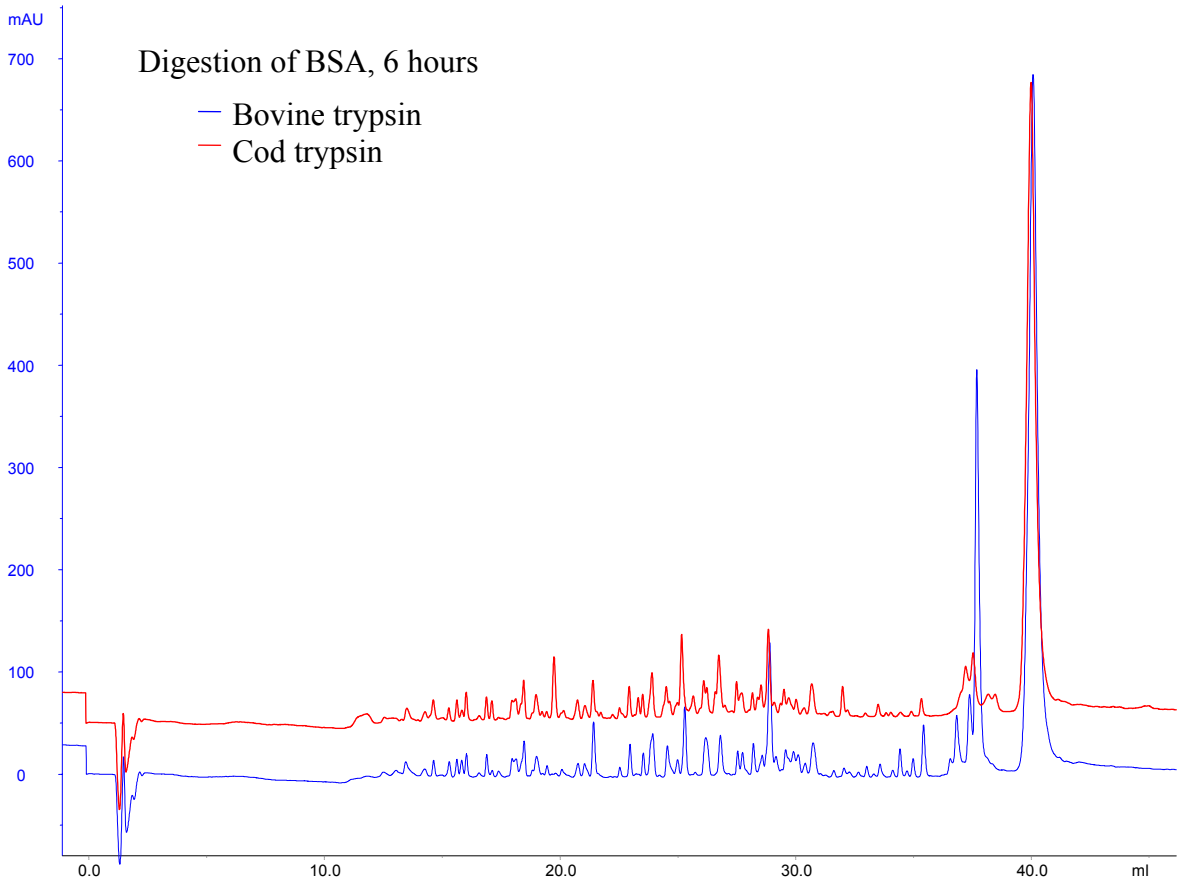

Figure 15. Degradation of BSA, 6 hours at 25°C.

Digestion of 50 µg of BSA with 2.0 µg of cod trypsin (red line) or with 2.4 µg bovine trypsin (blue line) after 6 hours at 25°C analyzed using reversed phase chromatography. Y axis indicates absorbance at 215 nm. X axis indicates the volume of eluent in mL.

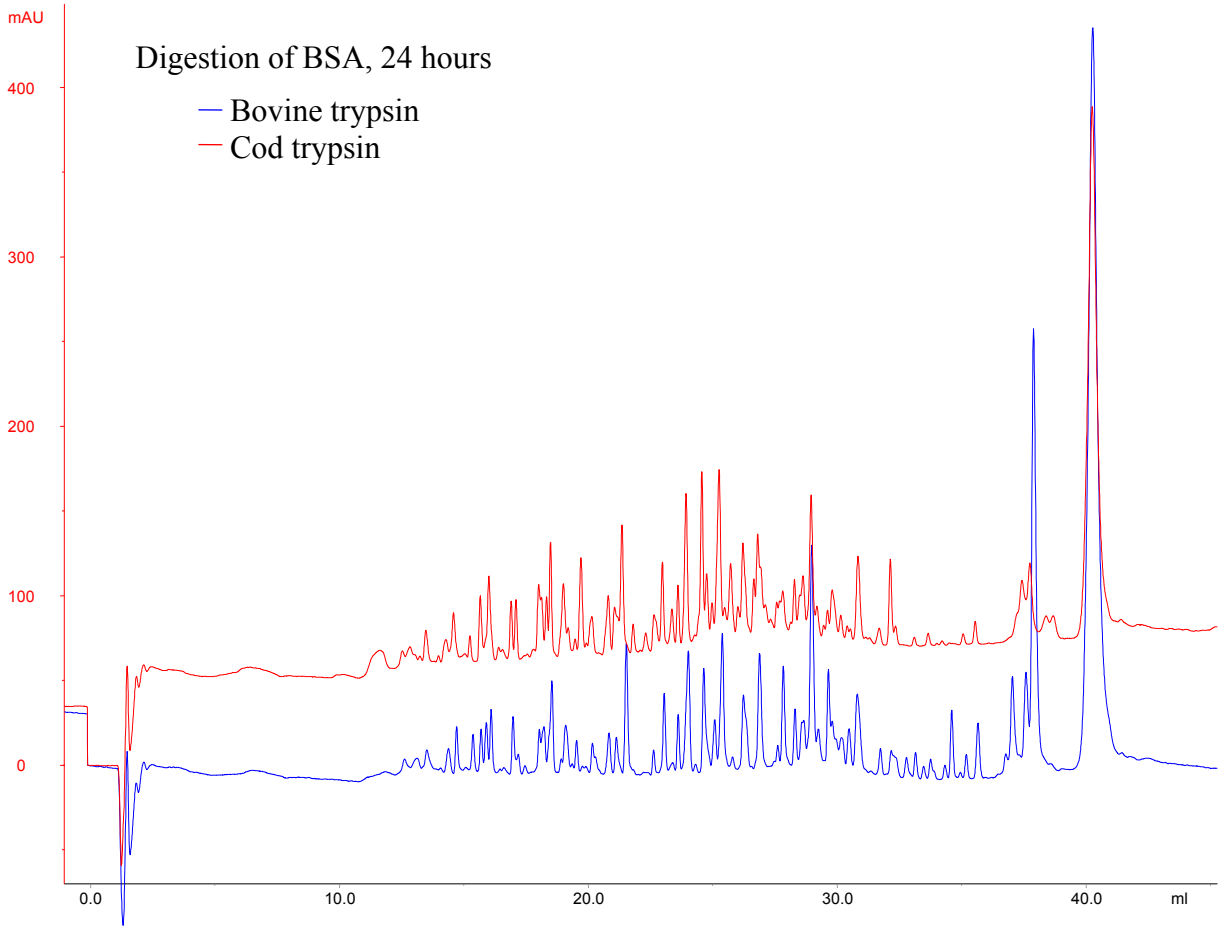

Figure 16. Degradation of BSA, 25 hours at 25°C

Digestion of 50 µg of BSA with 2.0 µg of cod trypsin (red line) or with 2.4 µg bovine trypsin (blue line) after 25 hours at 25°C analyzed using reversed phase chromatography. Y axis indicates absorbance at 215 nm. X axis indicates the volume of eluent in mL.

4.2.5. **Degradation of ICAM-1.**

Intercellular cell adhesion molecule-1 (ICAM-1; CD54) is a glycoprotein that is expressed on the cell surface of different cell lines such as endothelial cells, fibroblasts, epithelial cells and leukocytes. ICAM-1 belongs to the immunoglobulin superfamily (IgSF) of adhesion molecules (Hopkins et al., 2004). These molecules share a common structure and many of them are involved in cell-cell or cell-matrix adhesion. The main function of ICAM-1, and related cell adhesion molecules, is in the recruitment and trafficking of leukocytes (T cells and neutrophils) mediated by its interaction with leukocyte expressed integrins. This is important for both regular immune surveillance and during inflammatory responses. Pro inflammatory cytokines and bacterial products lead to upregulation of ICAM-1 which augments the immune response and leukocyte accumulation in inflamed tissues. The adhesive properties of ICAM-1 and related IgSF members have been exploited by pathogens to enhance or possibly mediate

their invasion. These pathogens include rhinoviruses, polioviruses, human immunodeficiency viruses (HIV) and plasmodium falciparum (Hopkins et al., 2004).

