

Purification of endogenous transglutaminase from daggertooth pike conger fish (*Muraenesox cinerus*) meat

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Abstract

Daggertooth pike conger fish (*Muraenesox cinerus*) has the potential to be used as a raw material for the surimi industry because it has high yield and white flesh. Surimi quality is not only influenced by the production process but also by the strength of the texture which is in turn influenced by the transglutaminase enzyme (TGase) in the fish meat. However, the activity and characteristics of TGase in the fish meat were not clearly known. The present work, therefore, aimed to characterise the TGase in daggertooth pike conger fish meat. Results showed that the crude extract of TGase had an activity of 0.240 U/mL, and increased following dialysis (0.823 U/mL); in other words, the activity underwent purity boosting as much as 1.983 times. Moreover, TGase in daggertooth pike conger fish meat was found to have an optimum temperature of 40 - 50°C and pH 8, with a molecular size of around 80 kDa. It was also found that the reactivity of TGase depended on Ca²⁺ ions and could be increased with Mg²⁺ ions, but could be inhibited by Fe²⁺ ions.

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Keywords

activity,
muraenesox,
purification,
transglutaminase

Introduction

Daggertooth pike conger fish (*Muraenesox cinerus*), locally known as *malong*, has a wide distribution in Indonesia. According to Satapoomin (2011), daggertooth pike conger fish can also be found in the waters of Indonesia, Malaysia, the Philippines, Thailand, and Japan. Daggertooth pike conger fish is elongated (70 to 80 cm) and round like an eel. Of its body, 50 - 60% is edible, thus suitable as raw material for further food processing (Marichamy *et al.*, 2012). Currently, daggertooth pike conger fish is used for daily consumption, and some industries use it as the raw material for meatballs. Its high meat yield and white flesh colour also has the potential to be used as raw material for making surimi.

Raw materials used by the surimi industries include threadfin bream (*Nemipterus hexodon*), Pacific whiting (*Merluccius productus*), blue whiting (*Micromesistis poutassou*), silver carp (*Hypophthalmichthys molitrix*), Alaska pollock (*Gadus chalcogrammus*), bigeye snapper (*Priacanthus tayenus*), and croaker (*Pennahia* spp. and *Johnius* spp.) (Benjakul *et al.*, 2004; Ramirez *et al.*, 2007; Li *et al.*, 2008; Bourtoom *et al.*, 2009; Piyadhamvi-boon and Yongsawatdigul, 2009; Nolsoe *et al.*, 2011). These fish species are selected based on the quality of

its meat gel (myofibril protein) in the form of surimi. Several studies have been conducted in this particular area. For instance, Fadda *et al.* (1999) studied the characteristics of myofibril and sarcoplasmic hydrolysed by the bacterium *Lactobacillus plantarum*. Verrez-Bagnis *et al.* (2002) studied the enzymatic effect (m-calpain) on myofibril and sarcoplasmic proteins during rigor mortis. Similarly, Ladrat *et al.* (2002) also studied the enzymatic effects on myofibril and sarcoplasmic proteins during rigor mortis, but with different enzymes (cathepsin B, D, and L). Another study conducted by Subagio *et al.* (2004) found that turmeric fish myofibril is better than big eye fish. Ayala *et al.* (2005) studied the changes in the structure and ultrastructure of sea bass (*Decentrarchus labrax* L.) after cooking and freezing.

Intrinsic factors determining the gel strength of fish meat include the amount of myofibril protein and transglutaminase (TGase) enzyme. The TGase catalyses the acyl transfer reaction by forming covalent bonds between proteins, especially peptide chains with various amine groups (Ohtsuka, 2001; Cui *et al.*, 2007). The TGase can form ties between the amino acids namely lysine and glutamine, thus making a protein polymer that gives the texture of the meat. Haard and Simpson (2000), Schmidt *et al.* (2008), and Mirzaei (2011) define TGase as a type of

