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Physicochemical and biochemical properties of intestinal trypsin from sturgeon fish

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Abstract

This work aimed to determine the physicochemical and biochemical characterization of the intestinal trypsin from beluga (*Huso huso*) and sevruga (*Acipenser stellatus*), two highly valuable sturgeon species, by a series of assays. According to the results obtained from casein-zymogram and inhibitory activity staining method, indicating the existence of trypsin in the intestinal crude extract of both species, molecular weight of the enzyme was estimated to be 27.5 and 29.5 kDa in sevruga and beluga, respectively. Optimum pH and temperature of both trypsins were recorded at 8.5 and 55°C by BAPNA (a specific substrate), respectively. The stability of both trypsins was well preserved at pH from 6.0 to11.0 and temperatures of up to 50°C. TLCK and SBTI, two specific trypsin inhibitors, showed a significant inhibitory effect on the enzymatic activity of both trypsins ($P < 0.05$). The enzyme activity was significantly increased in the presence of Ca^{+2} and surfactants and decreased by oxidizing agents, Cu^{+2}, Zn^{+2} and Co^{+2} ($P < 0.05$). However, univalent ions $Na^{+}$and K^{+} did not show any significant effect on the activity of both trypsins ($P > 0.05$). Therefore, the results of our study can contribute to the clear understanding of the intestinal trypsin activity in beluga and sevruga under evaluated experimental conditions.

Introduction

The beluga (*Huso huso*) and the sevruga (*Acipenser stellatus*) are of the most important species of sturgeon fish (Acipenseridae) inhabited in the Caspian Sea in the north of Iran with a high demand for products such as caviar, meat, skin, and cartilage (Ghomi et al. 2012; Asgari et al. 2013; Hashemi et al. 2018). Today, the sturgeons are considered as the vulnerable fish species for different reasons like overfishing for production of meat and caviar, water pollution and destruction of their natural habitat (Babaei et al. 2011; Ghasemi et al. 2020). Therefore, researchers have focused their studies on restocking and commercial rearing of sturgeon in recent years. According to the Iranian Fisheries Organization report (2022), the aquaculture production of sturgeon has risen from 363 t in 2009 to 2516 t in 2020. A very important aspect in sturgeon farming industry, affecting its production efficiency and long-term sustainability, is the development of the formulated diets with sufficient nutrient content. However, the ability of fish for digestion of formulated diets containing the different ingredients is mostly related to the existence of the digestive enzymes in different parts of the gastrointestinal tract (Furne et al. 2008). Digestive enzymes reflect the capability of digestion in the organism under study and can thus indicate the nutritional status at different stages of growth as explained by Kolkovski (2001) and Yufera and Darias (2007). Furthermore, the analysis of digestive enzymes activity is regarded as a simple and dependable biochemical procedure which can contribute to generate the valuable information for cognition of the physiology of digestion in fish (Twining et al. 1983). This important issue can also help to define the requirements of fish for essential nutrients such as proteins, lipids or carbohydrates (Bolasina et al. 2006). Digestion of protein in utilized food, recognizing as a key factor for fish growth, can be linked to the activity of proteolytic enzymes in the gastrointestinal tract and understanding of

properties and function of these enzymes (Glass et al. 1989; Eshel et al. 1993; Rungruangsak-Torrissen et al. 2006).

In fish viscera, especially pyloric caeca and intestine, one of the main serine endoproteinases is trypsin (EC 3.4.21.4) which catalyzes the hydrolysis of peptide bands of protein chain mainly at the C-terminal side of the amino acids lysine and arginine (Bernhard 1968). According to surveys conducted in various species of fish, trypsin indicating a wide distribution in fish intestine, plays an effective role in protein degradation of the consumed diet in the carnivorous fish up to 40–50% and participates in activating trypsinogen and other zymogens (Eshel et al. 1993; Krogdahl et al. 1994; Haard et al. 1996; Chong et al. 2002; Hedstrom 2002). Hence, a better understanding of the properties of trypsin is necessary to generate valuable information for protein degradation in the fish digestive tract. The characterization of trypsin, especially its physicochemical and biochemical properties, has been thoroughly studied from the intestine of various fish including grass carp, spotted goatfish, grey triggerfish, skipjack tuna, smooth hound and Brazilian flounder (Liu et al. 2007; Souza et al. 2007; Jellouli et al. 2009; Klomklao et al. 2009; Bougatef et al. 2010; Candiotto et al. 2017).

The activity of this enzyme among the various species of sturgeon has been mostly studied during larval ontogeny in the members of the genus *Acipenser* such as *A.transmontanus* (Buddington and Doroshov 1984), *A.fulvescens* (Buddington 1985), *A.oxyrinchus* (Dabrowski et al. 1985; Ostaszewska et al. 2011), A.*baerii* (Bardi et al. 1998), *A.medirostris* (Gisbert and Doroshov 2006), *A.persicus* (Babaei et al. 2011), A.*nacarii* (Camacho et al. 2011; Sanz et al. 2011), A.*stellatus* (Ghasemi et al. 2020) and genus *Huso* like *H.huso* (Asgari et al. 2013). Since the physicochemical and biochemical characteristics of trypsin from intestine of the beluga and the sevruga still remain unknown, this study attempts to characterize trypsin from intestine of the beluga and the sevruga and to provide the basic information about its physicochemical and biochemical properties.

