Review

MARINE BIOTECHNOLOGY

Atlantic Cod Trypsins: From Basic Research to Practical Applications

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Abstract

Atlantic cod trypsin I is an appropriate representative of the traditionally classified cold-adapted group I trypsins, and the recombinant form of cod trypsin Y is the only biochemically characterized member of the novel group III trypsins. Trypsin Y is adapted to lower temperatures than all other presently known trypsins. This review describes the basic characteristics of and practical uses for trypsins of Atlantic cod, as well as those of other organisms. Overexpression of the recombinant forms of cod trypsins I and Y in microorganisms is explained as well as the advantages of using site-directed mutagenesis to increase their stability toward autolysis and thermal inactivation. Trypsins appear to play a key role in the nutrition and development of marine fish. We discuss the potential use of cod trypsins as biomarkers to evaluate the nutritional status of cod larvae and describe the industrial applications of cod trypsin I and other trypsins.

Key words: trypsins — fish — cold-adapted — expression — practical applications

Introduction

This review spans over two decades of research on trypsins from the Atlantic cod (*Gadus morhua*). Its main goal is to shed light on the basic properties of these cold-adapted proteases as well as to view the numerous practical uses of trypsins from cod and other organisms. The Atlantic cod is an economically important fish species in Iceland and elsewhere in the Northern hemisphere. In 2002 cod products accounted for approximately 24% of the total value of exported goods from Iceland (Table 1). The decline of cod stocks in many areas has led to increased emphasis on cod farming. There is also heightened awareness in many countries of the importance of increasing the value of seafood products by advancing the utilization of by-products from the fishing industries (Nafstad et al., 2002; Rustad, 2003). The research presented here has been focused on that issue from the beginning.

The pyloric cecum, serving the role of a digestive organ in the Atlantic cod, is a by-product of the fishing industry found in abundance in Iceland. As would be expected, it is rich in digestive enzymes such as serine proteases (Ásgeirsson et al., 1989). The best known members of the serine protease family are trypsins, chymotrypsins, elastases, serine collagenases, and brachyurins (Halfon and Craik, 1998). The serine proteases play important roles in a number of biological functions. In digestion (Neurath, 1984) trypsin has a dual role in that it cleaves ingested proteins and activates the precursor forms of several other digestive proteases including chymotrypsin. Serine proteases from the Atlantic cod such as trypsin (Ásgeirsson et al., 1989), chymotrypsin (Ásgeirsson and Bjarnason, 1991), elastase (Ásgeirsson and Bjarnason, 1993), and serine collagenase (Kristjánsson et al., 1995) have typical characteristics of cold-adapted enzymes.

The Atlantic cod is known to produce numerous trypsin isozymes (Ásgeirsson et al., 1989; Gudmundsdóttir et al., 1993; Helgadóttir, 2002). Several of these have been isolated from their native source, with trypsin I being the most predominant one. It also has the highest catalytic efficiency and is by far the best characterized of the trypsin isozymes (Ásgeirsson et al., 1989; Jónsdóttir et al., 2004). The complementary DNAs of two trypsin isozymes (I and X) (Gudmundsdóttir et al., 1993), in addition to a novel trypsin termed trypsin Y (Spilliaert and Gudmundsdóttir, 1999),

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Table 1. Value of Exported Marine Products by Product Categories in 2002 Relative to All Exported Goods[a]

Product category	*Total (million ISK)*	*(%) Value*
All exported goods	204,000	100
Total marine products	129,000	63
Pelagic catch	25,000	12
Demersal catch	78,000	38
Cod products	49,000	24
Other marine products	26,000	13

[a]The value is given in million ISK (Icelandic kronas), fob. Also shown is the percentage value relative to all exported goods. Source: Statistics Iceland (External Trade, available at http://www.hagstofan.is).

have been isolated from a cod pyloric ceca cDNA library. Characterization of the recombinant trypsin Y polypeptide demonstrated that it is very different from the classical trypsins, such as trypsin I (Pálsdóttir and Gudmundsdóttir, 2004), and may be the digestive enzyme produced under cold-shock conditions (Roach 2002).

In general trypsins 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 et al., 1989; Gerday et al., 2000; Schrøder Leiros et al., 2000). These enzymes are also more sensitive to inactivation by heat, low pH, and autolysis than their mesophilic analogues (Simpson et al., 1989; Ásgeirsson et al., 1989). In addition, native proteins are easily hydrolyzed by the cold-adapted fish proteases. These traits and the fact that the cold-adapted enzymes function properly at low temperatures have stimulated interest in their commercial use, as they are generally better suited for enzymatic processes than their mesophilic counterparts (Bjarnason et al., 1993; Bjarnason, 2000; Bjarnason and Benediktsson, 2001; Shahidi and Janak Kamil, 2001).

The recent data presented on the expression of Atlantic cod trypsin I (Jónsdóttir et al., 2004) and trypsin Y (Pálsdóttir and Gudmundsdóttir, 2004) are, to our knowledge, the first reports on the expression of psychrophilic or cold-adapted proteolytic enzymes from fish in an active form. Difficulties related to the sensitivity of cold-adapted proteases to autolytic degradation, thermal inactivation, as well as molecular aggregation may account for the limited number of publications in this area (Ásgeirsson et al., 1989; Ásgeirsson and Bjarnason, 1991; Kristjánsson et al., 1995; Helgadóttir, 2002). Site-directed mutagenesis of their cDNAs may be used to produce new and improved recombinant enzyme variants (Narinx et al., 1997; Benjamin et al., 2001; Gerike et al., 2001).