In light of the importance of ICAM-1 and other IgSF members for immune response, inflammation and pathogen invasion, it was of interest to test the ability of cod trypsin in degrading ICAM-1. Recombinant ICAM-1 protein was obtained that contained the extracellular domain of ICAM-1 but not the transmembrane and cytoplasmic domain.

The extent of degradation of ICAM-1 by bovine trypsin or cod trypsin after 6 hours is shown in Table 6. Chromatograms of the degradation by cod trypsin (in red) and by bovine trypsin (in blue) are shown in Figure 18.

Table 6. Degradation of ICAM-1, 25°C.

Degradation of by bovine trypsin and cod trypsin analyzed using reversed phase chromatography. The length of the reaction (in hours), the percentage of undigested ICAM-1 of the total area of all the peaks and the standard deviation based on n number of experiments is shown.

Enzyme	*Reaction time (hours)*	*Undigested protein (%) (average)*	*STDEV*	*(n)*
Cod trypsin	6	7.62	-	1
Bovine trypsin	6	34.67	-	1

Digestion of ICAM-1 for 6 hours reveals that cod trypsin degrades ICAM-1 at a faster rate compared to bovine trypsin. As before, the digestion pattern is close to identical when comparing the resulting chromatograms of the digests of ICAM-1 by cod trypsin or bovine trypsin.

These results are particularly interesting since they might partly explain the observed immunomodulatory effects and disinfecting properties of cod trypsin. The portion of ICAM-1 that is most accessible to cod trypsin is most likely the extracellular domain of ICAM-1 which is readily degraded by cod trypsin. Therefore, the application of cod trypsin to wounds or inflamed areas might speed up recovery by causing degradation of ICAM-1. The degradation of ICAM-1 would lessen the risk of infection and dampen the inflammatory response which is many cases slows the healing process.

Structure of ICAM-1.

The N terminus of ICAM-1 consists of five extracellular immunoglobulin superfamily domains (IgSF domains) and the C terminus holds the hydrophobic transmembrane domain and a short cytoplasmic domain (Chen et al., 2007) (Figure 17 A). The IgSF domain type

shows sequence similarity to domains present in immunoglobulins (antibodies) and is composed of two sheets of antiparallel beta strands connected by conserved cysteines (Figure 17 B and C). The IgSF superfamily is now recognized as one of the largest in vertebrate genomes (Barclay, 2003) and it has been estimated that there are more than 850 immunoglobulin superfamily (IgSF) genes in the human genome (Barrow and Trowsdale, 2008). The main function of the IgSF domain is in protein interactions (it can bind other molecules such as carbohydrates) involved in the recognition, binding or adhesion processes of cells. Studies indicate that most of the IgSF members are associated with roles in the immune system.

Since the IgSF proteins share a common structure, the results obtained with ICAM-1 might indicate that cod trypsin is effective in degrading IgSF proteins in general. Because numerous IgSF proteins function in the immune system the ability of cod trypsin in modulating the immune systems may be caused by cleavage of multiple different IgSF proteins.

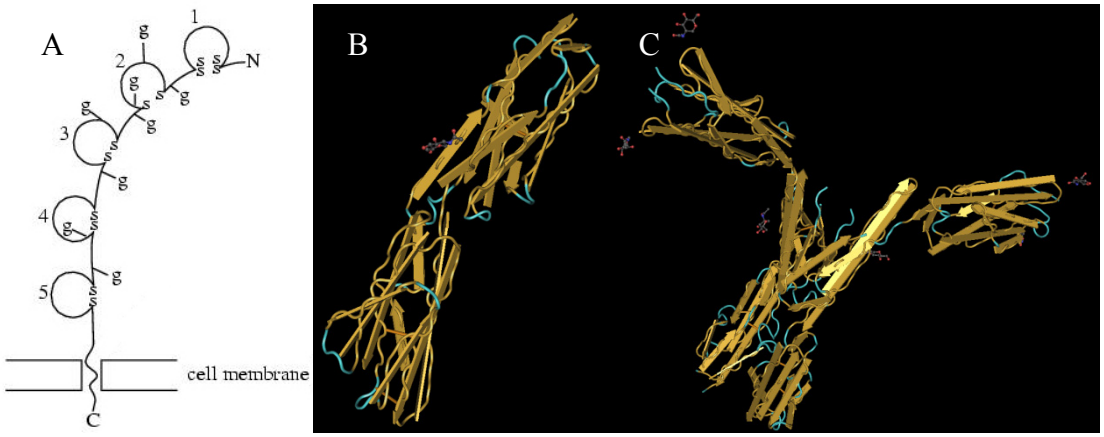

Figure 17. ICAM-1.

A. Schematic representation of ICAM-1, g=glycosilation, SS= disulfide bridge, domains are labeled from 1-5 (taken from (Witkowska and Borawska, 2004)). B. The structure of the two amino-terminal domains (1 and 2 in panel A) of human ICAM-1 (PDB ID: 1IAM). C. The Crystal Structure Of Icam-1 D3-D5 Fragment (3, 4 and 5 in panel A) (PDB ID: 1P53).

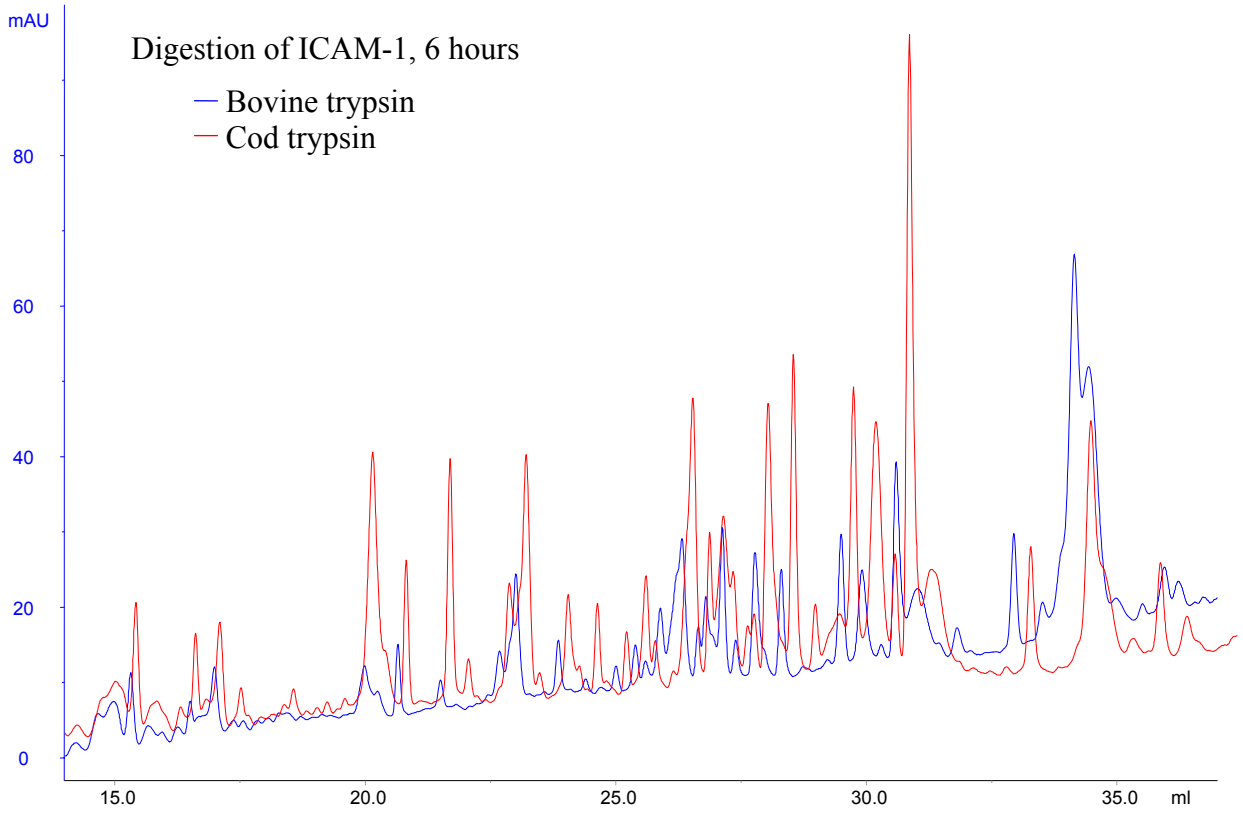

Figure 18. Degradation of ICAM-1, 25°C.

