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Thermal inactivation kinetics of proteases and polyphenoloxidase in brown shrimp (*Crangon crangon*)

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ABSTRACT

To optimize product quality of the cooked brown shrimp (*Crangon crangon*), quantitative data on the influence of all relevant process parameters (treatment time and temperature) on several quality attributes is required. Surprisingly, kinetic data and models on heat induced inactivation of important endogenous spoilage enzymes of the brown shrimp are not available today. In this study the thermal inactivation kinetics of the most important spoilage enzymes, proteases and polyphenoloxidase (PPO), were determined from isothermal heat treatments of enzyme extracts of the cephalothorax. For both enzymes, inactivation kinetics showed first order decay(s). Proteases showed two distinct stability fractions. A labile fraction, representing 42 ± 2% of the total activity with $k_{l,60\ ^\circ C}$ = 0.94 ± 0.14 min^{-1} and $E_{a,l}$ = 178 ± 8.5 kJ/mol, and a stable fraction, representing 58 ± 2%, with $k_{s,60\ ^\circ C}$ = 0.020 ± 0.002 min^{-1} and $E_{a,s}$ = 155 ± 7.0 kJ/mol. PPO showed a single fraction with $k_{60\ ^\circ C}$ = 1.58 ± 0.02 min^{-1} and E_a = 161 ± 2.2 kJ/mol. Based on these results, the proteolytic activity, in particular the thermostable fraction, should be considered as a target in thermal processing of brown shrimp in relation to enzyme induced product quality changes during storage.

1. Introduction

Brown shrimp (*Crangon crangon*) is in Europe – in terms of value – amongst the top 5 species for the Dutch, German and Belgian fishery fleet (Aviat, Diamantis, Neudecker, Berkenhagen, & Müller, 2011; Platteau, Van Gijseghem, & Van Bogaert, 2014). It is of significant economic and social importance in the North Sea fisheries (Aviat et al., 2011). Brown shrimp are traditionally cooked and cooled on board of the vessel using seawater. Subsequently, the shrimp are stored in plastic bags on ice in the ship hold (Bon, 1996; FAO, 2015). As fishermen do not apply a standard cooking process and the important cooking parameters (temperature, time and salt content) vary from batch to batch, major quality differences can occur. Reports on the influence of these cooking parameters on the brown shrimp quality are scarce and there is clearly room for quality improvement of the cooked shrimp (Aviat et al., 2011; Bon, 1996; Denton & Prout, 1993). Studies on *Penaeus* species show that intensive heating and high salt concentrations result in high cooking losses up to 20% (Jantakoson, Kijroongrojana, & Benjakul, 2012; Manheem, Benjakul, Kijroongrojana, & Visessanguan, 2012; Niamnuy, Devahastin, & Soponronnarit, 2008). Changing the cooking parameters to optimize yield, might create the risk of losing some of the benefits of the heating process. By cooking, shelf-life is prolonged and a ready-to-eat product is created. Shelf-life is extended by inactivation of spoilage bacteria and endogenous spoilage enzymes (Ashie & Simpson, 1996). The doneness of the product is determined by heat-induced denaturation of muscle proteins (texture) and by the release of free astaxanthine from the crustacyanin complex (color) (Brookmire, Mallikarjunan, Jahncke, & Grisso, 2013; Skipnes, Van der Plancken, Van Loey, & Hendrickx, 2008).

To ensure a certain shelf-life, spoilage bacteria and enzymes have to be inactivated sufficiently. For convenience foods it is commonly accepted that mild thermal processing of fish products should at least provide a 6 log inactivation of *Listeria monocytogenes*. Generally a minimal pasteurization value $P_{70\ ^\circ C}$ of 2 min is suggested (ECFF, 2006). Yet, no kinetic data considering heat induced inactivation of important endogenous spoilage enzymes of brown shrimp are available.

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Polyphenoloxidase (PPO) is one of the most reported spoilage enzymes in crustaceans (Kim, Marshall, & Wei, 2000; Manheem et al., 2012; Martínez-Alvarez, López-Caballero, Gómez-Guillén, & Montero, 2009; Nirmal & Benjakul, 2009; Zamorano, Martínez-Álvarez, Montero, & Gómez-Guillén, 2009). PPO plays an important role in shell hardening after molding (Cobb, 1976). Post mortem, PPO induces the oxidation of phenol substrates to quinones, which are then further oxidized to melanins, causing black spot (Kim et al., 2000). Melanosis or black spot has a negative impact on consumer acceptability (Ruddy, 2007).

Proteolytic enzymes constitute a second group of important endogenous spoilage enzymes. Post-mortem, these enzymes can deteriorate muscle tissue, causing rapid flesh softening and loss of water binding capacity by breakdown of muscle tissue (Ahmed, Donkor, Street, & Vasiljevic, 2013; Ashie & Simpson, 1996; Hagen, Solberg, & Johnston, 2008; Huss, 1995; Lakshmanan, Patterson, & Piggott, 2005). Furthermore, proteases are capable of accelerating microbial spoilage trough the release of small peptides and free amino acids, both diminishing the sensory quality (Springett, 1996; Zeng, Thorarinsdottir, & Olafsdottir, 2005). Proteolytic enzymes are also reported as precursors for PPO (Manheem et al., 2012).

No data is available regarding the influence of process time and temperature on the inactivation of these endogenous spoilage enzymes in brown shrimp. Therefore, the aim of this study was to quantify the inactivation kinetics of PPO and proteases during thermal processing of brown shrimp. Insight in the spoilage enzyme inactivation kinetics can be a first step in a total optimization of the heating process.

