3 Biotech (2018) 8:186
https://doi.org/10.1007/s13205-018-1208-0

REVIEW ARTICLE

Fish trypsins: potential applications in biomedicine and prospects for production

Kristal Jesús-de la Cruz[1] · Carlos Alfonso Álvarez-González[1] · Emyr Peña[1,3] · José Antonio Morales-Contreras[2] · Ángela Ávila-Fernández[2]

Received: 29 July 2017 / Accepted: 9 March 2018 / Published online: 16 March 2018

Abstract
In fishes, trypsins are adapted to different environmental conditions, and the biochemical and kinetic properties of a broad variety of native isoforms have been studied. Proteolytic enzymes remain in high demand in the detergent, food, and feed industries; however, our analysis of the literature showed that, in the last decade, some fish trypsins have been studied for the synthesis of industrial peptides and for specific biomedical uses as antipathogenic agents against viruses and bacteria, which have been recently patented. In addition, innovative strategies of trypsin administration have been studied to ensure that trypsins retain their properties until they exert their action. Biomedical uses require the production of high-quality enzymes. In this context, the production of recombinant trypsins is an alternative. For this purpose, *E. coli*-based systems have been tested for the production of fish trypsins; however, *P. pastoris*-based systems also seem to show great potential in the production of fish trypsins with higher production quality. On the other hand, there is a lack of information regarding the specific structures, biochemical and kinetic properties, and characteristics of trypsins produced using heterologous systems. This review describes the potential uses of fish trypsins in biomedicine and the enzymatic and structural properties of native and recombinant fish trypsins obtained to date, outlining some prospects for their study.

Keywords Fish trypsins · Recombinant enzymes · Aquaculture · Trypsin applications · Trypsin structure

Introduction

Potential applications of fish trypsins in biomedicine

Features of fish trypsins from different species and their potential applications in different kinds of industries have been widely reported. The applications of fish trypsins include the following: as ingredients in detergents (Espósito et al. 2009, 2010; Jellouli et al. 2009; Ktari et al. 2012; Nasri et al. 2012; Younes et al. 2015; Bkhairia et al. 2016), in the extraction of carotenoproteins from shrimp waste (Simpson et al. 1992; Klomklao et al. 2009a; Sila et al. 2012; Younes et al. 2015; Poonsin et al. 2017), in the production of protein hydrolysates as food ingredients (Kristinsson and Rasco 2000; He et al. 2013; Tavano 2013; Ketnawa et al. 2016; Villamil et al. 2017), and various applications in the food industry (Bougatef et al. 2007; Balti et al. 2009; Freitas et al. 2012).

The cold-adapted trypsin of Pacific cod (*Gadus macrocephalus*) has been proposed as an alternative to bovine trypsin in the synthesis of industrial peptides as its catalytic efficiency is 35 times greater than that of bovine trypsin (Fuchise et al. 2011). Both natural peptides and their synthetic analogues have interesting biological properties (stability, potency of action, biological specificity, etc.) and are useful in different fields as therapeutic agents, synthetic analogues of peptide hormones, and in pharmacological applications, among others (Korhonen and Pihlanto 2006).

✉ Ángela Ávila-Fernández
angela.avila@ujat.mx

[1] Laboratorio de Acuacultura, DACBiol-UJAT, Carr. Villahermosa-Cárdenas Km 0.5, 86139 Villahermosa, Tabasco, México

[2] Centro de Investigación, DACS-Universidad Juárez Autónoma de Tabasco, Av. Gregorio Méndez No. 2838-A. Col. Tamulté, 86150 Villahermosa, Tabasco, México

[3] Cátedra Consejo Nacional de Ciencia y Tecnología-UJAT, Villahermosa, Tabasco, México

A potential application for fish trypsins is as an antipathogenic agent. In in vitro assays, the trypsin of Atlantic cod (*Gadus morhua*) has shown a high antipathogenic efficacy against HSV-1 and respiratory syncytial virus (RSV), the two most prevalent pathogenic viruses in upper respiratory tract infections. In addition, in vivo assays have shown favorable results using Atlantic cod trypsin in formulations to heal wounds. In both cases, the high digestive capacity of the enzyme plays an important role in its effectiveness against pathogens. Furthermore, this enzyme was 3–12 times more effective in the degradation of large native proteins than bovine trypsin (Gudmundsdóttir et al. 2013). The trypsin from Atlantic cod has been proposed for the development of cosmetics and medicines and six patents related to its production and use for the prevention and treatment of diseases has been registered. Since these enzymes inactivate bacterial enterotoxins, inflammatory cytokines, and cell-surface receptors involved in cell adhesion, their therapeutic use as a topical agent against pain, acute and chronic inflammation, rheumatic and autoimmune diseases, allergies, microbial infections, dermatopathies, and dental plaque remover has been described in the patent WO2000078332A2 (Bjarnason 2000). In 2015, their use was extended as adjuvant in the prevention and treatment of microbial infections in people with an immunodeficiency and patented as a novel treatment (Clarsund and Blom 2015). In addition, novel trypsin isoforms called ZT have been described and patented. These isoforms preferentially hydrolyze Arg residues and are more efficient to cleave peptides that contain consecutive basic amino acid residues (Arg/Lys), which are frequently found in proteins involved in the pathogenicity of viruses, parasites and bacteria (Gudmundsdottir et al. 2017). Finally, a synergistic effect of Atlantic cod trypsin and conventional antibiotics for prevention, inhibition, and removal of bacterial biofilm in patients with recurrent and nosocomial infections has been described as a result of the ability of the trypsins to weaken the biofilm and allow the antibiotics to exert their function on bacteria (Gudmundsdottir and Scheving 2017; Gudmundsdottir et al. 2015).