Digestion of 15 µg of ICAM-1 with 0.6 µg of cod trypsin (red line) or with 0.72 µg bovine trypsin (blue line) after 6 hours at 25°C analyzed using reversed phase chromatography. Y axis indicates absorbance at 215 nm. X axis indicates the volume of eluent in mL.

4.3. Comparison of the ability of bovine trypsin and cod trypsin in cleaving simple globular proteins at 4°C

The Atlantic cod usually lives at 200-400 m depth around Iceland, at temperature of about 2-4°C (Pálsdóttir, 2006). In general trypsin from the Atlantic cod and other fish adapted to cold environments differ somewhat from their mammalian analogues in that they have higher catalytic efficiencies, especially at low temperatures (Ásgeirsson B, 1989, Gerday C, 2000, Schrøder Leiros HK, 2000). Cod trypsin I shows enzymatic activity at 4°C, reaching a maximum at 55°C and complete inactivation at 65°C (Ásgeirsson B, 1989) while mammalian trypsin like bovine trypsin are more active in the range from 60-80°C (Klomklao, 2009).

The ability of bovine trypsin and cod trypsin in cleaving lysozyme and lactoferrin was tested at 4° C. We chose those proteins because they shoved largest difference in digestion between bovine trypsin and cod trypsin at 25°C.

Here the degradation reaction was performed in a waterbath at 4°C and the length of the reaction is 72 hours. After the reaction the resulting peptides in the digest were separated using reversed phase chromatography column. For both the samples tested, the column was prepared with 0.1 % Trifluoroacetic Acid (TFA) in dH_2O. The samples were eluted from the column with 0-70% gradient (buffer A: 0.1 % TFA in dH_2O; buffer B: 0.1 % TFA, 80 % acetonitrile in dH_2O) with a flow rate of 1 mL/min.

4.3.1. **Degradation of lysozyme**

The extend of degradation of lysozyme at 4°C by bovine trypsin of cod trypsin at different time points is shown in Table 7. Representative chromatograms of the degradation at 4°C by cod trypsin (in red) and by bovine trypsin (in blue) are shown in Figure 19.

Table 7. Degradation of lysozyme, 4°C.

Degradation of lysozyme at 4°C by bovine trypsin and cod trypsin analyzed using reversed phase chromatography. The length of the reaction (in hours), the percentage of undigested lysozyme of the total area of all the peaks and the standard deviation based on n number of experiments is shown.

Enzyme	*Reaction time (hours)*	*Undigested protein (%) (average)*	*STDEV*	*(n)*
Cod trypsin	72	58,98	2,17	3
Bovine trypsin	72	90,37	1,12	3

After 72 hours 4°C waterbath there is a significant and statistical difference in the percentage of undigested protein left after digestion with cod trypsin or bovine trypsin. There is almost no digestion after 72 hours with bovine trypsin and there are nearly 90 % of undigested protein left but only 59 % with cod trypsin. This shows that cod trypsin is more efficient at 4°C in degrading lysozyme compared to bovine trypsin. That indicates the ability of cod trypsin in degrading lysozyme is much greater at lower temperature. Both cod trypsin and bovine trypsin shows grater digestion after 6 hours at 25°C than after 72 hours at 4°C. The catalytic efficiency shows about 2-fold decreases for every 10°C in decreased temperature.

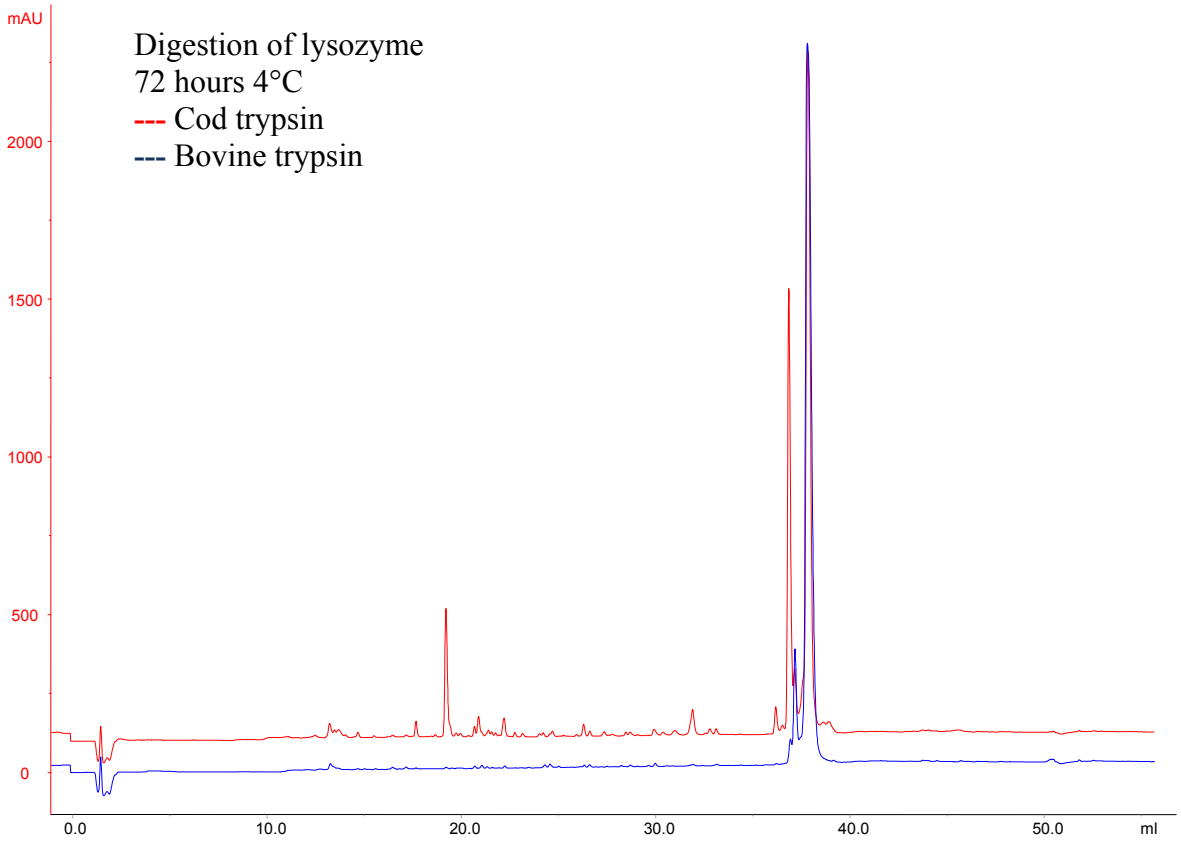

Figure 19. Degradation of lysozyme, 72 hours at 4°C

Digestion of 50 µg of lysozyme with 2.0 µg of cod trypsin (red line) or with 2.4 µg bovine trypsin (blue line) after 72 hours at 4°C analyzed using reversed phase chromatography. Y axis indicates absorbance at 215 nm. X axis indicates the volume of eluent in mL.

4.3.2. **Degradation of lactoferrin**

The extend of degradation of lactoferrin at 4°C by bovine trypsin of cod trypsin at different time points is shown in Table 8. Representative chromatograms of the degradation at 4°C by cod trypsin (in red) and by bovine trypsin (in blue) are shown in Figure 20.

Table 8. Degradation of lactoferrin at 4°C

Degradation of lactoferrin by bovine trypsin and cod trypsin analyzed using reversed phase chromatography. The length of the reaction (in hours), the percentage of undigested lysozyme of the total area of all the peaks and the standard deviation based on n number of experiments is shown.