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transferase enzyme with the systematic name of protein-glutamine γ -glutamyltransferase (EC 2.3.2.13). The TGase catalyses the acyl transfer reaction between the γ -carboxamide group on proteins, peptide bonds, and various types of primary amino acids. The lysine amino acid ϵ -amino group acts as the recipient of an acyl to form polymerisation and intra- or inter-molecular cross-bonds of proteins through the formation of ϵ -(γ -glutamyl)-lysine bonds. The bond causes changes in the lysine amino acid ϵ -amino group, and produces ammonia in the carboxamide group of glutamine in protein molecules. This mechanism will make fish protein to have high elastic properties and able to trap enough water to form a three-dimensional structure. If the substrates for TGase are slightly amine, the water available can be used by TGase as a replacement. The work process of the endogenous TGase is influenced by the presence of the mineral calcium (Ca), which enhances its performance. Potential utilisation of daggertooth pike conger fish as surimi raw material requires detailed information related to the endogenous role/capacity of TGase to form myosin heavy chain (MHC). Therefore, the present work aimed to characterise the endogenous TGase in the flesh of daggertooth pike conger fish.

Materials and methods

The experiment was done in Raw Material Laboratory, and Biochemistry Laboratory, Fisheries and Marine Faculty; and Spectrometry Laboratory, MIPA Faculty at Bogor Agricultural University.

Materials and instruments

Sampling of daggertooth pike conger fish was done in the Karangsong fish landing base (PPI) in Indramayu Regency, West Java, and the samples were transported using a cool box to the laboratory. The TGase extraction and characterisation used monodansylcadaverine (MDC) (Sigma-Aldrich, US), N'N-dimethyl casein (DMC) (Sigma-Aldrich, US), NaCl PA 99.5% (Merck, Germany), 2-mercaptoethanol (DMC) (Sigma-Aldrich, USA), EDTA (Titriplex III PA) (Merck, Germany), hydroxymethyl PA (Merck, Germany), and CaCl₂·2H₂O (Merck, Germany).

Tools including fluorometer (BMG LABTECH, Germany), cold centrifuge (Himac CR 21G, Japan), micropipette (Thermo Scientific Vantaa, Finland), spectrophotometer (Spectro UV-VIS 2500, Germany), pH meter (Thermo Electron, Finland), incubator (Thermoline), and electrophoresis (TV-100 YK, SCIENPLAS, UK)

were also utilised.

TGase extraction

Firstly, the daggertooth pike conger fish was deboned, skinned, gutted, and filleted. The fillet was then homogenised for 5 min using a homogeniser at 9,000 rpm using a buffer (10 mM NaCl, 5 mM EDTA, 2 mM 2-mercaptoethanol, 10 mM Tris-HCl, pH 7.5) at a fillet to buffer ratio of 1:4. The homogenate was then centrifuged at 9,700 g at 4°C for 30 min. The supernatant was re-centrifuged at 12,000 g at 4°C for another 30 min. This yielded the TGase crude extract (Binsi and Shamsundar, 2012).

TGase activity

The TGase activity was measured following the procedures described by Takagi *et al.* (1986) with slight modifications. Briefly, 0.1 mL extract of crude endogenous TGase was mixed with a solution containing 1 mg/mL N'N-dimethylated casein, 15 μ M monodansylcadaverine, 70 mM Tris-HCl (pH 7.5), 5 mM CaCl₂, and 3 mM 2-merkaptoethanol, and incubated at 37°C for 10 min. The reaction was then stopped by adding 0.1 M EDTA. Next, absorbance was measured with a spectrophotometer at 350 nm excitation wavelength, and 480 nm emission wavelength. A standard solution was prepared by the same procedure as the sample, except in the process of adding monodansylcadaverine, which was done after the addition of EDTA. A blank solution was made by replacing TGase using deionised water, and no incubation was conducted. The calculation of one unit of TGase is defined as the number of enzymes catalysed by 1 nmol when added with monodansylcadaverine (MDC) and N'N-dimethylated casein (DMC) for 1 min at 37°C.

Protein concentration

The protein concentration was determined by the Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as standard. Briefly, 0.1 mL sample (enzyme solution) was placed in a test tube. Then, 1 mL of Bradford reagent was added, and the mixture was incubated for 5 min. The absorbance was measured with a spectrophotometer at a wavelength of 595 nm.