Recently, innovative strategies of trypsin administration have been studied to ensure that trypsins retain their properties until they exert their action. To achieve this, trypsin was nano-encapsulated with chitosan and supplied to *Labeo rohita*, which led to an increase in the efficiencies of productive fish compared to the control fed with unencapsulated trypsin. A similar administration in humans could contribute to the treatment of some health disorders related to the deficiency of pancreatic enzymes, and in animals, this administration strategy could facilitate the digestion of feeds based on vegetable proteins (Kumari et al. 2013).

Sources and enzymatic properties of native fish trypsins

Fish trypsins are currently obtained from pyloric caeca (Kurtovic et al. 2006; Pálsdóttir and Gudmundsdóttir 2008; Khantaphant and Benjakul 2010; Freitas-Júnior et al. 2012), viscera (Klomklao et al. 2011; Sila et al. 2012) and intestines (Souza et al. 2007; Jellouli et al. 2009; Unajak et al. 2012). However, the process of obtaining the enzymes involves several constraints, such as (a) seasonal availability of raw materials (Simpson et al. 1992; Bougatef 2013); (b) heterogeneity in yield and/or enzymatic activity due to the nutritional status of the species and the degradation of tissues used as raw material (Simpson et al. 1992; Espósito et al. 2010); (c) production costs (Espósito et al. 2009; Bougatef 2013); and (d) low yields associated with the purification processes used. In addition, several steps are required for purification; for example, the use of affinity chromatography (Marcuschi et al. 2010; Fuchise et al. 2011), ion-exchange chromatography (Khangembam and Chakrabarti 2015; Bkhairia et al. 2016), and/or gel filtration (Bougatef et al. 2007; Silva et al. 2011; Costa et al. 2013) are usually employed; however, an optimal methodology has not been established and novel purification methods such as the use of ferromagnetic particles and the partitioning in aqueous two-phase system (ATPS) are being studied. The first one achieved about 60 times of purification from the crude extract, in a single step of purification (Menezes Estevam Alves et al. 2017; Poonsin et al. 2017).

The biochemical and kinetic properties of several native fish trypsins of different species have been reviewed by Bougatef (2013). In this context, the molecular weight of the enzymes reported was in the range of 22–30 kDa, while the optimum temperature was between 40 and 65 °C and the optimum pH was in the range of 8–11. The trypsins have esterase and amidase activity; in both cases, the mechanism involves the transfer of the acyl group to water. On their natural substrate, the trypsins catalyze the hydrolysis of peptide bonds at the carboxyl side of the lysine or arginine residues. Therefore, the most common substrates used for the kinetic characterization of fish trypsins are synthetic arginine substrates as *N*-α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) and *N*-α-tosyl-L-arginine methyl ester (TAME), which are used to measure amidase and esterase activity, respectively. However, some alternative substrates, such as, carbobenzoxy-Phe-Arg-7-amido-4-methylcoumarin (z-FR-MCA), casein, and azocasein have been used. The kinetic properties of the enzymes are analyzed in terms of Km, the affinity of the enzyme for the substrate; kcat, the number of substrate molecules that each enzyme converts per unit time; and kcat Km^{-1},

the rate of the reaction at negligible substrate concentration commonly named the catalytic efficiency. In the fish trypsins reviewed by Bougatef (2013), the Km on the BAPNA substrate varies in the range of 0.068–1.67 mM, while the catalytic efficiency (kcat Km^{-1}) varies in the range of 2.3–40.58 s^{-1} mM^{-1}. The highest affinity and catalytic efficiency were found for the trypsin from grey triggerfish (0.068 mM and 40.58 s^{-1} mM^{-1}, respectively) (Bougatef 2013).

In the last 12 years, some other native fish trypsins have been characterized and are summarized in Table 1. BAPNA and TAME remain the preferred substrates for the enzymatic characterization; however, alternative synthetic substrates, such as t-butyloxy-carbonyl-Phe-Ser-Arg-4-methyl-coumaryl-7-amide (Boc-Phe-Ser-Arg-MCA), *N*-benzoyl-L-arginine ethyl ester HCl (BAEE), and benzyloxycarbonyl-L-Phe-L-Arg-*p*-nitroanilide (CBZ-FR-pNA) and benzyloxycarbonyl-Gly-Pro-Arg *p*-nitroanilide acetate salt (CBZ-GPR-pNA), have been recently assayed (Liu et al. 2007; Lu et al. 2008; Cai et al. 2011; Stefansson et al. 2017). These fluorogenic substrates facilitate the quantification of enzymatic activity; however, it is unknown whether the features observed using these synthetic substrates resemble those that would be obtained using natural substrates.