Enzyme	*Reaction time (hours)*	*Undigested protein (%) (average)*	*STDEV*	*(n)*
Cod trypsin	72	20,16	1,09	3
Bovine trypsin	72	65,47	4,64	3

Digestion for 72 hours at 4°C further underscores how dramatic the difference is between the ability of cod trypsin and bovine trypsin in degrading lactoferrin. Only 20 % of undigested lactoferrin remains after proteolysis with cod trypsin wile 65% of undigested lactoferrin remains after proteolysis with bovine trypsin (see Figure 17 at 40 mL elution point). These results clearly show that cod trypsin is far superior in degrading lactoferrin at 4°C than bovine trypsin. The chromatogram of the digests of lactoferrin by cod trypsin or bovine trypsin (Figure 20) reveals that the fragmentation pattern is essentially the same. Therefore, as was true for the digestion of lactoferrin at 25°C, cod trypsin seems to cleave lactoferrin at the same sites as bovine trypsin but at a faster rate.

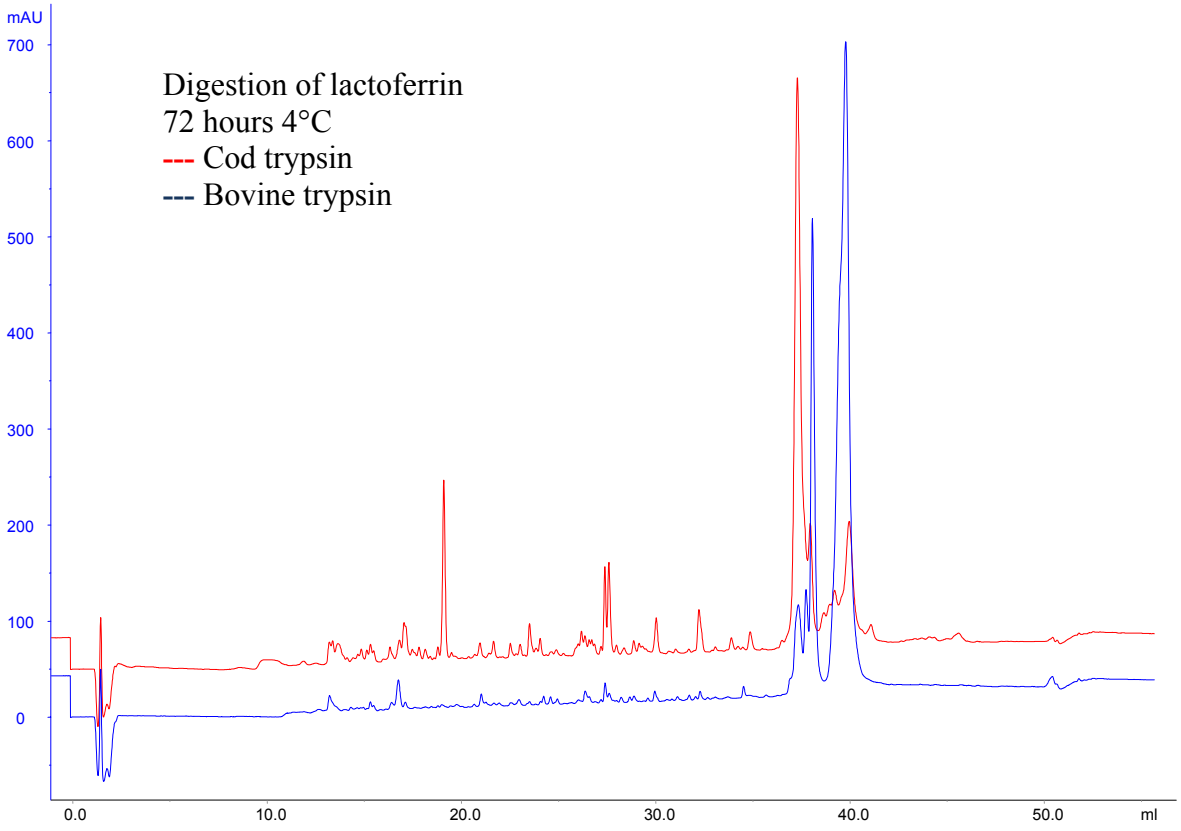

Figure 20 Degradation of lactoferrin, 72 hours at 4°C.

Digestion of 50 µg of lactoferrin with 2.0 µg of cod trypsin (red line) or with 2.4 µg bovine trypsin (blue line) after 72 hours at 4°C analyzed using reversed phase chromatography. Y axis indicates absorbance at 215 nm. X axis indicates the volume of eluent in mL.

4.4. Comparison of the ability of bovine trypsin and cod trypsin in cleaving simple globular proteins at 37°C

Here the degradation reaction was performed in a waterbath at 37°C but the length of the reaction and amount of enzyme used was dependent on the protein used as substrate. After the reaction the resulting peptides in the digest were separated using reversed phase chromatography column.

Therapeutic use of cod trypsin requires it to be active at the physiological temperature of humans. Therefore, it was of interest to compare the ability of bovine trypsin and cod trypsin in cleaving simple globular proteins at 37°C.

4.4.1. Degradation of lysozyme.

The extent of degradation of lysozyme at 37°C by bovine trypsin of cod trypsin at different time points is shown in Table 9. Representative chromatograms of the degradation at 37°C by cod trypsin (in red) and by bovine trypsin (in blue) are shown in Figure 21 and Figure 22.

Table 9. Degradation of lysozyme at 37°C.

Degradation at 37°C by bovine trypsin and cod trypsin analyzed using reversed phase chromatography. The length of the reaction (in hours), the percentage of undigested lysozyme of the total area of all the peaks and the standard deviation based on n number of experiments is shown.

Enzyme	*Reaction time (hours)*	*Undigested protein (%) (average)*	*STDEV*	*(n)*
Cod trypsin	3	47,45	4,35	3
Bovine trypsin	3	88,48	0,68	3
Cod trypsin	6	25,54	7,69	3
Bovine trypsin	6	83,89	0,76	3

Already, in 3 hours there is a significant and statistical difference in the percentage of undigested protein left after digestion with cod trypsin or bovine trypsin. Therefore, this shows that cod trypsin is more efficient in degrading lysozyme at 37°C compared to bovine trypsin. Longer digestion 6 hours further substantiates this observation. There was a small increase in the digestion of lysozyme with bovine trypsin after 6 hours compared to after 3 hours while digestion with cod trypsin showed a substantial increase in the digestion of

lysozyme when comparing digestion for 3 hours and 6 hours. In conclusion, the ability of cod trypsin in degrading lysozyme is much greater compared to bovine trypsin at 37°C.

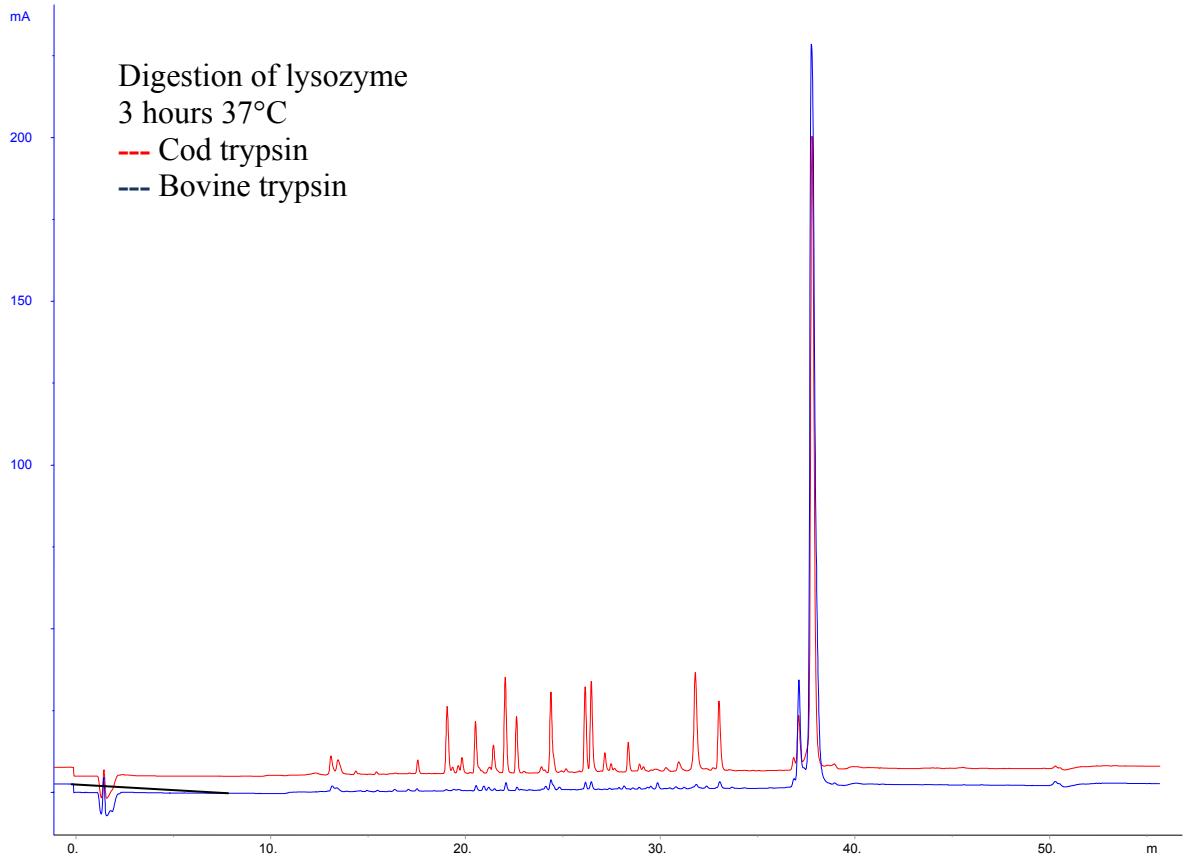

Figure 21. Degradation of lysozyme, 3 hours at 37°C.