Atlantic cod trypsin I has already proved useful in industrial applications (Bjarnason et al., 1993; Bjarnason and Benediktsson, 2001) and medical applications (Bjarnason, 2000), as will be discussed later in this review. Other cold-adapted proteolytic enzymes have been applied as processing aids in the food and feed industries, as was thoroughly described in a review by Shahidi and Janak Kamil (2001).

Sunde et al. (2001) demonstrated a significant correlation between trypsin activity and the specific growth rate of Atlantic salmon (*Salmon salar*). The secretion rate of trypsin and chymotrypsin in marine fish has also been shown to be related to feed intake and filling of the stomach (Einarsson et al., 1996). Moreover, fish development appears to be highly dependent on serine protease activity, and trypsin, in particular, has been suggested to play a key role in larval survival (Porter and Theilacker, 1999). This is due to the fact that trypsin activity, which is already present in fish larvae at first feeding or prior to the development of the digestive system, is mainly responsible for the digestion of ingested food (Kolovski, 2001). Trypsin is also a suitable short-term indicator of nutritional quality in both farm-raised and field-caught marine fish larvae (Nolting et al., 1999). Currently we are using knowledge about the Atlantic cod trypsins accumulated in our laboratory in the past in a research project aimed at increasing the survival rate of farm-raised Atlantic cod larvae.

Trypsins I and Y

Trypsin I. Most trypsins can be classified into 3 basic groups, termed I, II, and III, based on their amino acid sequence identities. The largest of these, group I includes members like Atlantic cod trypsin I and salmon trypsin I. All 3 trypsin groups, share the catalytic triad residues His57, Asp102, and Ser195 in addition to the obligatory Asp189 localized at the bottom of their substrate-binding pocket (Figure 1). They also contain the Gly216 and Gly226 residues lining the sides of the substrate-binding pocket and Tyr172, considered to be a key residue in trypsin substrate specificity (Hedstrom et al., 1994).

Three native trypsin isozymes, termed trypsins I, II, and III, were previously isolated from the pyloric ceca of Atlantic cod and purified (Ásgeirsson et al., 1989). Trypsin I, the most abundant and best characterized form, also shows the highest catalytic efficiency (k_{cat}/K_m), which is approximately 20 times higher than that of its mesophilic bovine analogue. The high catalytic efficiency and low thermal stability of Atlantic cod trypsin I makes it an interesting enzyme to study with respect to its structure-function relationship. Atlantic cod trypsin I and