The optimal temperature of trypsins from cold-zone fishes, such as Pacific cod (*G. macrocephalus*), saffron cod (*Eleginus gracilis*), walleye pollock *(Theragra chalcogramma*), and arabesque greenling (*Pleurogrammus azonus*), is, in general, around 50 °C, while the optimal temperature of temperate-zone fish trypsins, such as those obtained from jacopever (*Sebastes schlegelii*), spotted mackerel (*Scomber australasicus*), true sardine (*Sardinops melanostictus*), yellow tail (*Seriola quinqueradiata*), and Japanese anchovy (*Engraulis japonicus*), is slightly higher (60 °C). The optimal temperature of the temperate-zone trypsins is similar to those of tropical fish trypsins from Nile tilapia (*O. niloticus*), Atlantic bonito (*Sarda sarda*), skipjack tuna (*Katsuwonus pelamis*), bluefish (*Pomatomus saltatrix*), tongol tuna (*Thunnus tonggol*), and yellowfin tuna (*Thunnus albacores*), which have an optimal temperature of 55–65 °C (Kishimura et al. 2005, 2006b, c, 2007, Klomklao et al. 2006a, b, 2007a); some of these are reported in Table 1 and the others have been summarized by Bougatef (2013). Even though the optimal temperatures for cold-zone fish trypsins are high compared to the temperatures in which these organisms live (Kishimura et al. 2008), their thermal stabilities are lower and, in general, are less than 40 °C. Interestingly, the fish trypsin with the highest optimal temperature (80 °C) belongs to the Atlantic wolffish (*Anarhichas lupus*), a cold-zone fish, and its temperature stability drops to 60 °C (Desrosiers et al. 2008). Cold-adapted fishes have been found some trypsin isoforms that work at high temperature and are classified within the mesophilic group. Another example is the cationic salmon (*Salmo salar*) trypsin isoform (PDB: 1A0J) (Schrøder et al. 1998; Gorfe et al. 2000). On the other hand, the trypsin I proteins of the cold-zone fishes Atlantic salmon (*Salmo salar*) and Atlantic cod (*G. morhua*) have low thermal stability compared to bovine trypsin (Smalas et al. 1994; Outzen et al. 1996; Stefansson et al. 2010) and the skipjack tuna trypsins have lower stabilities than porcine trypsin. Studies have suggested the presence of stronger hydrophobic interactions that stabilize the structure and contribute to the increased stabilities of the enzymes (Klomklao et al. 2009b).

In general, fish trypsins have an optimal pH in the range of 7–11 and are stable at alkaline pH (7–9), but their stability decreases in acidic pH (less than 6) (Khandagale et al. 2017; Bougatef et al. 2010). However, some fish trypsins are stable over a wider pH range, such is the case of the Tunisian barbel (*Barbus callensis*) and striped seabream (*Lithognathus mormyrus*) trypsins that preserve above 90% of the enzymatic activity in a pH range of 5–12 (Sila et al. 2012; Elhadj-Ali et al. 2009); the zebra blenny trypsin (*Salaria basilisca*) that conserves 100% of the enzymatic activity in a pH range of 7.0–12.0; and the trypsins of pectoral rattail (*Coryphaenoides pectoralis*) and Bogue (*Boops boops*) that conserve above 70% of the enzymatic activity in a pH range of 6–11 (Ktari et al. 2012; Klomklao et al. 2009c; Barkia et al. 2010). In all of them, the stability test was carried out at 40 °C for 1 h. It has been proposed that the pH stability is related to the charge that the enzyme acquires at different pH values. In particular, the acidic conditions lead to a conformational change in the active site that cause an effect on substrate affinity, weakening the binding of the substrate to the active site, which is observed as low activity (Castillo-Yáñez et al. 2005; Bougatef et al. 2010; Klomklao et al. 2006b). This agrees with the biological activity of the enzyme, since trypsin is released in the small intestine, which has an alkaline environment, to carry out the hydrolysis of its specific substrates.

Some authors have mentioned that psychrophilic trypsins have higher catalytic efficiency than mesophilic ones, which could be due to the distribution of charged residues that favors a higher affinity towards the substrate (Smalas et al. 1994) or the structural flexibility that these enzymes have acquired as a result of the cold adaptation (Gudmundsdóttir et al. 2013). However, results are not consistent to this regard, as previously mentioned by Bougatef (2013). As shown in Table 2, the highest catalytic efficiency of fish trypsins on the BAPNA substrate was reported in 2012 for the carp *Catla catla* trypsin (310.16 s^{-1} mM^{-1}), which are not considered a psychrophilic trypsin. In contrast, the typical cold-adapted Atlantic cod trypsin X had a catalytic efficiency about nine times lower (3.5 s^{-1} mM^{-1}) on the same substrate (Stefansson et al. 2017). On the other hand, higher catalytic efficiencies have been reported for trypsins on esterase substrates. For example, the highest catalytic

Table 1 Properties and kinetic constants of native fish trypsins

Species	Molecular weight (kDa)	Optimum temperature (°C)	pH	Substrate assayed	Km (mM)	Kcat (s^{-1})	kcat Km^{-1} (s^{-1} mM^{-1})	References
Oil sardine (*Sardinella longiceps*)	24	60	8	BAPNA	0.0206	1.19	57.76	Khandagale et al. (2017)
Atlantic cod (*Gadus morhua*) trypsin X -variant 7	25	65	8.5–11	BAPNA	3.9	13.0	3.5	Stefansson et al. (2017)
				CBZ-FR-pNA	0.050	25.9	520	
				CBZ-GPR-pNA	0.008	64.9	8210	
Golden grey mullet (*Liza aurata*)	23	50	10	BAPNA	0.43	4.04	9.39	Bkhairia et al. (2016)
Lophiosilurus alexandri	24	50	9	BAPNA	0.517	5	9.67	Dos Santos et al. (2016)
Unicorn leatherjacket (*Aluterus monoceros*)	23.5	55	8	BAPNA	–	–	–	Zamani and Benjakul (2016)
Cirrhinus mrigala	21.7	30–40	7.6	BAPNA	0.0672	6.17	92.09	Khangembam and Chakrabarti (2015)
Thornback ray (*Raja clavata*)	–	50	8	CASEIN	–	–	–	Lassoued et al. (2015)
Scorpionfish (*Scorpaena scrofa*)	–	55	10	CASEIN	–	–	–	Younes et al. (2015)
Pacific saury (*Cololabis saira*)	24	55	8.5	TAME	0.19	210	1105	Klomklao et al. (2014)
Common kilka (*Clupeonella cultriventris caspia*)	23.2	60	8	BAPNA	0.10	7.93	79.3	Zamani et al. (2014)
	–	–	–	TAME	0.07	28.5	407.1	
Crevalle jack (*Caranx hippos*)	27.5	50	8	BAPNA	0.689	6.9	10	Costa et al. (2013)
Catshark (*Scyliorhinus canicula*)	28	55	8	BAPNA	0.104	0.23	2.21	Blanco et al. (2014)
Indian mackerel (*Rastrelliger kanagurta*)	26	50	9	BAPNA	0.430	0.77	1.78	Khandagale et al. (2013)
Sailfin Catfish (*Pterygoplichthys disjunctivus*)	27.5	40	9.5	BAPNA	0.13	1.46	11.24	Villalba-Villalba et al. (2013)
Nile Tilapia (*Oreochromis niloticus*) genetically improved	28	60	8	TAME	0.036	152	4242	Zhou et al. (2013)
Flatfish (*Paralichthys olivaceus*)	29.6	70	7.5	BAPNA	0.017	0.024	1.422	Kim and Jeong (2013)
Carp (*Catla catla*)	20.2	40	7	BAPNA	0.062	19.23	310.16	Khangembam et al. (2012)