Digestion of 50 µg of lysozyme with 2.0 µg of cod trypsin (red line) or with 2.4 µg bovine trypsin (blue line) after 3 hours at 37°C analyzed using reversed phase chromatography. Y axis indicates absorbance at 215 nm. X axis indicates the volume of eluent in mL.

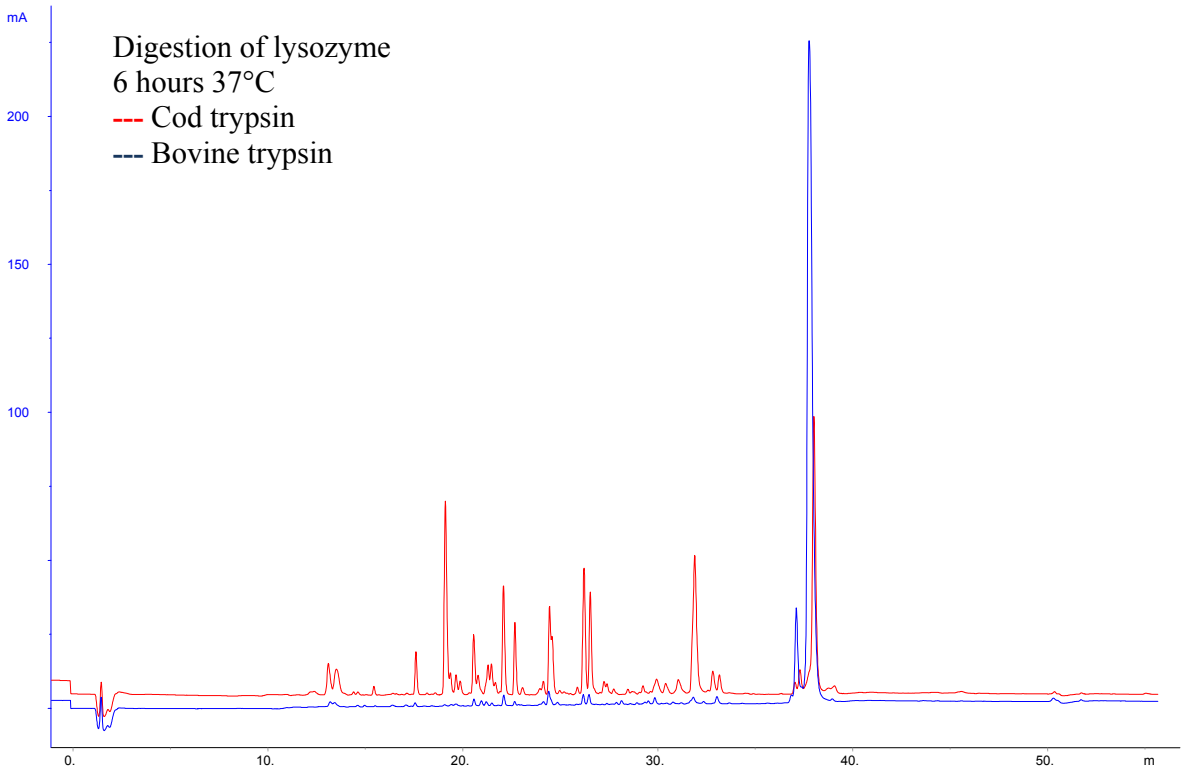

Figure 22. Degradation of lysozyme, 6 hours at 37°C.

Digestion of 50 µg of lysozyme with 2.0 µg of cod trypsin (red line) or with 2.4 µg bovine trypsin (blue line) after 6 hours at 37°C analyzed using reversed phase chromatography. Y axis indicates absorbance at 215 nm. X axis indicates the volume of eluent in mL.

4.4.2. **Degradation of lactoferrin.**

The extent of degradation of lactoferrin at 37°C by bovine trypsin of cod trypsin at different time points is shown in Table 10. Representative chromatograms of the degradation at 37°C by cod trypsin (in red) and by bovine trypsin (in blue) are shown in Figure 23.

Table 10. Degradation of lactoferrin

Degradation of lactoferrin at 37°C by bovine trypsin and cod trypsin analyzed using reversed phase chromatography. The length of the reaction (in hours), the percentage of undigested lysozyme of the total area of all the peaks and the standard deviation based on n number of experiments is shown.

Enzyme	*Reaction time (hours)*	*Undigested protein (%) (average)*	*STDEV*	*(n)*
Cod trypsin	2	8,92	0,55	3
Bovine trypsin	2	61,87	0,46	3

Digestion for 2 hours further underscores how dramatic the difference is between the ability of cod trypsin and bovine trypsin in degrading lactoferrin. At 37°C the peak for undigested lactoferrin had nearly disappeared in the cod trypsin digest whereas 62% of undigested

lactoferrin remained after digestion with bovine trypsin (see Figure 23 at 40 mL elution point). The extent of degradation of lysozyme d with bovine-trypsin after 2 hours at 37°C is very similar to digestion after 24 hours at 25°C and 72 hours at 4°C with bovine trypsin. That indicates that bovine trypsin seems to cleave lactoferrin better at higher temperature. These results clearly show that cod trypsin is far superior in degrading lactoferrin than bovine trypsin even at 37°C.

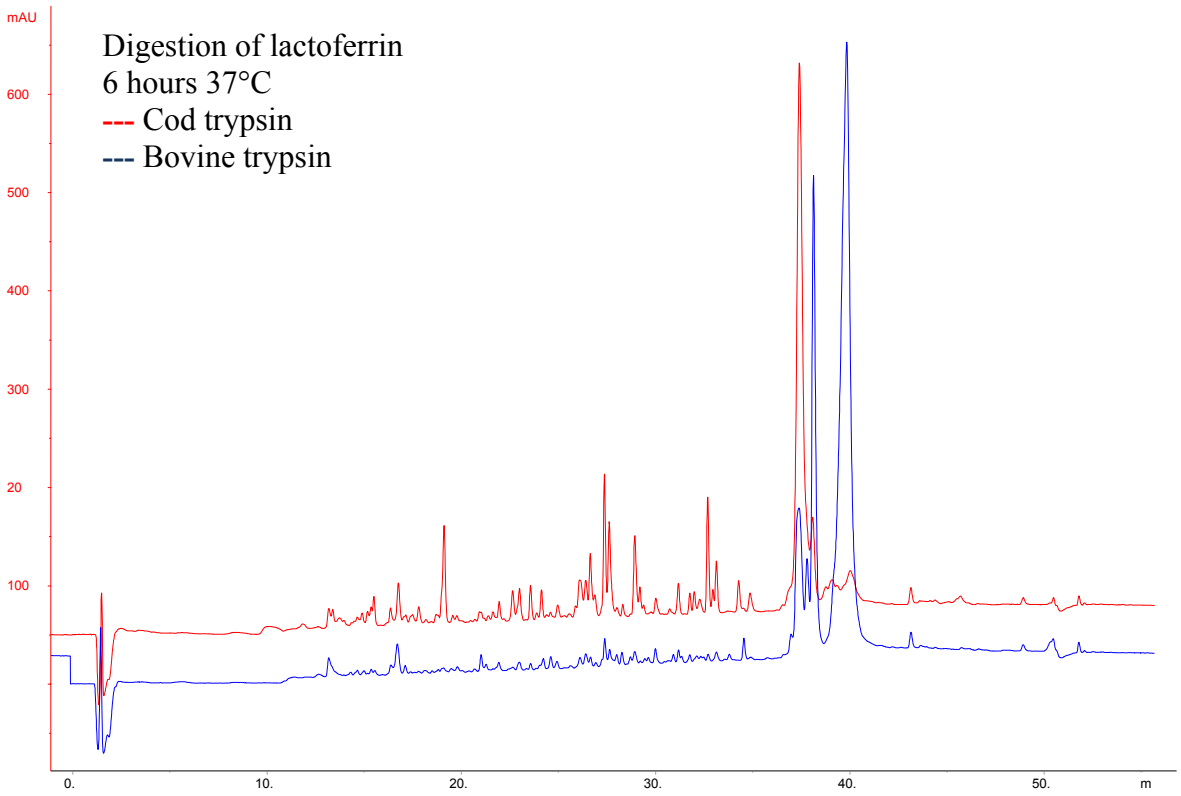

Figure 23. Degradation and lactoferrin, 2 hours at 37°C.