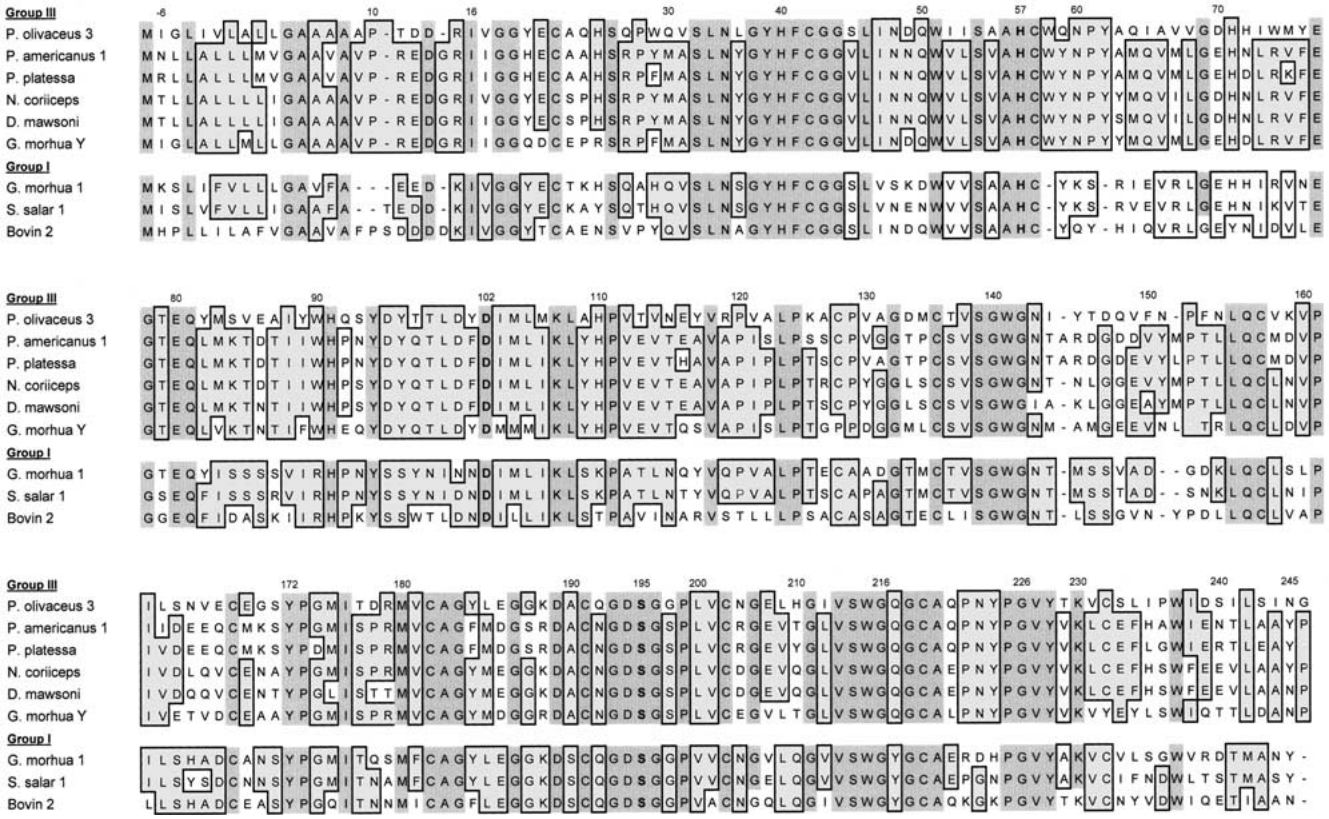

Fig. 1. Amino acid sequence alignments of group III and group I trypsins. The trypsin sequences shown in the upper row (group III trypsins) are those of bastard halibut (*P. olivaceus*, AB029752), winter flounder (*P. americanus*, AF012462), plaice (*P. platessa*, X56744), black rockcod (*N. coriiceps*, AF134323), Antarctic cod (*D. mawsoni*, U58945), and Atlantic cod (*G. morhua*, AJ459311). Sequences shown in the lower row (group I trypsins) are those of Atlantic cod trypsin (*G. morhua*, X76886), salmon trypsin (*S. salar*, X70075), and bovine trypsin 2 (*Bos taurus*, X54703). The sequences are numbered according to the chymotrypsinogen numbering system in which the N-terminal residue of the mature sequences, Ile16, is indicated by a number. The catalytic triad residues His57, Asp102, and Ser195 are indicated with boldface letters. Dark gray areas show residues that are identical in all the trypsins, whereas lighter gray boxed areas indicate residues that are identical within each of the 2 trypsin groups.

salmon trypsin I (Smalås et al., 1994) are typical of the traditionally classified psychrophilic or cold-adapted proteolytic vertebrate enzymes. These 2 trypsins have about 80% amino acid sequence identity (Gudmundsdóttir et al., 1993), and they appear to share a nearly identical 3-dimensional structure, as previously demonstrated by modeling studies (Smalås et al., 2000; Schrøder Leiros et al., 2000).

A cDNA clone corresponding to trypsin I was isolated earlier from an Atlantic cod pyloric ceca cDNA library and sequenced (X76886) (Gudmundsdóttir et al., 1993). Two other Atlantic cod trypsin clones, termed X (Gudmundsdóttir et al., 1993) and Y (Spilliaert and Gudmundsdóttir, 1999), have been identified. Trypsins I and X are isozymes that differ by a mere 8 amino acid residues, but trypsin Y has only about 45% amino acid sequence identity to the other 2 trypsins (Table 2).

The precursor form of the recombinant Atlantic cod trypsin I has been produced in a soluble form as a thioredoxin fusion protein in the His-Patch ThioFusion *Escherichia coli* expression system (Jónsdóttir et al., 2004). An active recombinant trypsin I was generated by cleavage of the purified fusion protein with a minute amount of native trypsin I, and the recombinant cod trypsin I was purified to homogeneity (Figure 2, A). At present about 50% of the native cod trypsin I sequence has been determined by N-terminal amino acid sequencing (Helgadóttir, 2002). This sequence is identical to the corresponding amino acid sequence deduced from the cDNA sequence of cod trypsin I (Gudmundsdóttir et al., 1993). This, along with the analysis of the recombinant cod trypsin I by MonoQ chromatography and sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using polyclonal antibodies raised against native trypsin I, as well as its binding to a trypsin-specific *p*-aminobenzamidine affinity column, strongly indicates that recombinant cod trypsin I is identical to native trypsin I from Atlantic cod.

Table 2. Percentage Amino Acid Sequence Identity Between Cod Trypsin I and Group III Trypsins Including Cod Trypsin Y

	Cod I	Cod Y	*D. maw*	*P. ame*	*N. cori*	*P. plat*	*P. oliv*
Cod I	—	45	47	48	49	47	53
Cod Y	45	—	73	74	75	74	57
D. maw	47	73	—	81	94	78	57
P. ame	48	74	81	—	83	92	57
N. cori	49	75	94	83	—	80	59
P. plat	47	74	78	92	80	—	57
P. oliv	53	57	57	57	59	57	—

Trypsin Y. The cDNA encoding trypsin Y (AJ459311) was previously isolated from an Atlantic cod cDNA library and characterized in a search for serine proteases with novel characteristics (Spilliaert and Gudmundsdóttir, 1999). Trypsin Y was classified into a new group of trypsins, termed group III, on the basis of its novel traits. The cDNAs of 7 other putative group III trypsins have been isolated and characterized from a variety of fish species that live at least part of their lives in cold environments (Spilliaert and Gudmundsdóttir, 1999; Roach, 2002). These include plaice (*Pleuronectes platessa*, X56744), winter flounder (*Pleuronectes americanus*, AF012462) (Douglas and Gallant, 1998), Antarctic cod (*Dissostichus mawsoni*, U58945) (Chen et al., 1997), black rockcod (*Notothenia coriiceps*, AF134323) (Cheng and Chen, 1999), bastard halibut (*Paralichthys olivaceus*, AB029752) (Suzuki et al., 2002) and Japanese pufferfish (*Fugu rubripes*), which has 2 isozymes from the novel clade (AY661445, AY661446).