Table 1 (continued)

Species	Molecular weight (kDa)	Optimum temperature (°C)	pH	Substrate assayed	Km (mM)	Kcat (s^{-1})	kcat Km^{-1} (s^{-1} mM^{-1})	References
Goby (*Zosterisessor ophiocephalus*)	23.2	60	9	BAPNA	0.312	2.03	6.51	Nasri et al. (2012)
Nile tilapia (*Oreochromis niloticus* L.)	22.39	55–60	9	BAPNA	0.16	23.8	238	Unajak et al. (2012)
Piracucu (*Arapaima gigas*)	28.0	65	9	BAPNA	0.47	1.33	2.82	Freitas-Júnior et al. (2012)
Tropical sierra (*Scomberomorus sierra*)	25.4	60	9	BAPNA	–	–	–	Valdez-Melchor et al. (2013)
Japanese sea bass (*Lateolabrax japonicus*)	21.0 (trypsin A) 21.5 (trypsin B)	40	9	Boc-Phe-Ser-Arg-MCA	1.12 0.70	72.08 67.79	64.16 92.55	Cai et al. (2011)
Silver mojarra (*Diapterus rhombeus*)	26.5	50–55	8.5	BAPNA	0.266	0.93	3.48	Silva et al. (2011)
Hybrid catfish (*Clarias macrocephalus/Clarias gariepinus*)	24	60	8	TAME	0.30	92.1	307	Klomklao et al. (2011)
Atlantic cod (*Gadus morhua*)	24.0 (trypsin I)	–	8	CBZ-Gly-Pro-Arg-pNA	0.007	53.1	7590	Stefansson et al. (2010)
Smooth hound (*Mustelus mustelus*)	24	50	8.5	BAPNA TAME	0.387 0.156	2.62 59.15	6.77 379.16	Bougatef et al. (2010)
Skipjack tuna (*Katsuwonus pelamis*)	24	55 (trypsin A) 60 (trypsin B)	9	TAME	0.22 0.31	82.5 69.5	266.13 315.91	Klomklao et al. (2009b)
Pacific cod (*Gadus macrocephalus*)	24	50	8	TAME	–	–	–	Fuchise et al. (2009)
Saffron cod (*Eleginus gracilis*)	24	50	8	TAME	–	–	–	Fuchise et al. (2009)
Sardinelle (*Sardinella aurita*)	24	55	8	BAPNA	1.67	3.87	2.31	Ben Khaled et al. (2008)
Mandarin fish (*Siniperca chuatsi*)	21.0 (trypsin A) 21.5 (trypsin B)	35 (A) 40 (B)	8.5	Boc-Phe-Ser-Arg-MCA	0.00218 0.00188	81.6 111.3	37431.19 59202.12	Lu et al. (2008)
Atlantic wolffish (*Anarhichas lupus*)	–	>80	9	BAPNA	0.55 0.56	–	–	Desrosiers et al. (2008)
Walleye pollock (*Theragra chalcogramma*)	24	50	8	TAME	–	–	–	Kishimura et al. (2008)
Atlantic bonito (*Sarda sarda*)	29	65	9	BAPNA	–	–	–	Klomklao et al. (2007b)
Skipjack tuna (*Katsuwonus pelamis*)	24	60	8.5	TAME	0.29 (trypsin A) 0.20 (trypsin B) 0.11 (trypsin C)	114 61.5 57.1	393.10 307.50 519.09	Klomklao et al. (2007c)

Table 1 (continued)

Species	Molecular weight (kDa)	Optimum temperature (°C)	pH	Substrate assayed	Km (mM)	Kcat (s^{-1})	kcat Km^{-1} (s^{-1} mM^{-1})	References
Grass carp (*Ctenopharyngodon idellus*)	30.7 (trypsin A) 26.4 (trypsin B)	38.5 44.0	8.0 8.5	BAEE	0.0212 0.0317	33.3 55.0	1572.32 1735.01	Liu et al. (2007)
Hoki fish (*Macruronus novaezelandiae*)	26	60	9	BAPNA TAME	0.06 2.08	0.33 19.00	5.50 9.13	Shi et al. (2007)
Spotted goatfish (*Pseudupeneus maculatus*)	24.5	55	9	BAPNA	1.82	–	–	Souza et al. (2007)
True sardine (*Sardinops melanostictus*)	24	60	8	TAME	–	–	–	Kishimura et al. (2006a)
Arabesque greenling (*Pleurogrammus azonus*)	24	50	8	TAME	–	–	–	Kishimura et al. (2006a)
Monterey sardine (*Sardinops Sagax Caerulea*)	25	50	8	BAPNA	0.051	2.12	41.0	Castillo-Yáñez et al. (2005)
Anchoa (*Engraulis japonicus*)	24.5 (trypsin II)	60	9.5	BAEE BAPNA	0.014 0.033	42.09 3.70	3006 112	Ahsan and Watabe (2001)
Anchoa (*Engraulis japonicus*)	24.5 (trypsin I)	60	9.5	BAEE BAPNA	0.031 0.037	34.56 1.94	1114 52	Ahsan and Watabe (2001)