Materials And Methods

Reagents

EDTA (Ethylenediaminetetraacetic acid), Pepstatin A, PMSF (phenylmethanesulfonyl fluoride) and sodium cholate were obtained from Molekula Co (Gillingham, U.K). BAPNA (Nα-benzoyl-DL-arginine-ρ-nitroanilide hydrochloride), ß-mercaptoethanol, BSA (bovine serum albumin), iodoacetic acid, saponin, SBTI (soybean trypsin inhibitor), TLCK (N-ρ-tosyl-L-lysine-chloromethyleketone) and TPCK (N-tosyl-L-phenylalanine chloromethyleketone) were purchased from Sigma Chemical Co (St. Louis, MO, USA). Molecular weight marker (PM 2700) was obtained from SMOBIO Technology, Inc (Hsinchu, Taiwan).

Fish samples

Viscera from farmed beluga and sevruga weighting approximately 8.0 kg and around 95 cm in length were obtained from a sturgeon rearing center in Mazandaran province, Iran. The polyethylene bags were used for packing those samples and carried on ice at a ratio of 1:2 (sample/ice) directly to the

laboratory. Upon arrival, intestine from the rest of the viscera in each species was separated, washed with cold distilled water (4°C) and stored at -80°C for further analysis.

Preparation of intestinal crude extract

The frozen intestine of beluga and sevruga was thawed at the refrigerator (4°C) for 2h. The samples were then cut into small pieces and defatted in cold acetone in the ratio of 1:3 (tissue: cold acetone) for 30 s by a tissue homogenizer (Heidolph Diax 900, Sigma Co. St. Louis, MO, US). The homogenate was then filtered with a Whatman filter paper (No.2) and the residue was dried overnight at room temperature as described by Zamani et al. (2014). For preparing the crude extract from intestine of each species, the dried samples were milled by a hand pounder and separately suspended in 50 volumes of 50 mM Tris–HCl buffer (pH 7.5, 10 mM $CaCl_2$, 0.5 M NaCl). After stirring the suspension for 3h at 4°C, the centrifugation was performed for 45 min at 4°C at 14000 × *g* by a refrigerated centrifuge (B-22M, Louisiana, USA). The resulting supernatant from each sample was collected, defined as intestinal crude extract (ICE) and then used throughout this study.

Trypsin assay

To measure the enzyme activity in ICE, BAPNA (as the substrate) was used at a concentration of 1 mM after preparing in 50mM Tris–HCl, 20 mM $CaCl_2$ (pH 8.5) according to Erlanger et al. (1961) method. Each ICE (25µL) was mixed with the prepared substrate (1250 µL) and incubated at 55°C for 20 min. The reaction was terminated by adding 30% (v/v) acetic acid (250 µL) to the mixture and followed by measuring the trypsin activity at absorbance of 410 nm using spectrophotometer (UV-1601, Shimadzu, Kyoto, Japan). One unit of activity was defined as 1 µmol of ρ-nitroaniline released per min and calculated with the following equation (Zamani et al. 2014):

$$\text{Trypsin activity (Unit/mL)} = \frac{\text{Absorbance 410 nm} \times 1000 \times \text{mixture volume (mL)}}{8800 \times \text{reaction time (min)} \times 0.025}$$

where 8800 (cm^{-1} M^{-1}) is the molar extinction coefficient of ρ-nitroaniline at 410 nm.

Protein assay

The concentration of protein in both ICEs was determined at 750 nm by using BSA (1mg / ml as the standard) and Folin-Ciocalteau reagent according to Lowry et al. (1951) method.

Characterization of trypsin by electrophoresis

SDS-PAGE electrophoresis was performed for determination of the protein pattern in both ICEs (Laemmli 1970). Each ICE was mixed at 2:1 (v/v) ratio with sample buffer (62.5 mM Tris–HCl pH 6.8, 2% SDS (w/v), 10% (v/v) glycerol, 0.3% (w/v) bromophenol blue and 5% (v/v) ß-mercaptoethanol) and boiled for

10 min. Thereafter, the ICEs (with protein concentration of 15 µg) were loaded on the gel made of 4% stacking gel and 12% separating gel and the electrophoresis was run at a constant current of 15 mA using a vertical electrophoresis system (Bio-Rad Laboratories, Inc.). After the run, protein bands present in the gel were stained with 0.1% Coomassie Brilliant Blue (G-250) in methanol (35%) and acetic acid (7.5%) and destained in methanol (35%) and acetic acid (7.5%).

Casein-zymography was performed after electrophoresis for detection of proteases in both ICEs by the method of Garcia-Carreno et al. (1993) as displayed in Fig. 1b. The both ICEs were submitted to native-PAGE electrophoresis in a same manner of SDS-PAGE expect that the samples were not boiled and SDS and reducing agent were removed. After the run, the gel was immersed in 50 ml of a casein solution (20 mg / ml in 50 mM Tris–HCl, pH 7.5) for 1h at 4°C with gentle agitation to allow diffusion of the casein into the gel. Thereafter, the gel was transferred to the another solution (50 ml) containing casein (20mg / ml in 50mM Tris–HCl, pH 8.5,10mM $CaCl_2$) for 20 min at 55°C with continuous agitation. The gel was then stained with 0.1% Coomassie Brilliant Blue (R-250) in methanol (35%) and acetic acid (7.5%) and destained in methanol (35%) and acetic acid (7.5%). The presence of proteolytic activities in both ICEs was indicated by appearing the clear zones on the blue background of the gel.