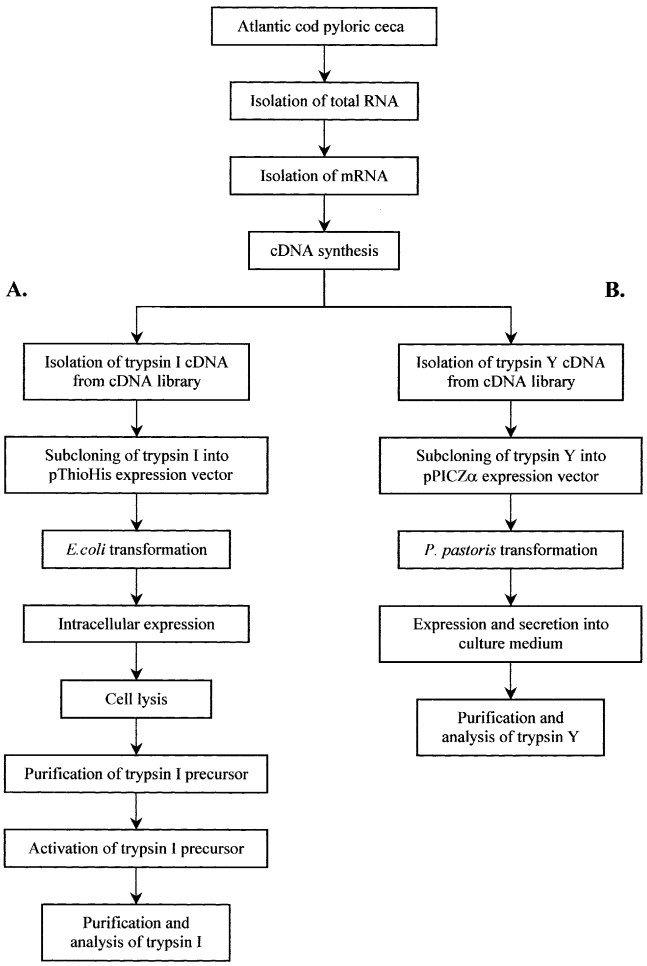

Fig. 2. Schematic diagram showing the cDNA cloning and overexpression of cod trypsins I and Y. **A:** Main steps involved in the expression of trypsin I in *E. coli*. **B:** Expression of trypsin Y in the yeast *Pichia pastoris*.

Amino acid sequence alignments of the putative group III polypeptide sequences, as deduced from their cDNA sequences, show that they are 57% to 94% identical (Table 2). The group III trypsins are more similar to vertebrate trypsins than to any other known proteins despite their relatively low sequence identity to the classical group I trypsins. Molecular modeling studies of Atlantic cod trypsins Y and I indicate that their overall 3-dimensional structures are similar, as shown in Figure 3. However, various residues known to contribute to the substrate specificity of the group I trypsins (Hedstrom et al, 1992, 1994; Perona and Craik, 1997) are not shared by the novel group III trypsins. For example, a difference is observed in surface loops that play a crucial role in determining substrate specificity (Hedstrom et al., 1992, 1994). Moreover, the group III trypsins contain a series of mutations localized around the highly conserved catalytic triad residues. In addition, some unique alterations are found in and around the substrate specificity pocket of the group III trypsins.

Apparently, the most important of those mutations is a Ser190Ala mutation shown to be localized at the bottom of the substrate-binding pocket in trypsins along with Asp189 (Figure 3). The Ser190 residue in the group I trypsins forms a hydrogen bond to substrates containing arginine and lysine (Perona and Craik, 1995) and thus modulates the specificity of substrates containing these 2 residues (Ruhlmann et al., 1973; Bode et al., 1984). An Ala190 residue replacing the Ser190 would not form a hydrogen

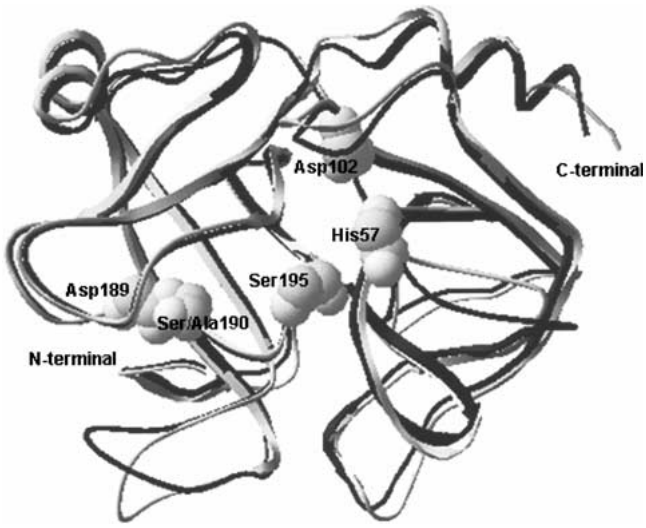

Fig. 3. Molecular model of trypsin Y (black) overlaying that of trypsin I (gray). Human trypsin (1TRN) was used for modeling of trypsin Y, whereas the model for trypsin I was built on the structure of salmon trypsin (1UTM). The catalytic triad residues His57, Asp102, and Ser195 are shown in gray along with residue Asp189 localized at the bottom of the substrate-binding pocket in trypsins. Also shown is residue 190, which is Ala in all known group III trypsins including trypsin Y. This residue is a Ser190 in all known group I trypsins.

bond to trypsin substrates. Therefore, the Ser190Ala mutation in trypsin Y and the other group III trypsins may result in impaired binding to trypsin substrates. This may possibly also lead to extended substrate specificity, as observed for the recombinant trypsin Y (Pálsdóttir and Gudmundsdóttir, 2004). The group III trypsins are 5 to 6 residues longer than the group I trypsins. Furthermore, their activation peptides, consisting of 6 residues preceding residue Ile16 (Figure 1), contain only 2 negatively charged residues instead of 4 (AspAspAspAsp) found in most group I trypsins, with the exception of Atlantic cod and salmon trypsins, which contain only 3 negative charges in their activation peptides (Figure 1). The 4 negatively charged residues present in the group I trypsin activation peptides are recognized by enterokinase, the enzyme responsible for activating the precursor forms of mammalian trypsins.