Table 2 Crystallographic structures of fish trypsins

Enzyme	*Species*	X-ray resolution (Å)	PDB code	References
Trypsin isoform 1 Trypsin isoform 2 Trypsin isoform 3	Chum Salmon *(Oncorhynchus keta)*	1.9 1.75 1.55	2ZPQ 2ZPR 2ZPS	Toyota et al. (2009)
Trypsin	Chum Salmon *(Oncorhynchus keta)*	1.8	1MBQ	Toyota et al. (2002)
Trypsin I	Atlantic salmon (*Salmo salar*)	1.83 1.53 1.7 1.5	1UTJ 1UTK 1UTL 1UTM	Leiros et al. (2004)
Trypsin I	Atlantic salmon (*Salmo salar*)	1.0	1HJ8	Leiros et al. (2001)
Anionic salmon trypsin in complex with squash seed inhibitor (*Cucurbita* pepo trypsin inhibitor II) Anionic salmon trypsin in complex with squash seed inhibitor (*Cucurbita* maxima trypsin inhibitor I)	Atlantic salmon (*Salmo salar*)	1.8	2STB 2STA	Helland et al. (1999)
Anionic salmon trypsin in complex with bovine pancreatic trypsin inhibitor	Atlantic salmon (*Salmo salar*)	2.1	1BZX	Helland et al. (1998)
Trypsin	Atlantic salmon (*Salmo salar*)	1.7	1A0 J	Schrøder et al. (1998)
Trypsin	Atlantic salmon (*Salmo salar*)	1.83	1BIT	Berglund et al. (1995)
Trypsin	Atlantic salmon (*Salmo salar*)	1.8	2TBS	Smalas et al. (1994)
Trypsin-1	Atlantic cod (*Gadus morhua*)	1.85	2EEK	Unpublished

efficiency for the Atlantic cod trypsin X using CBZ-GPR-pNA as a substrate was 8210 s^{-1} mM^{-1} (Stefansson et al. 2017), while that for the trypsin of the genetically improved Nile tilapia (*Oreochromis niloticus*) was 4242 s^{-1} mM^{-1} using TAME as substrate (Zhou et al. 2013). The trypsins have higher esterase than amidase activity; therefore, assays on esterase substrates are more sensitive. However, unless the enzymes are characterized on a common substrate, it will be difficult to establish, which of the enzymes has the best characteristics and whether the kinetic properties of fish trypsins and their biochemical features are interrelated.

The inhibitors are widely used for the characterization and differentiation among the different kind of proteases. The phenyl-methyl-sulfonyl fluoride inhibitor (PMSF) identifies the serine proteases; however, it does not discriminate between trypsins, chymotrypsins, and elastases. For this reason, specific inhibitors such as tosyl-lysin-chloromethyl ketone (TLCK) and tryptic soy inhibitor (SBTI) are used for identifying trypsin activity (Klomklao et al. 2006b). On the other hand, metalloprotease inhibitors, such as EDTA, identify enzymes whose activity depends on some metallic ions. The effect of these inhibitors on native fish trypsins activity has been assayed at 25 °C for 30 min. The 5 mM PMSF achieved an inhibition in the range of 31–81%, whereas the specific inhibitor for trypsin, SBTI 1 mg ml^{-1}, inhibited in the range of 72–100% (Khandagale et al. 2017; Bkhairia et al. 2016; Lassoued et al. 2015; Blanco et al. 2014). On the other hand, the EDTA achieved an inhibition in the range of 5–63.75% (Bkhairia et al. 2016; Khangembam and Chakrabarti 2015); however, the reaction conditions such as temperature and time of incubation and concentration of EDTA in the assays involving fish trypsins are heterogeneous.

The effect of metal ions, including Ca^{2+}, has been studied in numerous fish trypsins. Some authors suggest that the calcium-binding site in the fish trypsins has a stabilizing effect on the thermal denaturation and autolysis, probably because the Ca^{2+} increases the intramolecular interactions, binds to the autolysis sites, and results in a compact structure that favors optimal catalytic activity (Kim et al. 1994; Klomklao et al. 2004; Khangembam and Chakrabarti 2015; Blanco et al. 2014; Kishimura et al. 2007). This stabilizing effect has been observed in the true sardine (*S. melanostictus*), greenling arabesque (*P. azonus*) (Kishimura et al. 2006a), golden grey mullet (*Liza aurata*) (Bkhairia et al. 2016), and scorpionfish trypsin (*Scorpaena scrofa*) (Younes et al. 2015).

As has been reported, after the trypsinogen is proteolyzed to constitute the active form of the enzyme, the N-terminal of fish trypsins begins with seven conserved amino acid residues (IVGGYEC) (Bougatef 2013). Recently, some hypothetical sequences of fish trypsins, which have, in the N-terminal region, the conserved IIGG sequence instead of IVGG, have been reported in the database. Such is the case of the anionic trypsin 2 of yellow croaker (*Larimichthys crocea*), the anionic trypsin 1 of the European seabass (*Dicentrarchus labrax*), the Trypsin-3-like of the dragonfish (*Scleropages formosus*), and the trypsin of spotted gar (*Lepisosteus oculatus*). Among the fish trypsins characterized, the Atlantic cod (*G. morhua*) recombinant trypsin is the only one with the IIGG sequence as N-terminal. The biochemical properties of this enzyme have been characterized; however, its kinetic properties are unknown (Palsdottir and Gudmundsdottir 2004). Interestingly, this enzyme has dual activity as trypsin and chymotrypsin. Therefore, it would be interesting to know if these characteristics are conserved in the enzymes that have the N-terminal region IIGG and if their kinetic properties are different from the other fish trypsins reported.