TGase characterisation

The TGase was characterised following the procedures described by Hemung and Yongsawatdigul (2007). The determination of the optimum temperature of TGase was conducted by varying the incubation temperatures (20, 30, 40, 50, 60, and 70°C). The determination of the optimum pH was

conducted by varying the buffer pH's (pH 6 - 7 using 100 mM sodium acetate, pH 7.5 - 9 using 70 mM Tris-HCl, and pH 10 - 11 using 50 mM glycine). The effect of metal ions on TGase was tested with 10 mM metal ions in the form of a chloride salt solution of cations Mg^{2+} , Mn^{2+} , Fe^{2+} , Cu^{2+} , and Zn^{2+} . Testing was done by replacing $CaCl_2$ with 10 mM of the tested metal ions.

TGase molecular weight

The molecular weight of endogenous TGase was determined using SDS PAGE (Laemmli, 1970). The acrylamide concentration used in the analysis was 15% separating gel and 3% stacking gel. Sample (20 μ L) was then mixed with 5 μ L buffer, and loaded into the well. The loading marker was 10 μ L. The gel was installed in the electrophoresis device by pouring the electrophoresis buffer into the chamber. The electrophoresis process lasted for 65 min at 180 V and 50 mA in the electrophoresis device, after which the gel was removed from the glass plate. The gel was then soaked in a solution of a Coomassie gel stain for 2 h. Then the de-staining process was done until the protein bands were obtained.

Results and discussion

Characteristics of TGase crude extract

The characterisation of TGase was done on its crude extract. The TGase was extracted from daggertooth pike conger fish meat (entire body parts). The characterisation included determining the optimum temperature, optimum pH, and the influence of metal ions. The TGase was extracted using a Tris-HCl buffer containing NaCl, EDTA, and 2-mercaptoethanol, or so-called TEND buffer. EDTA inhibited the reaction of proteases, and 2-mercaptoethanol protected the extract from environmental influences. The extraction was carried out by centrifugation. The TGase crude extract activity was 0.240 ± 0.04 U/mL.

Optimum pH

The determination of optimum pH was done by adding a buffer in a pH range of 6 to 11 (Figure 1). The highest TGase activity was found to be in the Tris-HCl buffer ranging from pH 7.5 to 9. The optimum activity of TGase was at pH 8 with an activity of 0.190 U/mL. The same optimum pH range was also found in tilapia (*Oreochromis niloticus*), having a pH range from 7 to 9, and optimum at pH 7.5 (Worratao and Yongsawatdigul, 2003; 2005). Subramanian (2008) also observed that the optimum pH range for bluefish (*Pomatomus saltatrix*) TGase

was between pH 7 - 8, and optimum at pH 7.5. Similar result was also found in the TGase produced by *Streptomyces hygroscopicus*, which has an optimum pH range of 6 - 7.

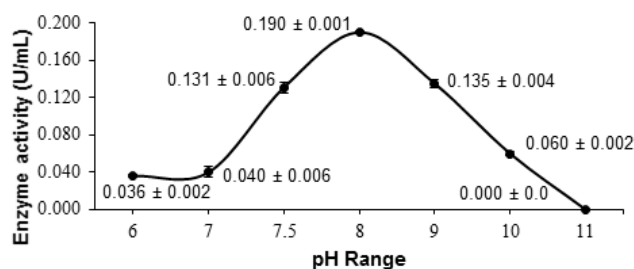


Figure 1. Transglutaminase activity in daggertooth pike conger fish meat at different pH ranges.

The optimum pH of daggertooth pike conger fish meat TGase was in the normal pH range, which suggests a distinct advantage. This is because it has high potential to facilitate the optimisation of its activities when utilising daggertooth pike conger fish meat. However, it also may result in easy loss or degradation of the enzyme during processing, due to the presence of other protease enzymes which also are active in the normal pH range. The TGase of daggertooth pike conger fish meat has low activity at pH below or above its optimum pH. This is in line with the study of Benjakul *et al.* (2004), stating that the activity of TGase remains unstable at very acidic and alkaline pH, due to the denaturation process under extreme pH conditions.

Optimum temperature

Temperature is a factor that affects the rate of catalysis in enzymatic reactions. An increase in temperature to a certain extent may increase the enzymatic activity; but, the increase in temperature above this limit can also cause a decrease in the enzyme activity. The optimisation was done in the temperature range of 20 to 70°C, and the results are depicted in Figure 2.