The recombinant form of cod trypsin Y was produced in the yeast *Pichia pastoris* (Figure 2, B) and characterized (Pálsdóttir and Gudmundsdóttir, 2004). The polypeptide was expressed with a HisMyc tag at its C terminus in order to facilitate its detection in Western blot analysis using an anti-myc antibody. The recombinant trypsin Y was purified by using Q-Sepharose ion-exchange and *p*-aminobenzamidine trypsin affinity chromatography. The enzyme was also found to bind to a 4-phenylbutylamine chymotrypsin affinity column.

Our data demonstrated that the putative recombinant trypsin Y has a dual substrate specificity: i.e., toward the trypsin-specific and chymotrypsin-specific synthetic substrates succinyl-Ala-Ala-Pro-Arg-*p*-nitroanilide and succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide, respectively. The fact that the recombinant trypsin Y binds to both trypsin-specific and chymotrypsin-specific affinity resins confirms its dual substrate specificity. Surprisingly, the chymotrypsin activity of the recombinant trypsin Y polypeptide was higher than the trypsin activity measured under the same conditions (Pálsdóttir and Gudmundsdóttir, 2004).

The group III trypsins have been suggested to play an important role in marine fish development and to represent extreme-cold-adapted enzymes. Roach (2002) demonstrated that the group III trypsins evolved relatively recently from other trypsins, possibly owing to selective pressure such as extreme cold adaptation. Our research shows that the recombinant trypsin Y may indeed be considered an extreme-cold-adapted enzyme, as it is active in the temperature range of 2° to 27°C when measured against the synthetic substrates for trypsin and chymotrypsin. The enzyme showed minor activity at 2°C, but its activity increased steeply, reaching a maximum at 21°C, followed by complete inactivation at 30°C (Pálsdóttir and Gudmundsdóttir, 2004). The activity profiles of chymotrypsin and trypsin with temperature changes have similar patterns, suggesting that the activities are localized in the same enzyme.

Notably, none of the putative group III trypsin polypeptides have been isolated from their biological sources (Roach, 2002). To our knowledge, neither were the recombinant forms of any of these enzymes produced and characterized prior to the expression of recombinant trypsin Y. Thus our data on the recombinant trypsin Y present the first biochemical data on a group III trypsin complementing the molecular description of these enzymes.

Cold-Adaptation Enzymes

Many cold-adapted proteolytic enzymes, such as trypsin I from Atlantic cod (Helgadóttir, 2002) and euphaulysin from Antarctic krill (*Euphausea superba*) (Kristjánsdóttir, 1999), have been shown to be more susceptible to autolysis than their mesophilic counterparts. This trait may be connected to their increased catalytic efficiency and decreased thermal and acid stability relative to their mesophilic analogues (Miyazaki et al., 2000; Shoicet et al.,

1995; Smalås et al., 2000; D'Amico et al., 2003; Feller, 2003).

Numerous authors (Schrøder Leiros et al., 2000; Smalås et al., 2000; Feller, 2003; Gerday et al., 2000; Aghajari et al., 2003) have pointed out that the mechanisms of cold adaptation are more complex than previously anticipated, as enzymes seem to have adapted to cold in different ways. One feature that appears to be common to cold-adapted enzymes is high molecular flexibility compared with their mesophilic analogues (Závodszky et al., 1998). Loops are flexible structures that seem to play important functions in substrate specificity as well as in the activity of enzymes (Perona et al., 1997). The cold-adapted triosephosphate isomerase from *Moraxella* (Rentier-Delrue et al., 1993) has an extended length of several surface loops relative to its mesophilic analogues. These extensions are presumed to play a role in its cold adaptation by increasing the flexibility of the molecule, possibly at the cost of thermal stability. Four extended regions may play a similar role in the cold adapted Antarctic krill euphaulysin molecule (Benjamin et al., 2001) analyzed in our laboratory. Structural analysis of cold-adapted trypsins (Schrøder Leiros et al., 2000) has indicated that the precise position of a particular residue ''determinant'' in their 3-dimensional structures is of primary importance. Also, interactions between structural regions of cold-adapted enzymes, in particular between domains, seem to be weaker than those between their mesophilic analogues. Weaker interactions appear to give rise to increased molecular flexibility and may in many cases cause thermal stability of cold-adapted enzymes to decrease.