Structural features of fish trypsins

Most trypsins can be classified into three basic groups (I, II, and III) based on the identity in their amino acid sequences. The three groups share the catalytic triad of His, Asp, and Ser (Gudmundsdóttir and Pálsdóttir 2005). To date, psychrophilic fish trypsins have shown high catalytic efficiency and high thermal sensitivity (Genicot et al. 1988; Smalas et al. 1994; Fuchise et al. 2011). In this context, Genicot et al. (1996) constructed a three-dimensional model of the trypsin from the Antarctic fish *Paranotothenia magellanica* to explain the structural parameters involved in the cold adaptation of this enzyme by comparing their model to the crystallographic structure of bovine trypsin. Some differences such as an increased hydrophilic surface, a consequently lower hydrophobicity, a decreased number of salt bridges, and an absence of Tyr residues in the substrate-binding pocket, among others, probably affect both the features and the flexibility of this enzyme (Genicot et al. 1996).

In the last 20 years, the crystallographic structures of fish trypsins, their isoforms and the complexes that fish trypsins form with their inhibitors have been reported and compared with the well-characterized structure of bovine trypsin (PDB: 1HJ9) to understand their reaction mechanisms and their affinities for certain substrates and to propose the reasons for their cold adaptation (Smalas et al. 1994; Gerday et al. 2000; Schrøder Leiros et al. 2000). The crystallographic structures of fish trypsins reported to date are summarized in Table 2.

The first reported crystallographic structure of a fish trypsin was that of the native Atlantic salmon (*S. salar*) trypsin. This protein sequence has an identity of 65% with the bovine trypsin. This trypsin form (STIIA) comprised 222 residues, one less than bovine trypsin; the missing residue is a Tyr located in the autolysis loop, a region composed of approximately 11 residues, which is a relatively sensitive structure that is considered a primary autolysis target. The

six-disulfide bridges present in bovine trypsin are conserved in salmon trypsin; however, the distribution of charged residues is different. Electrostatic interactions are an important feature of the cold adaptation of the anionic form of salmon trypsin (Smalas et al. 1994). Other studies of trypsins from organisms adapted to a wide range of environments have revealed some general patterns of variations in sequence and structure that enable the enzymes to perform similar catalytic functions under different environmental conditions (Leiros et al. 2001; Toyota et al. 2002). Leiros et al. (2001) obtained two trypsin structures of the anionic form of salmon and assessed the structural damage caused by radiation during X-ray crystallography. Both trypsin structures had broken disulfide bonds, demonstrating the particular sensitivity of some trypsins to the high intensity of X-rays. Hence, experimental conditions that minimize the damage to the protein crystal are necessary. When the crystal structure of chum salmon trypsin (CST) was studied, the primary structure was found to differ by only seven residues from the Atlantic salmon anionic trypsin (AST). The most notable difference was the absence of the last two residues in the C-terminus of the CST; this probably increases the flexibility of the enzyme due to the loss of important interactions between the N- and C-terminal domains (Leiros et al. 2001).

Similarly, compared to bovine trypsin, the absence of a Tyr residue in the autolysis loop probably increases the accessibility of the substrate to the binding pocket, while an Lys residue contributes to stabilize the loop. The comparison of CST, AST, and bovine trypsin structures has contributed to the understanding of the structural basis for differences in enzymatic activity between the enzymes of cold-adapted fish species and mammals. The reasons for cold adaptation could be diverse. For example, high molecular flexibility could be a strategy to increase the specificity of the enzyme at low temperatures (Gerday et al. 2000; Schrøder Leiros et al. 2000; Toyota et al. 2002).

Features of recombinant fish trypsins

Although fish trypsins can be obtained from natural sources, it is necessary to explore alternative sources to reduce production costs and due to the limitations mentioned above, particularly for the use of these enzymes in the food industry, in cosmetics and in biomedicine (Espósito et al. 2009; Bougatef 2013; Fornbacke and Clarsund 2013; Gudmundsdóttir et al. 2013). The production of recombinant trypsins is an alternative that offers some advantages: (a) this method is independent of the seasonality of the raw materials, (b) recombinant trypsins can be obtained using a controlled system that can be optimized to increase production yields, and (c) some purification strategies specifically designed for recombinant protein production systems can be used. To date, there has been little in-depth research on the feasibility of producing fish trypsins in heterologous systems. Some recombinant fish trypsins have been obtained using protein expression systems based on *Escherichia coli* and *Pichia pastoris* and are summarized in Table 3. The precursor form of Atlantic cod trypsin I was produced in soluble form as a thioredoxin fusion protein linked to the trypsin zymogen in the His-Patch ThioFusion *E. coli* expression system, and the active recombinant trypsin was obtained by cleavage of the purified fusion protein using a small amount of native trypsin I. The active enzyme was obtained at a low yield, probably due to poor folding of the enzyme or due to problems with autolysis during the excision of thioredoxin and the precursor form of recombinant trypsin I. However, the amount of enzyme obtained was enough to characterize the enzyme (Jónsdóttir et al. 2004). On the other hand, trypsinogen from *E. japonicus* was initially produced using the pETBlue-1 vector in the *E. coli* strain Tuner (DE3) pLacI. Preliminary assays showed a very low expression level in this strain even after optimization of the experimental conditions; however, when the plasmid was expressed in a different strain [Rosetta (DE3) pLacI], an increase was observed in the amount of enzyme obtained (Ahsan et al. 2005). Another fish trypsin that was successfully produced in *E. coli* was the trypsin Y of *G. morhua* (Pálsdóttir and Gudmundsdóttir 2007). This enzyme has been previously produced in the yeast *P. pastoris* system with satisfactory results (Palsdottir and Gudmundsdottir 2004); however, this system could not be used to produce Trypsin I of Atlantic cod due to unsuccessful enzyme purification and activation (Jónsdóttir et al. 2004). On the other hand, the trypsin of cunner (*Tautogolabrus adspersus*) produced in *P. pastoris* was active, although a low yield was obtained. The molecular weight (45 kDa) of the enzyme was almost double that obtained for recombinant fish trypsins produced in *E. coli* (≈ 25 kDa), suggesting that the enzyme was glycosylated when produced in *P. pastoris*; however, the effect of glycosylation on enzyme activity is unknown (Macouzet et al. 2005).