Digestion of 50 µg of lactoferrin with 2.0 µg of cod trypsin (red line) or with 2.4 µg bovine trypsin (blue line) after 2 hours at 37°C analyzed using reversed phase chromatography. Y axis indicates absorbance at 215 nm. X axis indicates the volume of eluent in mL.

The extent of degradation of different substrates with bovine trypsin or cod trypsin for 4, 25 and 37°C is summarized in table 11, 12 and 13.

Table 11. Summary of the results obtained with the degradation of different substrates at 25°C

with bovine trypsin and cod trypsin analyzed using reversed phase chromatography. The length of the reaction (in hours), the percentage of undigested substrate of the total area of all the peaks and the standard deviation based on n number of experiments is shown.

Substrate	*Enzyme*	*Reaction time (hours)*	*Undigested protein (%) (average)*	*STDEV*	*(n)*
Lysozyme	Cod trypsin	25	18.46	5.51	3
	Bovine trypsin	25	81	3.02	3
Lactoferrin	Cod trypsin	24	0,00	0	3
	Bovine trypsin	24	64,52	1,11	3
Myoglobin	Cod trypsin	48	36.61	3.56	3
	Bovine trypsin	48	55.60	5.59	3
BSA	Cod trypsin	24	37,00	3,95	3
	Bovine trypsin	24	48,51	6,95	3
ICAM-1	Cod trypsin	6	7.62	-	1
	Bovine trypsin	6	34.67	-	1

Table 12. Summary of the results obtained with the degradation of different substrates at 4°C

with bovine trypsin and cod trypsin analyzed using reversed phase chromatography. The length of the reaction (in hours), the percentage of undigested substrate of the total area of all the peaks and the standard deviation based on n number of experiments is shown.

Substrate	*Enzyme*	*Reaction time (hours)*	*Undigested protein (%) (average)*	*STDEV*	*(n)*
Lactoferrin	Cod trypsin	72	58,98	2,17	3
	Bovine trypsin	72	90,37	1,12	3
Lysozyme	Cod trypsin	72	20,16	1,09	3
	Bovine trypsin	72	65,47	4,64	3

Table 13. Summary of the results obtained with the degradation of different substrates at 37°C

with bovine trypsin and cod trypsin analyzed using reversed phase chromatography. The length of the reaction (in hours), the percentage of undigested substrate of the total area of all the peaks and the standard deviation based on n number of experiments is shown.

Substrate	*Enzyme*	*Reaction time (hours)*	*Undigested protein (%) (average)*	*STDEV*	*(n)*
Lactoferrin	Cod trypsin	2	8,92	0,55	3
	Bovine trypsin	2	61,87	0,46	3
Lysozyme	Cod trypsin	3	47,45	4,35	3
	Bovine trypsin	3	88,48	0,68	3
	Cod trypsin	6	25,54	7,69	3
	Bovine trypsin	6	83,89	0,76	3

4.5. **The ability of trypsin I in cleaving three amino acid para-nitroanilide substrates**

Which protein substrates and how well they bind to trypsin is dominated by the primary specificity pocket designated S_1 by convention (Schechter, 1967). The trypsin S_1-pocket has a hydrophobic wall so a lysine or an arginine has to be in substrate position P_1 for binding. To have a perfect hydrolysis the right amino acid has to be at site P_1, amino acids in site P_2 and P_3 have been shown to have an effect on catalysis by serine peptidases (Hedstrom, 1992, Hedstrom, 1994). It was of interest to see the effect on catalysis with cod trypsin I by having different amino acids in site P_2 and P_3.

The ability of MonoQ purified cod trypsin I in cleaving seven three amino acid *para*-nitroanilide (*p*NA) substrates was tested. The substrates were CBZ-Gly-Pro-Arg-*p*NA, Bz-Phe-Val-Arg-*p*NA, Tos-Gly-Pro-Lys-*p*NA, H-D-Val-Leu-Arg-*p*NA, Z-Val-Gly-Arg-*p*NA, D-Phe-Pro-Arg-*p*NA and MeoSuc-Ala-Ala-Arg-*p*NA. In all the substrates the *p*NA group is the leaving group in the P_{-1}-site. Measurements were performed as described in Materials and Methods. The steady-state kinetic constants k_{cat} and K_m were determined using Lineweaver-Burk plot, Hanes-Woolf plot, Direct-linear plot and by doing non-linear least-squares fit to the Michaelis Menten equation to find out catalytic efficiency (k_{cat}/ K_m) for each substrate. Lineweaver-Burk and Hanes-Woolf plots are not considered very accurate in determining k_{cat} and K_m because they do not handle experimental errors well and the substrates at low concentration have too much value. Fitting to the Michaelis-Menten equation is thought to be a good way to calculate K_m and k_{cat}.

4.5.1. **Activity measurements with CBZ-Gly-Pro-Arg-pNA**

The kinetic rate constants K_m and k_{cat} and the catalytic efficiency (k_{cat}/ K_m) for trypsin I obtained with CBZ-Gly-Pro-Arg-*p*NA is shown in Table 14.

Table 14. The kinetic rate constants and the catalytic efficiency for trypsin I obtained with CBZ-Gly-Pro-Arg-pNA.

CBZ-Gly-Pro-Arg-pNA was dissolved at 12,5 mM concentration in DMSO and used at a final concentration of 0,05-0,005 mM. Protein concentration of trypsin I was 20,7 µg/mL.

Substrate	*Method*	K_m *(mM)*	k_{cat} *(s^{-1})*	k_{cat}/K_m *($s^{-1}mM^{-1}$)*
CBZ-Gly-Pro-Arg-*p*NA	Michaelis-Menten	0,012	43,34	3514
	Lineweaver Burk	0,018	52,79	2879
	Hanes-Woolf	0,012	43,41	3427
	Direct-linear	0,015	48,96	3192

Trypsin cleaves on the C-side of Arg or Lys. Arg and Lys both have a net positive charge at neutral pH. Arg has a guanidinium group while Lys contains a protonated alkyl amino group (Garnett RH, 1995).

Enzymatic activity of the MonoQ purified trypsin I was measured toward the synthetic substrate CBZ-Gly-Pro-Arg-*p*NA. The measured catalytic efficiency (k_{cat}/ K_m) was 3514 $s^{-1}mM^{-1}$ based on fitting the data to the Michaelis-Menten equation with K_m of 0,012 mM and k_{cat} 43,34 s^{-1}. The catalytic efficiency (k_{cat}/ K_m) is low compared to Helgadottir (Helgadottir, 2002) were k_{cat}/ K_m was listed 7590 $s^{-1}mM^{-1}$ and K_m0,007 mM and k_{cat} 53 s^{-1} by using the same method. The difference lies in the value of K_m which is higher in this study and the k_{cat} is a little lower.

4.5.2. **Activity measurements with Tos-Gly-Pro-Lys-pNA**

The kinetic rate constants K_m and k_{cat} and the catalytic efficiency (k_{cat}/ K_m) for trypsin I obtained with Tos-Gly-Pro-Lys-*p*NA is shown in Table 15.

Table 15. The kinetic rate constants and the catalytic efficiency for trypsin I obtained with Tos-Gly-Pro-Lys-*p*NA.

Tos-Gly-Pro-Lys-*p*NA was dissolved at 12,5 mM concentration in DMSO and used at a final concentration of 0,05-0,005 mM. Protein concentration of trypsin I was 17,9 µg/mL.