To reveal the trypsin present in both ICEs, the inhibitory activity staining was used after the submission of both ICEs to native-PAGE electrophoresis as described by Ahmad and Benjakul (2011) with a slight modification. After the run, the gel was immersed in 30 ml of a SBTI solution (1mg / ml in 50 mM Tris–HCl, pH 8.5, 10 mM $CaCl_2$) for 30 min at 4°C to allow diffusion of SBTI into the gel. Thereafter, the incubation of gel was performed for 40 min at 55°C and followed by washing in cold distilled water and staining with 0.05% Coomassie Brilliant Blue (R-250) to appear inhibitory zones, indicating the presence of the trypsin in both ICEs. The molecular weight of the trypsin appeared in both ICEs was estimated using wide range molecular weight markers (PM2700, SMOBIO, Hsinchu, Taiwan) by calculating the trypsin Rf in comparison with those of protein markers.

Optimum temperature and thermostability

To determine the optimum temperature, the trypsin activity of both ICEs was measured at different temperatures including 10, 25, 35, 45, 50, 55, 60, 65 and 70°C after 20 min of incubation at pH 8.5 by 1mM BAPNA as a substrate. For thermostability test, both ICEs were incubated at the above mentioned temperatures for 30min and then cooled in an ice bath for assay of residual activity of the enzyme at pH 8.5 as described by Zamani et al. (2014).

Optimum pH and stability

Different buffers in the pH range of 4.0–11.0 (50 mM acetic acid-sodium acetate for pHs 4-6; 50 mM Tris-HCl for pHs 7-9 and 50 mM glycine-NaOH for pHs 10-11) were used for determining the optimum pH of the trypsin activity from both ICEs by 1mM BAPNA as a substrate after 20 min of incubation at 55°C. For pH stability test, the remaining activity of the trypsin from each ICE was measured using 1mM BAPNA as a substrate at 55°C after being incubated at the above mentioned pHs for 30 min (Zamani et al. 2014).

Effect of inhibitors

The various protease inhibitors (0.01 mM pepstatin A, 0.05mM SBTI, 1mM iodoacetic acid, 2mM EDTA, 5mM TLCK, 5mM TPCK, 5mM ß-mercaptoethanol and 10mM PMSF) were prepared in the relevant solvents and incubated with an equal volume of each ICE at room temperature for 15min. The remaining activity of the enzyme was then measured by 1 mM BAPNA as a substrate (at 55°C, pH 8.5) and the percent inhibition was calculated according to the method of Khantaphant and Benjakul (2010). The trypsin activity of control was measured in the same manner without the presence of inhibitors and scored as 100%.

Effect of metal ions

To investigate the effect of metal ions (5mM) on the trypsin activity of both ICEs, univalent (K^{+}, Na^{+}) and divalent (Ca^{+2}, Cu^{+2}, Zn^{+2} and Co^{+2}) cations were dissolved in 50mM Tris–HCl (pH 8.5) and then incubated with an equal volume of each ICE for 30 min at room temperature. The residual activity of the enzyme was determined using 1 mM BAPNA as a substrate at 55°C and pH 8.5 (Zamani et al. 2014). The enzymatic activity of control was assayed without the presence of metal ions and taken as 100%.

Effect of oxidizing agents and surfactants

The effect of surfactants (ionic: saponin and sodium choleate; non-ionic: SDS and Triton X-100, all at 1%) and oxidizing agents (sodium perborate at a concentration of 1% and H_2O_2 at three concentrations of 5%, 10%, and 15%) on the trypsin activity was measured by incubation of the above mentioned surfactants and oxidizing agents with an equal volume of each ICE for 1h at 40°C. The residual activity of the enzyme was then determined at 55°C and pH 8.5 using 1mM BAPNA as a substrate. The assessment of control enzymatic activity was conducted in the similar condition in the absence of chemicals and scored as 100% (Jellouli et al. 2009).

Statistical analysis

This study was conducted on the basis of a completely randomized design in triplicate and a one-way ANOVA was used for data analysis using SPSS package 22.0 (SPSS Inc. Chicago, IL, USA). Data was expressed as the means ± SD and the comparison of means was carried out by Duncan's multiple range tests with a statistical significance at $P< 0.05$.

Results And Discussion

Protein pattern, casein-zymography and inhibitory activity staining of ICEs

Protein pattern of ICE from beluga and sevruga is depicted in Fig. 1a. Based on results obtained from SDS-PAGE, a number of proteins with different molecular weights were shown in ICE of both species. The major bands of each ICE appeared between molecular weights of 10 and 60 kDa.

The protease activity of both ICEs was demonstrated by casein-zymography as illustrated in Fig. 1b. The clear bands, showing the presence of protease, appeared on the gel with different molecular weights. Based on zymogram pattern, proteases present in ICE from beluga were observed in the range of molecular weight of 19-35 kDa while those in ICE of sevruga were ranged from molecular weight of 19 to 45 kDa. Casein-zymogram can be used as a highly sensitive and fast assay method for detecting nanograms of protein.