Many other cold-adaptive determinants have been suggested for trypsins (Smalås et al., 2000) and other cold-active enzymes (Gerday et al., 2000) relative to their mesophilic analogues. Among these are a lower number of hydrogen bonds, less densely packed structures, increased surface hydrophilicity, a higher number of methionine residues, a different fold of the ''autolysis loop,'' as well as the carboxy-terminal region. In addition, some cold-adapted enzymes such as Atlantic cod trypsin I (Gudmundsdóttir et al, 1993) have reduced proline content at specific points in their structures. Others have an increased number of glycine residues or fewer disulfide bonds; for example, Atlantic cod trypsin Y contains only 5 of the 6 conserved disulfide bonds found in most trypsins, including all the other putative group III trypsins (Spilliaert and Gudmundsdóttir, 1999; Pálsdóttir and Gudmundsdóttir, 2004).

Interestingly, comparison of their amino acid compositions (Table 3) reveals that the group III trypsins have trends that are approximately opposite those of traditionally classified cold-adapted group. I trypsins (Roach, 2002; Spilliaert and Gudmundsdóttir, 1999). The group III trypsins have less net charge, fewer basic and polar residues, and more prolines and aromatic residues. Most of the changes are found in surface loops. The number of tyrosine residues exclusively found in the group III trypsins at certain spots in the molecules is striking (Figure 1) and may have a function in the extreme cold adaptation of these trypsins.

Table 3. Amino Acid Composition Differences Between Group III Trypsins and Cold-Adapted Group I Trypsins

Residues[a]	*Group III*	*Group I cold-adapted*
Proline	++	+
Methionine	++	+
Tryptophan	++	+
Tyrosine	++	+
Serine	+	++
Polar	+	++
Hydrophobic	++	+
Aromatic	++	+

Polar residues are Q+N+C+S+T+Y+R+K+H+D+E+G; hydrophobic residues, A+I+L+F+W+V+P+M; aromatic residues, F+W+Y; Two symbols (++) indicate more residues; one (+), fewer residues.

The term ''extreme'' cold-adapted has recently been introduced in connection with the molecular characterization of the group III trypsins (Roach, 2002). It basically refers to enzymes that are hypothetically active at subzero temperatures. Apparently, there are few reports in the literature on the traditionally termed cold-adapted enzymes showing measurements of enzymatic activity at temperatures below 4°C (Ásgeirsson et al., 1989; Raae, 1990; Feller, 2003; Smalås et al., 2000). Trypsin Y is active at 2°C, and the enzyme is completely inactivated at 30°C. For comparison, cod trypsin I shows enzymatic activity at 4°C, reaching a maximum at 55°C and complete inactivation at 65°C (Ásgeirsson et al., 1989). The relatively transient temperature range of enzymatic activity demonstrated by the recombinant trypsin Y (2°–30°C) could be a typical characteristic of the extreme-cold-adapted enzymes, as their primary role may be food digestion under cold-shock conditions (Pálsdóttir and Gudmundsdóttir, 2004). These enzymes may even contain antifreeze components in their structures and thus be able to function at subzero temperatures as previously suggested (Roach, 2002).

Expression in Microorganisms

The sensitivity of cold-adapted proteases to autolytic degradation, thermal inactivation, as well as

molecular aggregation, even at temperatures as low as 18° to 25°C may explain the problems observed with their expression, activation, and purification (Ásgeirsson et al., 1989; Ásgeirsson and Bjarnason, 1991; Kristjánsson et al, 1995; Helgadóttir, 2002). To our knowledge, there were no published reports on the expression of psychrophilic or cold-adapted proteolytic enzymes from fish prior to the recent data presented on the expression of Atlantic cod trypsin I (Jónsdóttir et al., 2004) and trypsin Y (Pálsdóttir and Gudmundsdóttir, 2004). Nevertheless, cold-adapted proteolytic enzymes from bacteria (Narinx et al., 1997; Taguchi et al., 1999; Gerday et al., 2000; Arnórsdóttir et al., 2002) and marine invertebrates (Kristjánsdóttir and Gudmundsdóttir, 2000) have been successfully expressed in microorganisms.

Before the discovery of the His-Patch ThioFusion *E. coli* expression system (LaVallie et al., 1993), production of trypsin I in its active form in mesophilic expression systems like *Pichia pastoris* or other *E. coli* seemed impossible. However, the inactive precursor form of trypsin I was readily produced in both expression systems. The His-Patch ThioFusion expression system (Figure 2, A) has many advantages for expressing sensitive enzymes such as cod trypsins I and Y. It offers milder environmental conditions and a shorter time of cell growth with an induction time of only 10 hours at 25°C compared with 3 to 5 days at 25°C in the *Pichia pastoris* expression system. Also, owing to the high amount of fusion protein produced in *E. coli*, the protein is easily detected during the expression process using either monoclonal antibody raised to the thioredoxin part of the fusion protein or polyclonal antibodies raised to the recombinant molecule. In general, recombinant proteins tend to be poorly soluble and prone to molecular aggregation (Feller et al., 2000). The presence of a highly soluble thioredoxin part of the fusion protein contained at the N-terminal part of the recombinant protein increases its solubility, as seen with the recombinant cod trypsin I molecule (Jónsdóttir et al., 2004). It also seemed to protect the N-terminal end of the recombinant cod trypsin I from proteolytic cleavage during expression and handling. In addition, it enabled purification of the fusion protein on a metal-chelating ProBond affinity column.

Expressing recombinant trypsin I in the His-Patch ThioFusion *E. coli* expression system resulted in a low yield of the active recombinant enzyme. A possible reason for this is incorrect folding of some of the recombinant trypsin I molecules. Also, cleavage of the thioredoxin part from the recombinant trypsin I may result in autolysis of the enzyme (Jónsdóttir et al., 2004).