Substrate	*Method*	*Km (mM)*	*kcat (s-1)*	*kcat/Km (s-1mM-1)*
Tos-Gly-Pro-Lys-*p*NA	Michaelis-Menten	0,0033	16,72	5125
	Lineweaver Burk	0,0038	16,88	4442
	Hanes-Woolf	0,0027	15,20	5563
	Direct-linear	0,0032	16,22	5123

Enzymatic activity of the MonoQ purified trypsin I was measured toward the synthetic substrate Tos-Gly-Pro-Lys-*p*NA. The measured catalytic efficiency (k_{cat}/ K_m) here is 5125 $s^{-1}mM^{-1}$ fitting the data to the Michaelis-Menten equation. K_m is 0,0033 mM and K_{cat} is 16,72 s^{-1}. There is about 3,6-fold increase in K_m compared to K_m obtained with the CBZ-Gly-Pro-Arg-*p*NA substrate and about 2-fold decrease in k_{cat}. The catalytic efficiency of trypsin I towards Tos-Gly-Pro-Lys-*p*NA is about 1.5 fold higher compared to the catalytic efficiency obtained with CBZ-Gly-Pro-Arg-*p*NA. The only difference in the amino acid sequence between these two substrates is the amino acid in site P_1, Lys in Tos-Gly-Pro-Lys-*p*NA and Arg in CBZ-Gly-Pro-Arg-*p*NA. Lys is smaller and has a simpler structure. The data indicates that Lys fits better into the binding pocket on trypsin I, and therefore leads to higher catalytic efficiency. According to Craik et al. (Carik, 1985) the catalytic efficiency for bovine trypsin towards substrates containing Arg at site P_1 is about 2- to 10-fold higher compared to substrates with Lys at site P_1. Our data therefore implies that the catalytic site and the binding pocket within cod trypsin I is somewhat different from the catalytic site and the binding pocket within mesophilic trypsin like bovine trypsin.

4.5.3. **Activity measurements with Bz-Phe-Val-Arg-pNA**

The kinetic rate constants K_m and k_{cat} and the catalytic efficiency (k_{cat}/ K_m) for trypsin I obtained with Bz-Phe-Val-Arg-*p*NA is shown in Table 16.

Table 16. The kinetic rate constants and the catalytic efficiency for trypsin I obtained with Phe-Val-Arg-*p*NA.

Phe-Val-Arg-*p*NA was dissolved at 50 mM concentration in DMSO and used at a final concentration of 0,05-0,005 mM. Protein concentration of trypsin I was 16,1 µg/mL.

Substrate	*Method*	*Km (mM)*	*kcat (s^{-1})*	*kcat/Km ($s^{-1}mM^{-1}$)*
Bz-Phe-Val-Arg-*p*NA	Michaelis-Menten	0,005	31,14	5774
	Lineweaver Burk	0,007	34,32	5022
	Hanes-Woolf	0,005	30,27	5878
	Direct-linear	0,006	31,53	5484

Enzymatic activity of the MonoQ purified trypsin I was measured toward the synthetic substrate Bz-Phe-Val-Arg-*p*NA. The measured catalytic efficiency (k_{cat}/ K_m) was 5774 $s^{-1}mM^{-1}$ according to Michaelis-Menten were K_m is 0,005 mM and k_{cat} is 31,14 s^{-1}. Using this substrate higher catalytic efficiency is obtained compared to CBZ-Gly-Pro-Arg-*p*NA. Therefore, the bulky hydrophobic amino acids in the Bz-Phe-Val-Arg-*p*NA substrate lead to a more productive binding than the smaller amino acids in CBZ-Gly-Pro-Arg-*p*NA. Both substrates have Arg in the P_1 site but Bz-Phe-Val-Arg-*p*NA has Valine (Val) in the P_2 site and Phenylalanine (Phe) in P_3 site and CBZ-Gly-Pro-Arg-*p*NA has Proline (Pro) in the P_2 site and Glycine (Gly) in P_3 site. Proline has an aliphatic side chain with a distinctive cyclic structure. The secondary amino group of proline residues is held in a rigid conformation that reduces the structural flexibility of polypeptide regions containing proline possible explaining the lower catalytic efficiency obtained with CBZ-Gly-Pro-Arg-*p*NA.

4.5.4. **Activity measurements with D-Phe-Pro-Arg-pNA**

The kinetic rate constants K_m and k_{cat} and the catalytic efficiency (k_{cat}/ K_m) for trypsin I obtained with Bz-Phe-Val-Arg-*p*NA is shown in Table 17.

Table 17. The kinetic rate constants and the catalytic efficiency for trypsin I obtained with Phe-Pro-Arg-*p*NA.

Phe-Pro-Arg-*p*NA was dissolved at 50 mM concentration in DMSO and used at a final concentration of 0,05-0,005 mM. Protein concentration of trypsin I was 18,1 µg/mL.

Substrate	*Method*	*Km (mM)*	*kcat (s^{-1})*	*kcat/Km ($s^{-1}mM^{-1}$)*
D-Phe-Pro-Arg-*p*NA	Michaelis-Menten	0,007	44,42	6886
	Lineweaver Burk	0,007	45,42	6631
	Hanes-Woolf	0,006	44,22	7104
	Direct-linear	0,007	45,25	6830

Enzymatic activity of the MonoQ purified trypsin I was measured toward the synthetic substrate D-Phe-Pro-Arg-*p*NA. The measured catalytic efficiency (k_{cat}/ K_m) was 5774 $s^{-1}mM^{-1}$ according to Michaelis-Menten were K_m is 0,005 mM and k_{cat} 31,14 s^{-1}. The catalytic efficiency is higher with D-Phe-Pro-Arg-*p*NA compared to CBZ-Gly-Pro-Arg-*p*NA and Bz-Phe-Val-Arg-*p*NA. Nearly 2-fold increase is in the catalytic efficiency compared to CBZ-Gly-Pro-Arg-*p*NA were the only difference is in the P_3-site were D-Phe-Pro-Arg-*p*NA has a phenylalanine (Phe) and CBZ-Gly-Pro-Arg-*p*NA has a glycine (Gly). Since k_{cat} obtained with both these substrates is nearly the same but the K_m value is about two-times higher obtained with CBZ-Gly-Pro-Arg-*p*NA it is thought that trypsin I is better adapted to bind substrates with Phe in the P_3 site rather than Pro. Again there is a higher catalytic efficiency obtained with a substrate containing a bulkier amino acid residue.

The difference in catalytic efficiency between having Pro in the P_2 site compared to having Val in the same site is not much. There is a slight increase in K_m and a little more increase in k_{cat} obtained with D-Phe-Pro-Arg-*p*NA that leads to a small increase in k_{cat}/ K_m compared to Bz-Phe-Val-Arg-*p*NA.

4.5.5. **Activity measurements with H-D-Val-Leu-Arg-pNA**

The kinetic rate constants K_m and k_{cat} and the catalytic efficiency (k_{cat}/ K_m) for trypsin I obtained with H-D-Val-Lau-Arg-*p*NA is shown in Table 18.

Table 18. The kinetic rate constants and the catalytic efficiency for trypsin I obtained with H-D-Val-Leu-Arg-*p*NA.

H-D-Val-Leu-Arg-*p*NA was dissolved at 37,9 mM concentration in DMSO and used at a final concentration of 0,05-0,005 mM. Protein concentration of trypsin I was 18,4 µg/mL.

Substrate	*Method*	*Km (mM)*	*kcat (s-1)*	*kcat/Km (s-1mM-1)*
H-D-Val-Leu-Arg-*p*NA	Michaelis-Menten	0,0016	14,54	9280
	Lineweaver Burk	0,0014	14,38	10270
	Hanes-Woolf	0,0015	14,51	9680
	Direct-linear	0,0014	14,60	10686

Enzymatic activity of the MonoQ purified trypsin I was measured toward the synthetic substrate H-D-Val-Leu-Arg-*p*NA. The measured catalytic efficiency (k_{cat}/ K_m) was 9280 $s^{-1}mM^{-1}$ according to Michaelis-Menten were K_m is 0,0016 mM and k_{cat} is 14,54 s^{-1}. This substrate gave the highest catalytic efficiency compared to all the other substrates that were used in this study. H-D-Val-Leu-Arg-*p*NA has Val in the P_3 site and Leucine (Leu) in the P_2 site. Both amino acids are nonpolar and tend to cluster together within proteins, stabilizing protein structure by means of hydrophobic interactions. The data obtained with H-D-Val-Leu-Arg-*p*NA follows the trend that hydrophobic and bulky amino acids at site P_2 and P_3 in the substrate lead to higher catalytic efficiency with cod trypsin I. Again the increase in catalytic efficiency is explained with a lower K_m value obtained but interestingly there is also a substantial drop in k_{cat} compared to the k_{cat} obtained with the other substrates.