Inhibitory activity staining for detection of the trypsin in ICEs is depicted in Fig. 1c. The results showed that a single band for each ICE clearly appeared on the gel with a molecular weight of 27.5 and 29.5 kDa for sevruga and beluga, respectively. Different molecular weight for trypsins was reported in various fish such as 21.7 kDa for mrigal carp (Khangembam and Chakrabarti 2015), 23.2 kDa for common kilka (Zamani et al. 2014), 23.5 kDa for pirarucu (De Freitas-Junior et al. 2021), 24 kDa for small red scorpion fish (Aissaoui et al. 2017), 21 and 24 kDa for liver of albacore tuna (Klomklao and Benjakul 2018), 24 kDa for catfish (Dos Santos et al. 2016), 24.4 kDa for gulf corvina (Gonzalez-Felix et al. 2020), 25 kDa for monterey sardine (Castillo-Yanez et al. 2005), 26 kDa for common dolphinfish (Dos Santos et al. 2020), 27 kDa for zebra blenny (Ktari et al. 2012), 28.8 kDa for sardinelle (Ben Khaled et al. 2011), 29 kDa for Atlantic bonito (Klomklao et al. 2007), 30 kDa for spleen of albacore tuna (Poonsin et al. 2019), 38.5 kDa for tambaqui (Bezerra et al. 2001) and 42 kDa for skipjack tuna (Klomklao et al. 2006). In general, trypsins have exhibited to have the molecular weights in the range of 20-30 kDa (Gendry and Launay 1992). However, some reasons such as different habitat and climate, autolytic degradation and genetic variation among fish species may explain why trypsins from various sources have the different molecular weight (Klomklao et al. 2006; Lu et al. 2008).

Optimum temperature and thermostability

Enzymes are one of the main biological macromolecules that their maximum activity depend on an optimum temperature to make them functional. Fig. 2a revealed that optimum temperature of the trypsin in ICE prepared from beluga and sevruga was found to be 55°C, although 92.70% of the maximum activity of enzyme was still maintained at 60°C for both trypsins. However, an obvious decrease in the trypsin activity of both ICEs was observed at temperatures above 60°C, probably due to thermal inactivation of enzyme (Zamani et al. 2014) causing by unfolding of the molecules. Similar optimum temperature (55 °C) was recorded for trypsins from skipjack tuna (Klomklao et al. 2009), sardinelle (Ben Khaled et al. 2011), silver mojarra (Silva et al. 2011) and spleen of albacore tuna (Poonsin et al. 2019). Optimum temperature of both trypsins was higher than that of trypsins reported for cold-water fish, indicating optimum temperatures over a range of 40-45°C (Simpson 2000). These differences could be attributed to the temperature of fish habitat or experimental conditions used in assessments (Kishimura et al. 2008). Although fish trypsins are mostly unstable at temperatures higher than 40-50°C, their thermal stability are well known to be at temperatures below 40°C (Klomklao et al. 2014). Trypsin thermal stability from ICE of beluga and sevruga is displayed in Fig. 2b. As can be observed in this figure, the stability of both trypsins was highly maintained up to 50°C with a remaining activity of 90.2% and 91.7% for sevruga and beluga, respectively. A gradual decrease in the activity of both trypsins was recorded at 55°C,

whereas enzymatic activity sharply decreased at 60°C. After heating the ICEs at 70°C, the relative activities for both trypsins were about 0.9% and 1.6% of their initial activity for sevruga and beluga, respectively. These results were in accordance with those of sardinelle, common kilka, mrigal carp and pirarucu which exhibited to be stable up to 50°C (Ben Khaled et al. 2011; Zamani et al. 2014; Khangembam and Chakrabarti 2015; De Freitas-Junior et al. 2021). The trypsins from beluga and sevruga showed to be more stable at high temperatures in comparison with those reported for the monterey sardine, chinook salmon, bluefish, Tunisian barbell and common dolphin fish that the enzymatic activity was rapidly lost at temperatures above 40°C (Castillo-Yanez et al. 2005; Kurtovic et al. 2006; Klomklao et al. 2007; Sila et al. 2012; Dos Santos et al. 2020). In general, thermostability of the trypsin enzyme might vary by some factors such as fish species and experimental conditions (Liu et al. 2007; Kanno et al. 2011).

Effect of pH on activity and stability

The results observed from the effect of pH on the activity and stability of the trypsins from beluga and sevruga are illustrated in Fig. 3. Trypsins from both species indicated the maximal activity at pH 8.5 (Fig. 3a). An appreciable inactivity was resulted in pH 4.0-5.0. Our results showed that the stability of both trypsins was highly preserved at pH 5.0-11.0 by 75% for beluga and 80% for sevruga. The high ranges of pH may change the net charge and conformation of an enzyme and inhibit to bind to substrate properly, resulting in the abrupt loss of enzymatic activity (Klomklao et al. 2006; Wu et al. 2018). Trypsins are mainly known to be more active at pH 7.5-10.5 (Simpson 2000). The optimum pH (8.5) recorded for trypsin in both ICEs was similar with results reported for trypsins from the brownstripe red snapper viscera and the albacore tuna liver (Khantaphant and Benjakul 2010; Klomkalo and Benjakul 2018), whereas both trypsins indicated the lower optimum temperature than those recorded for the Japanese sea bass, the albacore tuna spleen and the pirarucu (Cai et al. 2011; Poonsin et al. 2019; De Freitas-Junior et al. 2021). However, optimum pH may differ depending upon the experimental conditions such as concentration and type of substrate, temperature and type of metal ions (Klomkalo et al. 2006). To give an instance, Martinez et al. (1988) showed that the trypsin from pyloric caeca of the anchovy had the optimum pHs of 8.0 and 9.5 for the hydrolysis of BAPNA and casein, respectively.