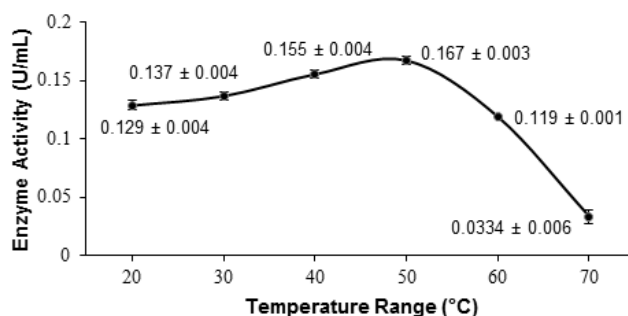


Figure 2. The effect of temperature on the activity of transglutaminase in daggertooth pike conger fish meat.

It was apparent that daggertooth pike conger fish meat TGase activity increased from 20 to 50°C, and decreased from 60 to 70°C. The optimum temperature ranged from 40 to 50°C, with enzyme activity values of 0.155 and 0.167 U/mL, respectively. This is in agreement with the study conducted by Worratao and Yongsawatdigul (2005), who indicated that pure TGase in tilapia had optimal conditions in cross-linking activity at the temperature of 50°C. Meanwhile, Klesk *et al.* (2000) found that surimi made of tilapia had a "setting" temperature of 40°C, but not at low temperatures such as 5°C and 25°C. Surimi "setting" is catalysed by the endogenous TGase (Joseph *et al.*, 1994). The TGase in tilapia can largely catalyse cross-linking bonds of meat protein at 40°C than at lower temperatures, which tend to make covalent bonds. This finding suggests that the loss of TGase activity occurs at higher levels when higher "setting" temperatures are applied.

Binsi and Shamsundar (2012) stated that the optimum temperature difference for TGase activities of several fish species depends on the temperature of their habitat, and the level of enzyme purity. Hemung and Yongstwadigul (2007) stated that the temperature difference is related to the temperature stability of fish meat. Meat protein in tropical fish needs a higher temperature which allows for interaction with hydrophobic bonds and the catalytic reaction of TGase. Higher "setting" temperatures are found in surimi from tropical fish because of the optimum temperature of the tropical environment is higher than that of the subtropical region.

Effects of the metal ion on TGase activity

Activators and inhibitors cannot be

distinguished chemically; instead, they can be distinguished by interacting them with enzymes. Activators bind to enzymes and cause an increase in the enzyme reaction speed, whereas inhibitors bind to enzymes and cause a decrease in the reaction speed (Suhartono, 1989). There are some metal ions that help bind enzymes to the substrate; some would bind to the enzyme directly so that the active conformation of the enzyme becomes stable, and some would bind to the enzyme inhibitor so that it affects the inhibitory action of the enzyme. Results showed that the addition of metal ions affected the activity of TGase, which could either decrease or increase the activity unit value (Figure 3). The TGase activity in daggertooth pike conger fish meat was influenced the greatest (two-fold increase) by Mg²⁺ metal ion with 0.584 U/mL. This was lower, however, when compared to the control using Ca²⁺. The TGase had no activity with Fe²⁺ metal ions, which suggests that these metal ions could become TGase inhibitors. Metal ions that also reduced the relative activity of daggertooth pike conger fish meat TGase included Zn²⁺ and Cu²⁺. This could be due to the fact that the metal ions can bind thiol groups from cysteine residues, which are the active site of TGase in daggertooth pike conger fish meat. A study of Kumazawa *et al.* (1996) showed that the activity of TGase in walleye pollock livers can be decreased by including Zn²⁺ metal ions, and increased by adding Mg²⁺ metal ions. The binding reaction was effected by metal ion due to the amino protein in the meat. As previously mentioned, TGase is an enzyme that catalyses acyl transfer reaction between the γ -carboxamide group on proteins, peptide bonds, and various types of primary amino acids. The transfer of

