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Proteolytic activities in fillets of selected underutilized Australian fish species

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ABSTRACT

The hydrolytic activity of major endogenous proteases, responsible for proteolysis of myofibrillar proteins during *post-mortem* storage, may be an indicator of the textural quality of fish which influences consumer purchasing behaviour and thus market value of the final product. Furthermore, it may also influence the type and bioactive properties of the peptides released during *post-mortem* proteolysis of myofibrillar proteins. This study compared the activities of cathepsins B, B+L, D, H and calpain-like enzymes in crude muscle extracted from 16 Australian underutilized fish species. Fish species had a significant effect on the activity of these enzymes with barracouta showing the highest cathepsins B, B+L, D and H activities. Activities of cathepsins B and B+L were higher than cathepsin H for all studied species. The more commercially important rock ling and tiger flathead demonstrated higher cathepsin B+L activity, whereas gemfish and eastern school whiting showed higher activity towards cathepsin B. Underutilized fish species showing higher endogenous protease activities may be suitable for fish sauce production, whereas those with lower protease activities for surimi processing.

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1. Introduction

The world's fisheries production increased from 16.8 million tonnes in 1950 to 86.4 million tonnes in 1996, and then declined to 77.4 million tonnes in 2010. The stocks of many traditional marine species have diminished worldwide over the last few years and the proportion of overexploited marine stocks increased, which demonstrates that the state of world marine fisheries is worsening (Food and Agriculture Organization of the United Nations (FAO), 2012). Furthermore, a rise in population and improved consumer awareness towards the health benefits of seafood increased the demand for fish and fishery products (He, Chen, Sun, Zhang, & Gao, 2006). Nonetheless, while target fish species continue to be exploited, many non-target species of almost no commercial interest still remain underutilized (Huang & Liu, 2010). Majority of these underutilized species are discarded due to certain technological inconveniences, which include poor sensory quality and/or functional attributes, making a large number of these species mainly utilised for animal feed and/or fertilizers (Geirsdottir et al., 2011; Mazorra-Manzano, Pacheco-Aguilar, Ramirez-Suarez, & García-Sánchez, 2008). The Food and Agriculture Organization of the United Nations (FAO) estimates that about 7.3 million tonnes of fish was discarded annually by marine fisheries throughout the world in the 1992–2001 period (Zeller & Pauly, 2005). Discarded marine species create a substantial waste of valuable living resources, which may be better utilised for human consumption, for economic and environmental reasons. For instance, undervalued species may be formulated and used to develop various high value-added seafood products, particularly since some of these species have nutritional (He et al., 2006), physiological (Medenieks & Vasiljevic, 2008) and functional properties (Geirsdottir et al., 2011) which may be comparable to those of traditional target species.

Enzymatic hydrolysis of fish proteins may be used to improve utilization of currently available underutilized fish resources for human consumption. The hydrolytic process releases a mixture of peptides depending on the enzyme specificity and the extent of protein hydrolysis (Geirsdottir et al., 2011). It involves using endogenous digestive enzymes and/or exogenous enzymes to hydrolyse specific peptide bonds in the protein molecule (Khalil, Metwalli, & El-Sebaiy, 1987) producing protein hydrolysates with enhanced physiological and/or functional properties. Higher autolytic activity of major muscle endogenous proteases induces hydrolysis of key myofibrillar proteins, and thus contributes to weakening of the myofibril structure during *post-mortem* storage. This accelerates textural deterioration in fish. The main proteolytic systems involved in hydrolysis of myofibrillar proteins during *post-mortem* storage of meat from mammalian and fish muscles are the cytoplasmic calpains and the lysosomal cathepsins (Yamashita & Konagaya, 1991). Calpains are optimally active at neutral pH, whereas cathepsins are optimally active in acidic environments

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(Stagg, Amato, Giesbrecht, & Lanier, 2012). The main cathepsins involved in proteolysis of key myofibrillar proteins are cathepsins B (EC 3.4.22.1), cathepsin L (EC 3.4.22.15), cathepsin H (EC 3.4.22.16) and cathepsin D (EC 3.4.22.5) (Aoki & Ueno, 1997). Generally, higher activity of endogenous muscle proteases during initial days of refrigerated storage may be an indicator of texture-associated degradation (Delbarre-Ladrat, Verrez-Bagnis, Noël, & Fleurence, 2004).

The contribution of proteolysis to textural properties of fish fillets during iced storage has been widely studied, but there exists a gap in knowledge regarding products of proteolysis during *post-mortem* ageing or storage and cooking, especially small peptides (Bauchart et al., 2007) which may possess different biological effects *in vitro* and *in vivo*. Proteolysis of fish proteins is an important process to liberate peptides and enhance the bioactivity of the protein source (Korhonen & Pihlanto, 2003). Peptides can be present in fish muscle *intra vitam* (e.g. anserine, glutathione) and/or could be generated during *post-mortem* storage via proteolytic digestion of fish myofibrillar proteins (Bauchart et al., 2007). Endogenous muscle proteases (cathepsins B, D, H, and L, and calpains) may initially act on myofibrillar proteins during meat tenderization process to produce a great number of oligopeptides (Arihara, 2006), small peptides and free amino acids (Toldrá and Flores, 1998). In addition, enzymatic digestion of fish muscle was reported to release peptides with improved bioactivity (Bauchart et al., 2007; Hasan et al., 2006; Samaranayaka, Kitts, & Li-Chan, 2010). Fish protein hydrolysates were reported to release peptides that exhibit anti-oxidative (Je, Qian, Byun, & Kim, 2007; Ovissipour et al., 2012; Wu, Chen, & Shiau, 2003), anti-cancer effects (Picot et al., 2006) and have the capacity to lower plasma cholesterol level (Wergedahl et al., 2004). These studies indicate that fish proteins may be partially degraded during *post-mortem* storage by endogenous muscle proteases releasing a mixture of polypeptides and oligopeptides which, due to the action of human digestive enzymes upon oral administration, may generate a range of small peptides that may possess physiologically important activities. Limited information is available in the literature on the physiological benefits upon gastrointestinal digestion of fresh fish as a whole meal. Thus, the results from the current study may pave the way for future studies in examining the feasibility of fish autolytic process as a means to release bioactive peptides, and improve the value of underutilized fish species.