The effect of pH on the trypsin stability in both ICEs is displayed in Fig. 3b. Stability of both enzymes was considerably retained at pH 6.0-11.0. The enzyme lost about 63.15% and 69.26% of its activity at pH 4.0 for *sevruga* and beluga, respectively, while the loss of the enzyme activity at pH 5.0 was recorded by 34.13% and 35.84% for *sevruga* and beluga, respectively. However, trypsin in ICE of *sevruga* and beluga lost only 1.19% and 1.51% of its activity at pH 8.5, respectively. A same behavior was reported for trypsins from zebra blenny (Ktari et al. 2012), common kilka (Zamani et al. 2014), albacore tuna (Poonsin et al. 2019) spleen and common dolphinfish (Dos Santos et al. 2020) which 80-100% of the activity was retained at pH ranges of neutral and alkaline. The high catalytic activity of the trypsin is observed in alkaline pHs and its stability at a particular pH may be linked to the net charge of the enzyme at that pH (Mamimin et al. 2016).

Effect of inhibitors on trypsin activity

The sensitivity of protease enzymes to various inhibitors can properly help to characterize them. Table 1 shows the effect of different inhibitors on the activity of both trypsins. As can be seen in this table, a serine protease inhibitor (PMSF) inhibited 39.11% and 36.29% the trypsin activity from *sevruga* and beluga, respectively. Both enzymes were completely inhibited by trypsin specific inhibitors (SBTI and TLCK) while a chymotrypsin specific inhibitor (TPCK) did not show any inhibitory effect on the enzymatic activity of both trypsins (P>0.05). Further, a metalloproteinase inhibitor (EDTA) and a disulfide bond reducing agent (ß-mercaptoethanol) had the partial inhibitory effect on the enzymatic activity both trypsins, in which trypsin from *sevruga* was inhibited by ß-mercaptoethanol and EDTA more than that of trypsin from beluga without significant differences (P>0.05). However, an aspartic proteinase inhibitor (Pepstatin A) and a cysteine proteinase inhibitor (iodoacetic acid) exhibited a negligible inhibitory effect on the trypsin activity of both species. The inactivation of the trypsin by the above mentioned inhibitors was reported for trypsins from fish species showing the similarities between them. Khangembam and Chakrabarti (2015) reported that the trypsin activity from the digestive system of mrigral carp was effectively inhibited by SBTI and TLCK. SBTI is a single polypeptide chain that acts as a reversible competitive inhibitor of trypsin and forms a stable, enzymatically inactive complex with trypsin, resulting in reduction of the enzyme availability (Senphan et al. 2015). TLCK is an irreversible inhibitor of trypsin and trypsin-like serine protease that deactivates these enzymes through the formation of a covalent bond with histidine residue in the catalytic site of the enzyme and blocks the active center of the enzyme for binding to substrate (Choi et al. 1998). As reported by Ktari et al. (2012), Zamani et al. (2014) and De Freitas-Junior et al. (2021), PMSF strongly inhibited the activity of trypsin from viscera of zebra blenny, common kilka and pirarucu, respectively, whereas TPCK had no effect on the enzyme activity from common kilka (Zamani et al. 2014) and spleen of albacore tuna (Poonsin et al. 2019). The trypsin activity from intestine of common dolphinfish was partially inhibited by β-mercaptoethanol and EDTA (Dos Santos et al. 2020), while the trypsin activity from liver of albacore tuna was not reduced in the presence of pepstatin A and iodoacetic acid (Klomklao and Benjakul 2018).

Effect of metal ions

Metal ions have a key role in the activity regulation of many enzyme-catalyzed reactions (Page and Di Cera 2006). Our results on the effect of metal ions on the trypsin activity in *beluga* and sevruga are detailed in Table 2. No significant effect on the activity of *both enzymes* was found in the presence of univalent cations Na^{+} and K^{+}. The enzymatic activity of trypsin in both species was significantly reduced by divalent cations Cu^{+2}, Zn^{+2} and Co^{+2}, whereas Ca^{2+} significantly enhanced the activity of both trypsins (P<0.05). Similar results on the effect of Ca^{2+} on the trypsin activity were also observed in zebra blenny, common kilka and common dolphinfish (Ktari et al. 2012; Zamani et al. 2014; Dos Santos et al. 2020). Attachment of Ca^{+2} to the active site of serine proteases like trypsin not only increases the stability of the enzyme structure but also protects the enzyme from self-digestion (Fu et al. 2005; Kishimura et al. 2008; Sila et al. 2012). The enzymatic activity of trypsin in common dolphinfish

was reduced by 82% and 81% by Zn^{+2} and Cu^{+2}, respectively (Dos Santos et al. 2020), while 100% of enzymatic activity of tryspin from zebra blenny was lost in the presence of Zn^{+2} and Cu^{+2} (Ktari et al. 2012). In common kilka (Zamani et al. 2014), no inhibition was observed in the trypsin activity in presence of Na^{+} and K^{+}. Differences in percent inhibition might be linked to species diversity, environmental adaptations and dietary behaviors of fish (Bougatef 2013).