Although the *Pichia pastoris* expression system did not seem to be suitable for producing recombinant trypsin I, the recombinant form trypsin Y was successfully expressed in *Pichia pastoris* (Figure 2, B) (Pálsdóttir and Gudmundsdóttir, 2004). The recombinant enzyme was fused to a C-terminal peptide containing a *myc* epitope for detection with anti-myc antibodies and a polyhistidine tag for purification on a metal-chelating resin. The enzyme was produced with an α-factor secretion signal attached to the N terminus. This signal allows for efficient secretion of the protein from the *P. pastoris* cells into the culture medium. Because *P. pastoris* secretes only low levels of endogenous proteins, the recombinant trypsin Y comprises the vast majority of proteins in the medium. Thus the secretion serves as a major first step in the purification process.

Site-directed mutagenesis of the cDNAs encoding enzymes offers possibilities of producing new and improved recombinant enzyme variants without sacrificing their catalytic efficiencies (Narinx et al., 1997; Benjamin et al., 2001; Gerike et al., 2001). However, expression of the enzymes involved is required to fulfil that goal. It is therefore of great importance to continue experiments leading to improvements in the expression of cold-adapted proteolytic enzymes. The fact that the problems with the expression of cod trypsins I and Y have been largely overcome opens a new era for future studies and applications of these enzymes, in particular in the pharmaceutical field (Jónsdóttir et al., 2004; Pálsdóttir and Gudmundsdóttir, 2004).

Improvements by Site-Directed Mutagenesis

The thermal stability of proteins is determined by factors such as structural stability as well as stability or resistance to chemical degradation processes. In the case of proteases, autolysis also plays a major role in thermal denaturation (Abraham and Breuil, 1995). autolysis is known to be one of the regulatory mechanisms for the protease activity of serine proteases (Halfon and Craik, 1998). The autolysis loop in trypsins, composed of residues 144 to 154, is a relatively flexible structure considered to be a primary autolysis target (Smalås et al., 1994). The autolysis loop of the cold-adapted salmon trypsin has a different fold than that of its mesophilic analogues (Smalås et al., 1990). Atlantic cod trypsin I is cleaved by autolysis, predominantly at residue Lys154 in its autolysis loop (Helgadóttir, 2002). A Lysl54Gln mutation has already been introduced into the cod trypsin I cDNA sequence. Preliminary data demonstrate that this mutation has a stabilizing effect on the recombinant trypsin I molecule. Future experi-

ments will focus on further improvements of the recombinant cod trypsin I and trypsin Y by site-directed mutagenesis of their cDNAs with the aim of enhancing the stability of the recombinant enzymes against autolytic cleavage and thermal inactivation. Such derivatives may broaden the commercial applicability of the recombinant enzymes.

Our previous research involved increasing the thermal stability of a multifunctional proteolytic enzyme from Antarctic krill (*Euphausia superba*), termed euphaulysin, through point mutations of its cDNA sequence (Benjamin et al., 2001). Euphaulysin has a high sequence identity to a protease previously isolated from fiddler crab (*Uca pugilator*), termed crab collagenase I (Eisen et al., 1973). These 2 enzymes have been classified as brachyurins, a subclass of serine proteases, because of their broad substrate specificity and ability to cleave collagen (Gudmundsdóttir, 2002). Loop D, extending from residues 143 to 153 in crab collagenase I (Perona et al., 1997) and euphaulysin, is analogous to the autolysis loop of trypsins. A molecular model of euphaulysin, based on the known 3-dimensional structure of crab collagenase I, was used to guide the design of amino acid substitutions in the molecule that could increase the thermal stability of the enzyme by decreasing its susceptibility to autolysis (Benjamin et al., 2001). The model revealed that 2 residues of loop D, Lys143 and Phe149, might be easy targets for autolysis because euphaulysin has a high affinity toward Phe, Lys, and Arg residues (Kristjánsdóttir, 1999). The Phe149 residue appeared to be more exposed on the surface of the molecule than the Lys143 residue, and euphaulysin is the only type I brachyurin known to contain a Phe residue at position 149. This difference may in part be responsible for the decreased stability of the cold-adapted euphaulysin relative to crab collagenase I. Euphaulysin had previously been shown to have a low affinity for Asp and Glu residues (Kristjánsdóttir, 1999).

Thus 2-changes were incorporated into the cDNA encoding the precursor form of recombinant euphaulysin, resulting in Phe149Glu and Phe149Asp substitutions in the enzyme. The precursor forms of the 2 mutants were expressed in a *Pichia pastoris* expression system and fully activated by cod trypsin, as previously described (Kristjánsdóttir and Gudmundsdóttir, 2000) for the wild-type recombinant euphaulysin. The amounts of the mutated recombinant euphaulysin forms recovered in the expression experiments were approximately 2 times higher than that of the wild-type recombinant euphaulysin under the same growth conditions. This indicates that the Phe149Asp and Phe149Glu mutants are more stable against autolysis during expression than the wild-type form of the enzyme. The melting temperature, T_m, of the Phe149Glu mutant (48°C) was approximately 5°C higher than that of the wild-type recombinant euphaulysin (43°C). For comparison the T_m of the Phe149Asp mutant was around 2.5°C higher than that of the wild-type recombinant enzyme or the native enzyme.