The production of cold-adapted trypsins I and Y of Atlantic cod as soluble proteins in the *E. coli* expression system was made possible by the His-Patch ThioFusion system. In addition, heterologous expression systems require mild environmental conditions for trypsin production; compared to *P. pastoris,* the *E. coli* expression system requires a shorter growing time; induction times of 10–12 h are used in *E. coli*; and 3–5 days are required in *P. pastoris*. Heterologous expression systems also facilitate enzyme detection by western blotting using polyclonal or monoclonal antibodies directed against thioredoxin, His-tag or any other fusion protein, or by simple polyacrylamide gel electrophoresis (SDS-PAGE) when a clear overexpression is achieved (Jónsdóttir et al. 2004; Palsdottir and Gudmundsdottir 2004; Gudmundsdóttir and Pálsdóttir 2005; Macouzet et al. 2005; Pálsdóttir and Gudmundsdóttir 2007). Some problems in

Table 3 Recombinant fish trypsins obtained in different expression systems

Species	Enzyme	Expression vector	Expression system	Yield	Molecular weight (kDa)	Substrates assayed	References
Medaka *(Oryzias latipes)*	Trypsin	pET30a (Novagen, Madison, WI)	*E. coli*	–	38 Trypsinogen fusion protein 24	Boc-Gln-Ala Arg-MCA	Rajapakse et al. (2014)
Engraulis japonicus (Anchoa)	Trypsin	pETBlue1	*E. coli* Rosetta (DE3) pLacI Tuner (DE3) pLacI	12 mg L^{-1}	24	BAPNA	Ahsan et al. (2005)
Tautoga americana/ cunner-fish *(Tautogolabrus adspersus)*	Trypsin	pPICZ y pPICZα	*Pichia pastoris* Cepa X-33 *(mut$^+$his$^+$)*	Low (NE)	45 (glycosylation) 35.4 (rCFT fused to the myc epitope)	BAPNA	Macouzet et al. (2005)
Atlantic cod *(Gadus morhua)*	Trypsin Y	pThioHis A	*E. coli* TOP10	0.15 mg L^{-1} (Mono Q)	40 (HP-thioredoxin-trypsin Y) 25	N-CBZ-Gly-Pro-Arg-pNA Suc-Ala-Ala-Pro-Phe-pNA	Pálsdóttir and Gudmundsdóttir (2007)
Atlantic cod *(Gadus morhua)*	Trypsin Y	pPiczα A	*Pichia pastoris* Cepa X-33 *(mut$^+$his+)*	300 mg L^{-1} (Fermentor)	30 (r-trypsin Y-HisMyc polypeptide) 27	Suc-Ala-Ala-Pro-Arg-p-nitroanilide N-CBZ-Gly-Pro-Arg-pNA Suc-Ala-Ala-Pro-Phe-pNA	Palsdottir and Gudmundsdottir (2004)
Atlantic cod *(Gadus morhua)*	Trypsin I (cold-adapted)	pThioHisA	*E. coli* Top 10	0.038 mg L^{-1} (Mono Q) 300 mg L^{-1} (Fermentor)	39 (HP-thioredoxin-trypsin I) 24 (Native trypsin I)	N-CBZ-Gly-Pro-Arg-pNA	(Jónsdóttir et al. 2004)

the expression, activation, and purification of these types of proteases have been observed, including autolysis, heat and low pH inactivation, and the formation of inclusion bodies. This may be because cold-adapted proteases are highly sensitive to temperature, even in a range of 18–25 °C (Atlantic cod Trypsin I), compared to their mesophilic counterparts (Jónsdóttir et al. 2004; Ahsan et al. 2005; Stefansson et al. 2010; Gudmundsdóttir et al. 2013). In addition, the Atlantic cod trypsin produced in *P. pastoris* lost all activity at 30 °C, and its optimum temperature of expression was 20 °C (Palsdottir and Gudmundsdottir 2004). This could explain why the recombinant cold-adapted trypsin from *T. adspersus*, produced at 30 °C in the same system, also had a low yield and activity (Macouzet et al. 2005). It is worth mentioning that the stability and catalytic properties of the Atlantic cod trypsin were improved by producing the enzyme as a recombinant protein and the production strategy and uses were patented (Clarsund et al. 2015).