4.5.6. **Activity measurements with Z-Val-Gly-Arg-*p*NA**

The kinetic rate constants K_m and k_{cat} and the catalytic efficiency (k_{cat}/ K_m) for trypsin I obtained with H-D-Val-Lau-Arg-*p*NA is shown in Table 19.

Table 19. The kinetic rate constants and the catalytic efficiency for trypsin I obtained with Z-Val-Gly-Arg-*p*NA.

Z-Val-Gly-Arg-*p*NA was dissolved at 25 mM concentration in DMSO and used at a final concentration of 0,05-0,005 mM. Protein concentration of trypsin I was 17,1 µg/mL.

Substrate	*Method*	*Km (mM)*	*kcat (s^{-1})*	*kcat/Km ($s^{-1}mM^{-1}$)*
Z-Val-Gly-Arg-*p*NA	Michaelis-Menten	0,014	51,43	3588
	Lineweaver Burk	0,016	53,43	3340
	Hanes-Woolf	0,014	50,93	3638
	Direct-linear	0,015	52,32	3412

Enzymatic activity of the MonoQ purified trypsin I was measured toward the synthetic substrate Z-Val-Gly-Arg-*p*NA. The measured catalytic efficiency (k_{cat}/ K_m) was found to be 3588 $s^{-1}mM^{-1}$ according to Michaelis-Menten fitting to the data were K_m is 0,014 mM and k_{cat} is 51,43 s^{-1}. There is a about 2,5 fold drop in catalytic efficiency obtained with Z-Val-Gly-Arg-*p*NA compared to H-D-Val-Leu-Arg-pNA. Therefore, by exchanging the bulky hydrophobic (compared to Gly) Leu amino acid in the P_2 site with Gly there is a substantial decrease in catalytic efficiency because of a large increase in K_m.

4.5.7. **Activity measurements with MeoSuc-Ala-Ala-Arg-*p*NA**

The kinetic rate constants K_m and k_{cat} and the catalytic efficiency (k_{cat}/ K_m) for trypsin I obtained with MeoSuc-Ala-Ala-Arg-*p*NA is shown in Table 20.

Table 20. The kinetic rate constants and the catalytic efficiency for trypsin I obtained with MeoSuc-Ala-Ala-Arg-*p*NA.

Z-Val-Gly-Arg-*p*NA was dissolved at 50 mM concentration in DMSO and used at a final concentration of 0,05-0,005 mM. Protein concentration of trypsin I was 17,1 µg/mL.

Substrate	*Method*	*Km (mM)*	*kcat (s^{-1})*	*kcat/Km ($s^{-1}mM^{-1}$)*
MeoSuc-Ala-Ala-Arg-*p*NA	Michaelis-Menten	0,013	33,56	2581
	Lineweaver Burk	0,025	47,28	1866
	Hanes-Woolf	0,014	33,58	2410
	Direct-linear	0,014	33,79	2414

Enzymatic activity of the MonoQ purified trypsin I was measured toward the synthetic substrate MeoSuc-Ala-Ala-Arg-*p*NA. The measured catalytic efficiency (k_{cat}/ K_m) was 2581 $s^{-1}mM^{-1}$ by fitting the data to the Michaelis-Menten equation. K_m was 0,013 mM and k_{cat}

33,56 s^{-1}. This substrate gave the lowest catalytic efficiency compared to all the other substrate that were used in this study. Alanine (Ala) which is a small nonpolar amino acid is in both the P_2 and P_3 site in the MeoSuc-Ala-Ala-Arg-pNA substrate. Most notably, a substrate with amino acids with a small side chain in site P_2 and P_3 gives a low catalytic efficiency. This is in line with the catalytic efficiency obtained with other substrates were the catalytic efficiency seems to increase as the side chain in amino acids at site P_2 and P_3 is larger.

Table 21 is a summary of the results obtained with all seven X-X-Arg/Lys-pNA substrates used in this study.

Table 21. K_m and k_{cat} constants and the catalytic efficiency (k_{cat}/K_m) for cod trypsin I obtained with seven X-X-Arg/Lys-pNA substrates.

All the kinetic constants were determined by fitting the data to the Michaelis Menten equation.

Substrate	*Method*	*K_m (mM)*	*k_{cat} (s^{-1})*	*k_{cat}/K_m ($s^{-1}mM^{-1}$)*
CBZ-Gly-Pro-Arg-*p*NA	Michaelis-Menten	0,012	43,34	3514
Tos-Gly-Pro-Lys-*p*NA	Michaelis-Menten	0,0033	16,72	5125
Bz-Phe-Val-Arg-*p*NA	Michaelis-Menten	0,005	31,14	5774
D-Phe-Pro-Arg-*p*NA	Michaelis-Menten	0,007	44,42	6886
H-D-Val-Leu-Arg-*p*NA	Michaelis-Menten	0,0016	14,54	9280
Z-Val-Gly-Arg-*p*NA	Michaelis-Menten	0,014	51,43	3588
MeoSuc-Ala-Ala-Arg-*p*NA	Michaelis-Menten	0,013	33,56	2581

5. Conclusion

One of the aims of this study was to increase the knowledge on the efficacy of cod trypsin to cleave proteins in their native form. This study shows that cod trypsin outperforms bovine trypsin in cleaving pathological proteins and other proteins in their native form. This lends a stronger scientific basis to the observed clinical efficacies of cod trypsin. Five proteins in their native form with variable structures were used as substrates for cod trypsin or bovine trypsin. Four of the substrates are extracellular or membrane proteins (lysozyme, lactoferrin, BSA and ICAM-1). These proteins are considered excellent model substrates, when it comes to testing the ability of cod trypsin to cleave native proteins, since cod trypsin is topically applied when used for therapeutic purposes. Furthermore, the proteins tested have quite different structures, which is important for demonstrating the ability of cod trypsin to cleave different native proteins. The extent of degradation of all the substrates was greater with cod trypsin compared to bovine trypsin except for BSA where it was about the same. Extensive difference was observed with three substrates, with one being ICAM-1. This is particularly intriguing since ICAM-1 plays an important role in immune response, inflammation and pathogen invasion.

Close inspection of the chromatograms of the digests of the five substrates by cod trypsin or bovine trypsin reveals that the fragmentation pattern is practically the same when comparing digestion with bovine trypsin and cod trypsin. This indicates that cod trypsin is not cleaving the substrates at different sites compared to bovine trypsin. Therefore, the increased ability of cod trypsin in digesting the substrates seems not to be caused by novel cleavage sites within the substrates compared to bovine trypsin. As a result, cod trypsin seems to cleave the substrates at the same sites as bovine trypsin but at a faster rate. Therefore it was deemed unnecessary to determine the cleavage sites by cod trypsin within the substrates.

To investigate the effects of the amino acids in the peptide chain in small substrates, on the steady state kinetic parameters of cod trypsin I, seven commercially available para-nitroanalide peptide substrate were analyzed. Trypsin I is the most abundant trypsin isoform in cod trypsin. The data shows that the size of the amino acid side chain at the P_2 and P_3site in the substrate has an effect on the kinetic constants and the catalytic efficiency. Of the substrates tested, larger hydrophobic amino acid resides at site P_2 and P_3 lead to a lower K_m value obtained for cod trypsin I. The biggest difference in the K_m constant between substrates was almost 9 fold. At the same time the change in k_{cat} is relatively small between all the substrates were largest difference was about 3,5 fold. As well, it is difficult to see a trend

based on the difference in the amino acids at site P_2 and P_3 within the substrates when looking at k_{cat}. The catalytic efficiency seems to be higher in substrates containing amino acids with larger side chains at site P_2 and P_3 in the substrate largely because of lower K_m value. Therefore, it is concluded that trypsin I binds bigger amino acids better than small amino acids leading to higher catalytic efficiency.

Future studies should focus on elucidated the structural aspect of cod trypsin and the catalytic mechanism as to why cod trypsin is better able to degrade proteins than mesophilic trypsin like bovine trypsin. In that regard the binding pocket of cod trypsin is of special interest because the data precented here indicates that cod trypsin binds Lys better than Arg but studys on mesophilic trypsins shows othervise.

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