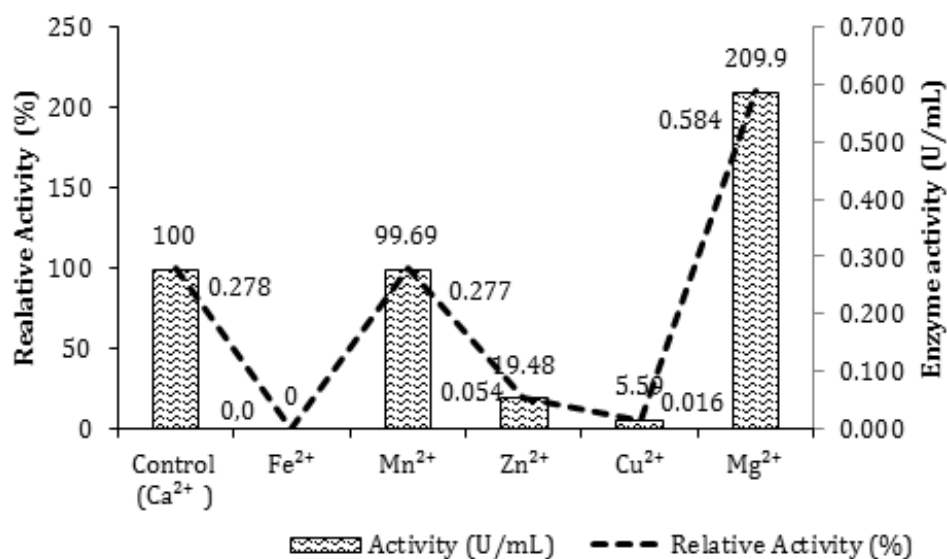


Figure 3. The effect of metal ions on the activity and relative activity of transglutaminase in daggertooth pike conger fish meat.

acyl is due to lysine and glutamine. Furthermore, the amount of these amino acids is also very important. Laksono *et al.* (2019a) found that the amount of lysine in daggertooth pike conger fish meat fish was 1.57%, glycine 0.87%, and glutamic acid 2.68% (wet basis).

TGase molecular weight

The molecular weight of enzyme is determined by electrophoresis, that is, by removing the charged particles from a mixture based on the movement of charged colloidal particles under the influence of an electric field (Suhartono, 1989). Electrophoresis uses sodium dodecyl sulphate-polyacrylamide separating gel to separate the charged protein based on its molecular weight. Protein with small molecular weight will move faster than the larger ones. The results of molecular weight measurements with SDS-PAGE crude extracts of TGase from daggertooth pike conger fish meat are presented in Figure 4. It was found that the TGase of daggertooth pike conger fish meat had many protein bands in the range of 20 to 100 kDa. This might be due to the presence of impurities in the crude extract. This finding is similar to a study by Worratao and Yongsawatdigul (2005). However, based on the

shape of the tape obtained, it could be seen that the molecular size was 80 kDa. This is also almost similar to the findings of Worratao and Yongsawatdigul (2005), which showed that the TGase of tilapia was in the range of 85 kDa.

Purification of crude TGase from daggertooth pike conger fish meat

The TGase in daggertooth pike conger fish meat was extracted using centrifugation. The results of enzyme activity and protein content of daggertooth pike conger fish meat can be seen in Table 1. The TGase crude extract activity was 0.240 ± 0.04 U/mL, with a specific activity of 1.983 U/mg. The TGase activity in daggertooth pike conger fish meat was 0.187 ± 0.02 U/mg, which was greater than TGase activity in *cunang* fish liver (Sidauruk *et al.*, 2017). A study by Subramanian (2008) indicated that the activity of TGase crude extract in the meat of bluefish (*Pomatomus saltatrix*) was 0.321 U/mg, and in the liver was 0.230 U/mg. This finding suggested that TGase has more impacts in the fish meat than in other organs. TGase is useful in the osmoregulation and motoric formation of an actomyosin complex functioned in the activity of living things, including fish.