Medenieks and Vasiljevic (2008) assessed the bioactive potential of three underutilized Australian fish species: silver warehou, barracouta and Australian salmon by *in vitro* assays. Processing conditions and seasonal variations affected the peptide profiles, and consequently the biological activity of the peptides released. Fewer peptides were produced following simulated gastrointestinal digestion of fish harvested in winter than those harvested in summer. The higher proteolytic activities of endogenous enzymes in the summer-harvested species contributed to greater release of polypeptide products and oligopeptides. The study demonstrated that the physiological properties of bioactive peptides released from myofibrillar proteins of these underutilized fish species, was affected by the fish species and most importantly catch season, which may have been influenced by the activity of endogenous proteases. Therefore, the proteolytic activity of major muscle endogenous proteases may affect the biological properties of the peptides released, as a result of proteolytic degradation of myofibrillar proteins from underutilized fish species. Other factors that influence the bioactive properties of the peptides released include the type of protease(s) used, processing conditions and the molecular size of the resulting peptides, which greatly affects their absorption across the gastrointestinal tract and bioavailability in target tissues (Udenigwe and Aluko, 2012). Freshness of the raw material is a critical parameter during processing of seafood products, for example, raw materials showing minimum *post-mortem* deterioration effects are selected for surimi processing (Martín-Sánchez, Navarro, Pérez-Álvarez, & Kuri, 2009). The use of underutilized Australian fish species as fresh fillets or as ingredients to incorporate into value-added seafood products may be restricted. This could be due to the lack of knowledge on the activity of the major proteases which participate to a different extent to the degradation of myofibrillar proteins and thus, influence the bioactive properties of the proteolytic products released. Therefore, the objective of this study was to establish the proteolytic activity of cathepsins B, D, H, and L as well as calpain-like enzymes in sixteen underutilized Australian fish species. This knowledge may be particularly useful when considering the feasibility of using these species as raw materials, during processing of different seafood products, that may be dependent on their endogenous protease activities to improve their commercial value.

2. Materials & methods

2.1. Materials

Underutilized Australian fish species used for this study included Australian salmon (*Arripis trutta*), barracouta (*Thyrsites atun*), bight redfish (*Centroberyx gerrardi*), deepwater flathead (*Neoplatycephalus conatus*), eastern school whiting (*Sillago flindersi*), gemfish (*Rexea solandri*), jackass morwong (*Nemadactylus macropterus*), mirror dory (*Zenopsis nebulos*), nannygai (*Centroberyx affinis*), ocean perch (*Helicolenus barathri*), ribbon fish (*Lepidopus caudatus*), rock ling (*Genypterus tigerinus*), silver trevally (*Pseudocaranx dentex*), silver warehou (*Seriolella punctata*), tiger flathead (*Neoplatycephalus richardsoni*) and yellowspotted boarfish (*Paristiopterus gallipavo*) (Yearsley, Last, & Ward, 1999). The samples were kindly supplied by Barwon Foods (Geelong North, VIC, Australia) and obtained through their supply chain. Fish were delivered fresh within 24 h from catch to our laboratory and stored on ice in a cold room. The samples were processed immediately upon receipt, usually within a couple of hours. Each species of fish was obtained on two separate occasions and assessed individually forming the two replicates for each species.

2.2. Assay of enzymatic activities

2.2.1. Activity measurements of calpain

The fish were filleted and minced for 30–60 s on ice using a food processor. Crude extract for calpain-like activity assay was obtained by homogenising the minced fish (30 g in 75 ml cold extraction buffer) in Tris-buffered saline (100 mM Tris–HCl, 145 mM sodium chloride (NaCl), 10 mM ethylenediaminetetraacetic acid (EDTA), pH 7.3) for 1 min on ice. The homogenate was centrifuged (Beckman J2-HS centrifuge, JA-20 rotor, Palo Alto, CA, USA) at 17,000×g for 45 min at 4 °C. The supernatant was filtered (Whatman, Qualitative 185 mm, # 1001185, Maidstone, UK), preserved with 5% trehalose and stored at −20 °C until further analysis.

In this study, the enzymes obtained in the crude extracts were not purified. Thus, the detected activity in the assay for calpain is reported as calpain-like (CA-like). The activity determination of calpain-like enzyme was performed using L-Methionine 7-amido-4-methylcoumarin trifluoroacetate salt from Sigma–Aldrich (St. Louis, MO, USA) as the substrate (Lakshmanan, Patterson, & Piggott, 2005) with slight modifications. Enzyme extract (1.0 ml) and 1.0 ml of substrate solution (0.125 mM L-Methionine-7-amido-4-methylcoumarin triflouroacetic salt in 100 mM Bis–Tris, 5 mM calcium chloride ($CaCl_2$), pH 6.5) were incubated for 10 min at 25 °C. By adding 2.0 ml of stopping reagent, consisting of 100 mM sodium monochloroacetate, 70 mM acetic acid and 30 mM sodium acetate at pH 4.3, the reaction was arrested. A

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