The purification of the recombinant trypsins reported, as well as that of native proteins, was performed by different chromatographic methods. Affinity chromatography using a *p*-aminobenzamidine column followed by ion-exchange chromatography was used to purify the recombinant trypsin I of Atlantic cod (*G. morhua*) (Jónsdóttir et al. 2004). Partial purification of recombinant trypsin from *T. adspersus* was performed using metal-ion affinity chromatography (IMAC) (Macouzet et al. 2005). Recombinant trypsin Y from *G. morhua* was purified by ion-exchange chromatography followed by affinity chromatography (Palsdottir and Gudmundsdottir 2004; Pálsdóttir and Gudmundsdóttir 2007), and *E. japonicus* trypsin was purified only by ion-exchange chromatography (Ahsan et al. 2005). Enzyme yields were variable (Table 3), showing a remarkable difference between the trypsins produced in *E. coli* (0.15 mg L^{-1}) systems and those produced in *P. pastoris* (300 mg L^{-1}) systems. In the last case, a bioreactor was used, which led to better expression.

The molecular weight range of the recombinant fish trypsins characterized is 24–45 kDa, as shown in Table 3. The substrates used for the characterizations have been mainly synthetic and include the following: the fluorogenic substrate (Boc)-Gln-Ala-Arg-MCA and chromogenic substrates N-CBZ-Gly-Pro-Arg-pNa and succinyl-Ala-Ala-Pro-Arg-p-nitroanilide to measure trypsin activity and the

synthetic substrate succinyl-Ala-Pro-Phe-p-nitroanilide to quantify chymotrypsin activity. A disadvantage of these small synthetic substrates for the characterization of protease activity is that they do not represent natural proteins at all. Therefore, it is difficult to establish a physiological conclusion from the data, because, even though the proteases can cleave these small synthetic substrates easily, the access to the active site for native proteins could be blocked (Hortin et al. 2001).

The biochemical and kinetic properties of the recombinant trypsin from *E. japonicus* produced in *E. coli* were similar to those determined for the native enzyme. The catalytic efficiency of this recombinant enzyme was 58 s^{-1} mM^{-1}, which is slightly lower than that of the native enzyme (112 s^{-1} mM^{-1}), and the affinities for the substrate (BAPNA) of the recombinant and native enzymes were 0.045 and 0.033 mM, respectively. The substrate specificity and cleavage site remained unchanged in the recombinant enzyme compared to the native enzyme, and no change attributable to the refolding protocol was observed (Ahsan et al. 2005). Another recombinant fish trypsin partially characterized is the one obtained from Medaka (*Oryzias latipes*) and produced in *E. coli*. The Km and catalytic efficiency of this recombinant enzyme were 0.10 mM and 7.15 s^{-1} mM^{-1}, respectively, using Boc-Gln-Ala Arg-MCA as substrate (Rajapakse et al. 2014). It is worth mentioning that a complete biochemical and kinetic characterization has not been performed on most of the recombinant fish trypsins, making their comparison with native trypsins difficult. In some cases, thermal stability was assayed at different temperatures, but the half-life was not reported, making it difficult to compare them and draw conclusions. The thermal instability of cold-adapted trypsins represents a drawback for their practical use. As a result, efforts have now been made to immobilize the enzyme to improve its stability (Fuchise et al. 2011). A complete characterization of recombinant fish trypsins is required to determine their potential use in specific industrial and biomedical applications (Palsdottir and Gudmundsdottir 2004; Macouzet et al. 2005). For example, it is unknown if recombinant fish trypsins are salt tolerant, which is desirable for their use in sauce formulations. This characteristic has been observed in the native trypsin of skipjack tuna and Atlantic wolffish (Desrosiers et al. 2008; Klomklao et al. 2009b).

Conclusions

The use of heterologous protein expression systems is a promising alternative for the production of high-quality fish trypsins and to minimize the main drawbacks in their production. In addition, these methods could facilitate the development of structure–function studies aimed at increasing their thermal stabilities and improving the properties of these enzymes, particularly for trypsins from cold-adapted species, which have a naturally low thermostability compared to their mesophilic analogues.

Escherichia coli has been the expression system most commonly tested for the production of fish trypsins due to the advantages of this system, such as ease of manipulation, rapid growth, low cost, and short production times (Ahsan et al. 2005). However, in *E. coli*, the expression of proteins requiring disulfide bonds usually leads to the precipitation of these proteins as insoluble aggregates called "inclusion bodies", from which the active proteins can be obtained only by solubilization in denaturing agents followed by careful refolding of the denatured proteins (Fischer et al. 1993; Clark 2001), which is an expensive and time-consuming process. The addition of thioredoxin as a fusion protein using specific expression vectors has contributed to obtain soluble and active fish trypsins; however, the yields using this method have been low.

The yeast (*P. pastoris)* system seems to have great potential in the generation of fish trypsins at higher yields. This system offers a suitable environment for the folding of eukaryotic proteins, since it develops some post-translational modifications, such as disulfide bond formation, thereby avoiding the expensive solubilization and renaturation processes required to obtain soluble and active proteins from inclusion bodies. In addition, this system can secrete the recombinant proteins into the culture medium efficiently, and due to the low secretion of endogenous proteins, the subsequent purification of the recombinant proteins is facilitated; this is particularly desirable for biomedical applications (Fornbacke and Clarsund 2013; Gudmundsdóttir et al. 2013).

It is necessary to know if the structural features and biochemical properties of native fish trypsins are preserved in the recombinant proteins produced in both the *E. coli* and *P. pastoris* systems. To date, the crystallographic structures of recombinant fish trypsins have not been determined. There is a lack of information comparing the biochemical and kinetic properties of native and recombinant fish trypsins. It is not known if *E. coli* strains with oxidizing cytoplasmic environments can produce fish trypsins more efficiently. In addition, the conditions for the production and purification of fish trypsins, in either case, require optimization.

Acknowledgements This work was supported by the CONACYT Grant 173065 and institutional project UJAT-2016-PFI-DACS-07. The authors thank José Natividad Arias Jiménez for support during the preparation of the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest in the publication.

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