Effect of surfactants and oxidizing agents

The results on the effect of various surfactants and oxidizing agents on the trypsin activity in *sevruga* and beluga are shown in Table 3. A significant increase in the activity of both trypsins was observed after incubation for 1 h at 40 °C in the presence of surfactants tested including saponin, sodium choleate and Triton X-100 at final concentrations of 1% (P<0.05). Both trypsins were highly unstable against sodium dodecyl sulfate (SDS), in which trypsins from *sevruga* and beluga significantly lost about 94% and 97% of their activity in the presence of 0.1% SDS, respectively (P<0.05). Similar results were found with trypsins of other fish species in the presence of saponin, sodium choleate, Triton X-100 and SDS (Ktari et al. 2012; Zamani et al. 2014). The obtained results on the effect of oxidizing agents on both trypsins showed that the enzymatic activity was reduced in presence of sodium perborate (1%) in sevruga and beluga by 22.23 and 24.37 %, respectively. The activity of both enzymes was also decreased significantly with an increase in H_2O_2 concentrations from 5% to 15%, as described in Table 3 (P<0.05). These results showed that trypsins form *sevruga* and beluga were more stable against H_2O_2 than trypsins from grey triggerfish and zebra blenny (Jellouli et al. 2009; Ktari et al. 2012). The most proteases have shown to be unstable in the presence of oxidizing agents like hydrogen peroxide (Jellouli et al. 2009).

Conclusions

Our results indicated that intestinal trypsin from beluga and sevruga had an optimum temperature of 55°C and thermal stability of enzyme was maintained over 90% up to 50°C. The enzymes had an optimum pH of 8.5 and showed to be tolerant in the pH range of 6.0 to 11.0. The molecular weight of trypsin for sevruga and beluga was estimated to be 27.5 and 29.5 kDa, respectively, by inhibitory activity staining. The both trypsins were inhibited by main specific inhibitors, SBTI and TLCK. Additionally, the enzymatic activity of trypsins was still detected after 1 h in the presence of surfactants and oxidative agents. The obtained results from this study could be considered as a reference to monitor the trypsin activity in relation to different physicochemical condition.

Declarations

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Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Code availability Not applicable.

Ethics approval This study conformed to the current Iranian law regarding the care and use of laboratory animals, and was approved by the Animal Ethical Committee of Malayer University (Malayer, Hamedan, Iran).

Consent to participate Not applicable.

Conflict of interest The authors declare that there is no conflict of interest.

Consent for publication The manuscript has never been published in other journals.

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Tables

Table 1 Effect of various inhibitors on the activity of intestinal trypsin from *Huso huso* and *Acipenser stellatus.*

Inhibitors	Concentration	Inhibition (%)	
		Huso huso	*Acipenser stellatus*
Control	-	$0.0 \pm 0.00A^{a}$	$0.0 \pm 0.00A^{a}$
PMSF	10 mM	$36.29 \pm 0.39A^{c}$	$39.111 \pm 0.45A^{c}$
SBTI	0.05 mM	$99.81 \pm 0.54A^{d}$	$99.29 \pm 0.49A^{d}$
TLCK	5 mM	$99.19 \pm 0.57A^{d}$	$99.51 \pm 0.47A^{d}$
TPCK	5 mM	$0.00 \pm 0.00A^{a}$	$0.00 \pm 0.00A^{a}$
Pepstatine A	0.01mM	$0.51 \pm 0.003A^{a}$	$0.89 \pm 0.002A^{a}$
Iodoacetic acid	1 mM	$0.25 \pm .002A^{a}$	$0.44 \pm .003A^{a}$
EDTA	2 mM	$21.06 \pm 0.36A^{b}$	$23.55 \pm 0.51A^{b}$
ß-Mercaptoethanol	5 mM	$22.84 \pm 0.41A^{b}$	$25.33 \pm 0.37A^{b}$

Each intestinal crude extract (ICE) was incubated with same volume of inhibitor at room temperature for 15 min and the enzyme activity was determined at pH 8.5 and 55°C. The control was prepared with similar conditions in absence of inhibitors. Data were represented by mean ± standard deviation and compared by Duncan's multiple-range test. Different capital letters in the same row and different superscripts in the same column show statistical difference (P<0.05).

Table 2 Effect of various metal ions on activity of intestinal trypsin from *Huso huso* and *Acipenser stellatus.*

Metal ions	Concentration	Residual activity (%)	
		Huso huso	*Acipenser stellatus*
Control	-	$100 \pm 0.00A^{d}$	$100 \pm 0.00A^{d}$
K^{+}	5 mM	$99.6 \pm 1.05A^{d}$	$99.2 \pm 0.92A^{d}$
Na^{+}	5 mM	$99.3 \pm 1.03A^{d}$	$99.5 \pm 1.01A^{d}$
Ca^{+2}	5 mM	$107.21 \pm 0.81A^{e}$	$105.32 \pm 0.98A^{e}$
Cu^{+2}	5 mM	$48.31 \pm 0.88A^{a}$	$44.58 \pm 0.96A^{a}$
Zn^{+2}	5 mM	$67.79 \pm 1.15A^{b}$	$64.83 \pm 1.23A^{b}$
Co^{+2}	5 mM	$76.69 \pm 0.85A^{c}$	$74.77 \pm 1.05A^{c}$

Univalent and divalent metal ions were incubated with same volume of each intestinal crude extract (ICE) at room temperature for 30 min and residual activity of the enzyme was measured at pH 8.5 and 55°C. The control was prepared with similar conditions without metal ions and scored as 100%. Data were represented by mean ± standard deviation and compared by Duncan's multiple-range test. Different capital letters in the same row and different superscripts in the same column show statistical difference (P<0.05).