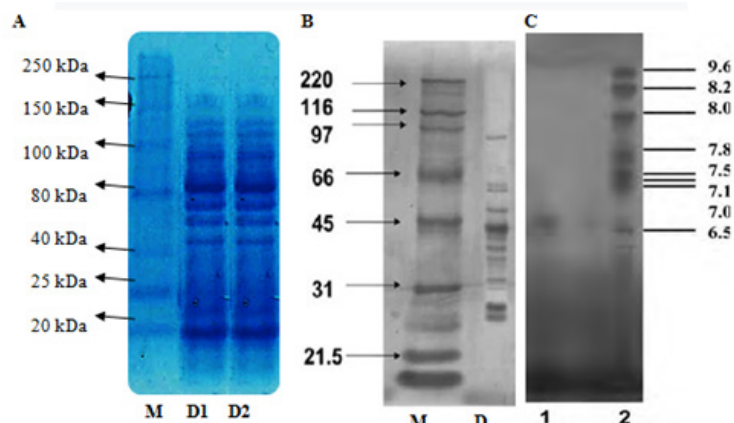


Figure 4. Electrophoresis results of molecular weight crude extract of transglutaminase: (A) TGase crude extract of daggertooth pike conger fish meat (D1 = repetition 1, D2 = repetition 2, M = marker); (B) crude extract of tilapia TGase; and (C) TGase after purification (Worratao and Yongsawatdigul, 2005).

Table 1. Concentrations of transglutaminase and proteins from daggertooth pike conger fish meat.

Sample (stages of the process)	Volume (mL)	Protein (mg/mL)	Protein (mg)	Activity (U/mL)	Activity (U)	Specific Activity (U/mg)	Multiple	Yield (%)
Meat	50	0.121 ± 0.02	6.050	0.240 ± 0.04	12	1.983	1	100
Precipitation	3.8	1.946 ± 0.43	7.395	2.157 ± 0.6	8.197	1.108	0.559	68.308
Dialysis	2.5	0.225 ± 0.01	0.563	0.823 ± 1.17	2.057	3.654	1.983	17.142

The specific activity of the TGase crude extract of daggertooth pike conger fish has considerably good value with its specific activity of 1.983 U/mL. This is almost the same as that of TGase from tilapia, which was 2.82 U/mg (Worratao and Yongsawatdigul, 2005). This finding suggested that the meat of daggertooth pike conger fish has a good ability of gel formation because the quality of its flesh is almost similar to tilapia meat, which has a good gel structure. However, the activity in the flesh of daggertooth pike conger fish was still inferior as compared to TGase activity in the liver of threadfin bream fish which was 45.45 U/mg (Hemung and Yongsawatdigul, 2007). The amount of endogenous TGase activity in daggertooth pike conger fish meat has the potential to be used as raw materials for surimi because it can be optimised by activating the enzyme with Ca^{2+} ions. According to Hemung and Yongsawatdigul (2007), TGase indicated no activity without the presence of Ca^{2+} ions, and increased in the presence of Ca^{2+} ions at the concentrations of 1 - 5 nM. Another study conducted by Laksono *et al.* (2019b) showed that Ca^{2+} ions combined with sodium tripolyphosphate (STPP) could increase the folding test in surimi of daggertooth pike conger fish.

The purification of TGase in daggertooth pike conger fish meat could increase its activity by dialysis technique. A research done by Worratao and Yongsawatdigul (2005) showed a very high purification multiplication with DEAE-Sephacel (12 times), 9.94 times with Sephacryl S-200, and 69.6 times with HiTrap Heparin technique. El-Hofi *et al.* (2014) purified TGase from rosemary (*Rosmarinus officinalis* L.) flowers with sulphuric acid precipitation, and obtained a multiplication of 1.68 times. The multiplication with precipitation demonstrated in the present work was only 0.559 times.

Conclusion

The present work succeeded in obtaining the activity of the crude extract transglutaminase (TGase) from daggertooth pike conger fish (0.240 U/mL) with a specific activity of 1.983 U/mg. The results of purification by dialysis were able to increase the activity of TGase crude extract to 0.823 U/mL, with a specific activity of 3.654 U/mg. The TGase in daggertooth pike conger fish meat had an optimum pH of 8, and a temperature range of 40 - 50°C, with a molecular size of around 80 kDa. The endogenous TGase was Ca^{2+} dependent and could be increased with Mg^{2+} ions, but could be inhibited by Fe^{2+} ions.

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