Practical Applications

Fish Nutrition and Development. Starvation has been suggested to be a major cause of the high mortalities that occur during the larval period of marine fish and thus may affect recruitment (Theilacker, 1986). Trypsin is known to play a vital role in digestion and the survival of marine fish larvae. Theilacker and Porter (1995) reported that the larvae of walleye pollock lack energy reserves at first feeding. Therefore availability of nutritious prey and the presence of trypsin activity at this time are critical for larval survival. Studies involving larvae of other marine fish have found similar results (Theilacker, 1986). Trypsin activity in fish larvae is therefore an appropriate indicator of digestive capacity. It can be used as a biomarker to measure larval condition in response to changing environmental factors such as the quality and quantity of ingested food. Thus low or moderate trypsin activity indicates inadequate feeding or starvation conditions. Trypsin is also a useful indicator for the evaluation of digestive processes and the nutritional condition of field-caught marine larvae (Ueberschär, 1995; Hjelmeland et al., 1984, 1988).

It is therefore challenging to analyze the possible role of cod trypsins I and Y in the survival rate of farm-raised Atlantic cod larvae using the biochemical and molecular biology methods already developed for these enzymes. Measurement of trypsin activity is a useful tool for studying development and nutritional requirements in the early life of marine fish (Ueberschär et al., 1992; Nolting et al., 1999). Alternative or supplementary methods such as the real-time quantitative reverse transcriptase polymerase chain reaction technique (real-time qRT-PCR) can be used to measure the expression of trypsin genes under different environmental conditions such as a change in temperature or feeding activity (Bustin, 2004). The real-time qRT-PCR is a highly sensitive technique to measure, compare, and quantify mRNA levels among samples. Both methods are currently employed to monitor the response of trypsins I and Y in cod larvae to varying environmental conditions. The role of trypsin Y and the

Table 4. Examples of Clinical Studies in Humans and Animals on Therapeutic Use of Trypsin[a]

Clinical indication	*Application*	*Clinical studies*
Inflammation	Systemic	Humphries, 1971; Martin et al., 1955
Wound healing	Topical	Rodeheaver et al, 1974; Lecht and Stephenson, 1968
Burn	Topical/systemic	Stucke, 1949; RaviKumar et al., 2001
Postoperative complications	Systemic/topical	Krause, 1961
Arthritis	Systemic	Mazourov et al., 1997; Rovenska et al., 1999[b]
Repair between cartilage and bone	Implant pretreated with trypsin	Chen et al., 2000[b]

[a]Trypsin was used either by itself or in a trypsin-chymotrypsin combination.
[b]Clinical studies in animals.

other novel group III trypsins in the development of marine fish larvae is of particular interest. These trypsins are thought to be important for the viability of marine fish larvae, but no biochemical data are yet available to support this hypothesis (Roach, 2002).

Commercial Use. Consumers worldwide are increasingly questioning the safety of their foods. This appears to be reflected in an increased demand for foods and biologically active ingredients from marine sources, as pathogens originating from marine organisms are less likely to be transmitted to humans than those from livestock.

Food Industry. The high catalytic efficiency of Atlantic cod trypsin I is especially useful in the processing of fresh foods in which protein digestion at low temperatures is required (Bjarnason et al., 1993; Bjarnason and Benediktsson, 2001). Food processing at low temperatures minimizes undesirable chemical reactions as well as bacterial contamination, which may indeed be elevated at higher temperatures. When enzymatic activity needs to be controlled, the cold-adapted enzymes are easily inactivated by relatively low heat. Also, cold-adapted proteolytic enzymes are in most cases more economical as their high catalytic efficiencies facilitate the use of smaller amounts of enzymes than are required using analogous mesophilic enzymes.

Atlantic cod trypsin I has already proved its usefulness in a variety of industrial applications (Bjarnason et al., 1993; Bjarnason and Benediktsson, 2001). Presently, the natural form of trypsin derived from cod is being used for these applications. The industrial application of cod trypsin involves the production of an all-natural seafood flavorant from lobster, shrimp, crab, and other seafood. These products are already the international market. Other uses of cold-adapted proteolytic fish enzymes in the food and feed industries have been reviewed by Shahidi and Janak Kamil (2001).

Skin Care and Medical Applications. Proteolytic enzymes such as trypsin and chymotrypsin from the pancreas of cattle or pigs have been used as therapeutic agents in humans and animals for over 100 years (Morris, 1891). In general, the proteolytic enzymes have been used therapeutically in 4 areas: (1) as oral agents for some gastrointestinal disorders, (2) as local agents to debride or solubilize collections of proteinaceous materials that either foster or cause disease, (3) as anti-inflammatory agents, and (4) as thrombolytic agents in the treatment of thromboembolic disorders (Sherry and Fletcher, 1960). Mammalian trypsins and chymotrypsins have been used with good results for wound healing (Spittler and Parmenter, 1954) and as an anti-inflammatory agent (Martin et al., 1955) in veterinary medicine (Lecht and Stephenson, 1968) and human medicine (Leipner and Saller, 2000). Table 4 gives a few examples of clinical studies in humans and animals demonstrating the therapeutic use of trypsins.

The medical application of trypsin I and other cold-adapted serine proteases from the Atlantic cod includes the use of the native trypsin I for inflammation, fungal diseases, acne, wound healing, and other dermatologic indications (psoriasis, and eczema), as well as for general and dental hygiene (Bjarnason, 2000). Already a natural skin care product is being marketed in several countries in Europe, America, and Asia, and clinical studies are ongoing to verify the medical efficacy of cod trypsin.

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