Table 3 Intestinal trypsin activity from *Huso huso* and *Acipenser stellatus* in presence of surfactants and oxidizing agents.

Each intestinal crude extract (ICE) was incubated with same volume of the chemicals for 1 h at 40°C and residual activity of the enzyme was measured at pH 8.5 and 55°C. The control was prepared with similar conditions without chemicals and scored as 100%. Data were represented by mean ± standard deviation and compared by Duncan's multiple-range test. Different capital letters in the same row and different superscripts in the same column show statistical difference (P<0.05).

Figures

Chemicals		Concentration	Residual activity %	
			Huso huso	*Acipenser stellatus*
Surfactants	Control	-	100 ± 0.00A^{d}	100 ± 0.00A^{d}
	Triton X - 100	1%	108.81 ± 1.62A^{e}	112.55 ± 1.11A^{e}
	SDS	1%	2.79 ± 0.21A^{a}	5.77 ± 0.33B^{a}
	Sodium cholate	1%	132.23 ± 1.12A^{j}	197.77 ± 0.99B^{j}
	Saponin	1%	126.14 ± 1.06A^{f}	128.44 ± 1.46A^{f}
Oxidising agents	Sodium perborate	1%	75.63 ± 1.07A^{c}	77.77 ± 0.97A^{b}
	H_2O_2	5%	70.30 ± 1.02A^{c}	87.55 ± 1.02B^{c}
		10%	64.97 ± 1.19A^{c}	84.55 ± 0.73B^{c}
		15%	40.35 ± 0.65A^{b}	73.77 ± 0.91B^{b}

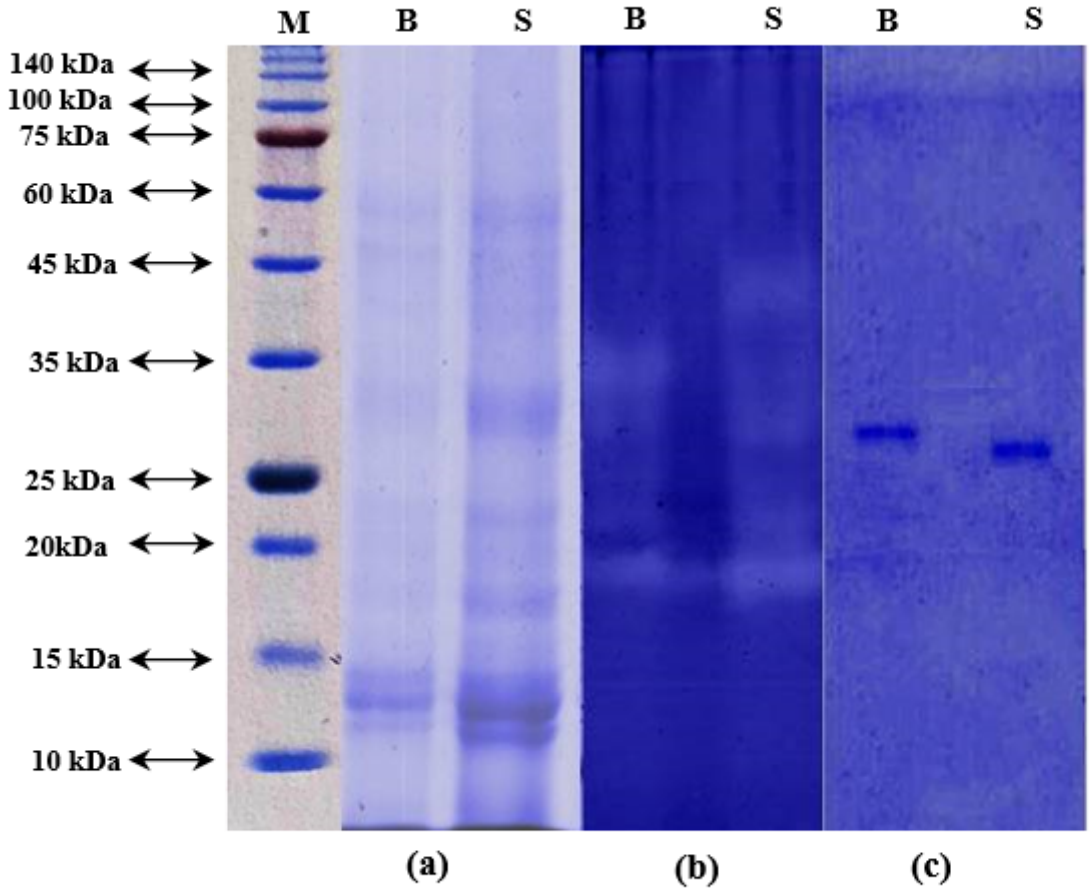

Figure 1

SDS–PAGE (a), zymography (b) and inhibitory activity staining (c) of the intestinal crude extract (ICE) of *Huso huso* and *Acipenser stellatus*. (a): molecular weight marker (M); ICE from *Huso huso* intestine (B); ICE from *Acipenser stellatus* intestine (S). Coomassie blue G-250 (0.1%) was used for staining proteins from SDS-PAGE. Zymogram activity and inhibitory activity staining were stained by using 0.1% coomassie blue R-250