2. Materials and methods

2.1. Chemicals

Disodiumhydrogenphosphate, trichloroacetic acid (TCA), acetic acid, sodium acetate, and Tween20 were obtained from Merck (Darmstadt, Germany). Citric acid, casein sodium salt from bovine milk, polyvinylpolypyrrolidone (PVPP) and 3,4-dihydroxy-DL-phenylalanine (DL-DOPA) were purchased from Sigma–Aldrich (St. Louis, MO, USA). All above mentioned chemicals were of analytical grade. Liquid nitrogen was purchased from Air Liquide (Paris, France).

2.2. Sample collection and preparation

Live brown shrimp with a minimal carapax width of 6.8 mm were bought from a local fisherman in Nieuwpoort, Belgium. The shrimp were transported at room temperature to the Institute for Agricultural and Fisheries Research within one hour. On arrival, the live shrimp were selected and decapitated. The cephalothorax was immediately frozen in liquid nitrogen. Preliminary experiments (data not shown) show, in accordance to Zamorano et al. (2009), highest proteolytic and PPO activity in the cephalothorax. 50 frozen cephalothoraxes were milled under liquid nitrogen by means of a centrifugal mill, equipped with a 0.75 mm sieve (Retsch Grindomix ZM 100, Retsch, Haan, Germany). The homogeneous powder was stored at −80 °C until further analysis.

2.3. Enzyme extraction

Proteases were extracted by dissolving the frozen powder in an extraction buffer (140 mM disodiumhydrogenphosphate, 37 mM citric acid, pH 6.4) at 4 °C in a 1:10 ratio. The mixture was centrifuged at 10,000*g* for 20 min at 4 °C (Sorvall LYNCX 6000 centrifuge, Thermo Scientific, Waltham, MA, USA). The supernatant was used as protease extract. The extract was stored on ice and used the same day.

PPO was extracted according to Simpson, Marshall, and Otwell (1988) with slight modifications. Frozen powder was dissolved in an extraction buffer (140 mM disodiumhydrogenphosphate, 37 mM citric acid, 2% PVPP, 0.2% Tween 20, pH 6.4) at 4 °C in a 1:4 ratio. The mixture was stirred for 30 min at 4 °C and centrifuged at 20,000*g* for 20 min at 4 °C. The supernatant was used as PPO extract. The extract was frozen in liquid nitrogen and stored at −80 °C until further use.

2.4. Heat treatments

Enzyme extracts were heated in capillary tubes (150 × 1.65 × 2.35 mm, Hirschmann, Eberstadt, Germany) to approach isothermal temperature profiles, in this way heating and cooling lags were minimized. The tubes were filled with 200 µl protease extract or 100 µl PPO extract and carefully sealed using a Bunsen burner. For the heat treatment, the capillary tubes were completely submerged in a water bath, of which the temperature was monitored using a calibrated thermometer. After the desired heating time, the tubes were removed from the water bath and immediately cooled in ice water for 1 min to stop any further inactivation. The tubes were broken and the treated samples were transferred to 2 ml micro centrifuge tubes (Eppendorf, Hamburg, Germany). All treated samples were frozen in liquid nitrogen and stored at −80 °C until further analysis. As a reference, two untreated samples were included for each treatment temperature.

To determine the relevant treatment temperatures for a detailed kinetic study, a preliminary screening was performed. Inactivation of both enzymes was screened by heating the extracts for 2 min at different temperatures (45–80 °C) in triplicate. Based on the results of these screening studies, a detailed kinetic study was performed by treating enzyme extracts isothermally at different relevant time–temperature combinations (0.5–30 min at 40–80 °C for proteases, 0.5–20 min at 45–65 °C for PPO).

2.5. Determination of enzyme activity

Proteolytic activity was analyzed according to Decker (1974) with slight modifications. A total volume of 75 µl protease extract was added to 265 µl substrate buffer (1.15% casein sodium salt from bovine milk, 80 mM disodiumhydrogenphosphate, 21 mM citric acid, pH 6.4) and incubated at 15 °C. After 60 min, enzyme activity was terminated by adding 750 µl of a stop solution (3.6% TCA, 3.6% sodium acetate, 4% acetic acid). This mixture was incubated for 15 min at 15 °C followed by cooling in ice water. Blanks were created by incubating substrate buffer without sample, 75 µl sample was added after the addition of the stop solution. In that way, proteolytic activity was avoided. After cooling, the samples and blanks were filtered over a PVDF 0.22 µm syringe filter (Merck). Activity was determined by measuring the difference in absorbance between a treated sample and its corresponding blank using a spectrophotometer (Jasco V660, Jasco, Easton, MD, USA) at 275 nm (A_{275}). Proteolytic activity was defined as the increase in absorbance per gram tissue per minute.

Thermally treated PPO extracts were defrosted at room temperature and centrifuged (Eppendorf Centrifuge 5417C, Eppendorf) at 20,000*g* for 5 min at room temperature. PPO activity was measured as described by Simpson et al. (1988). An aliquot of 50 µl of the supernatant was added to 700 µl of substrate buffer (10 mM DL-DOPA, 140 mM disodiumhydrogenphosphate, 37 mM citric acid, pH 6.4). This mixture was incubated at 20 °C for 20 min in a spectrophotometer (Jasco V660, Jasco) equipped with a thermostatic controller (Julabo MH-F25, Julabo, Seelbach, Germany). Absorbance was measured every 17.5 s at 475 nm (A_{475}). PPO activity