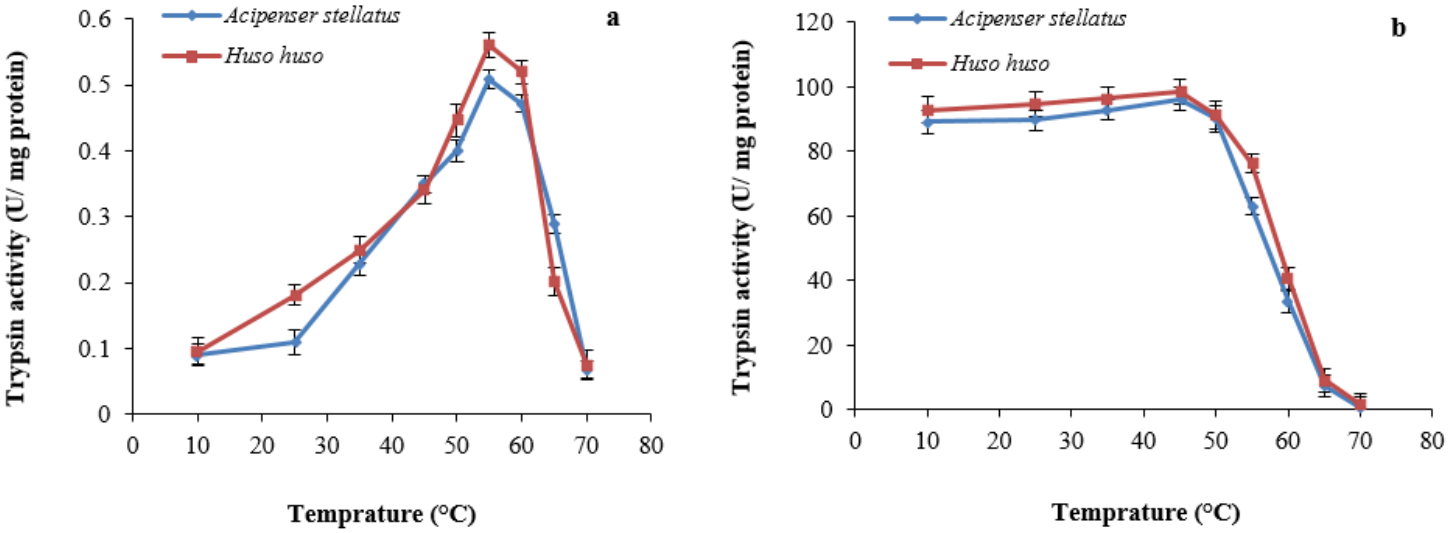

Figure 2

(a) Optimum temperature: the enzymatic activity of both trypsins was measured at different temperatures (the highest activity at 55°C was scored as 100%); (b) Thermostability: residual activity of both enzymes was determined at 55°C and pH 8.5 after incubating at different temperatures for 30 min

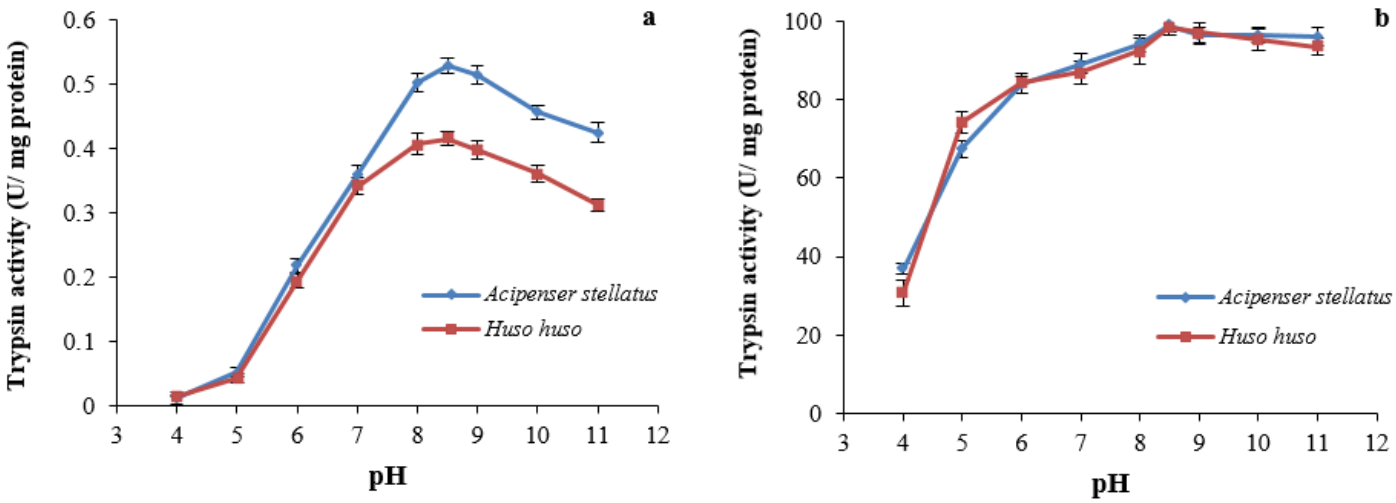

Figure 3

(a) Optimum pH: the enzymatic activity of both trypsins was determined at different pHs (the highest activity at pH 8.5 was scored as 100%); (b) pH stability: residual activity of both enzymes was measured at 55°C and pH 8.5 after incubating at different pHs for 30 min