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Occurrence of aspartyl proteases in brine after herring marinating

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ABSTRACT

Herrings are marinated in a brine consisting of salt and acetic acid. During marinating, various nitrogen fractions diffuse from fish flesh to the brine, causing significant nutritional quality losses of the raw material. In this study, it has been demonstrated for the first time that proteases diffuse from the fish to the marinating brine. Using ammonium sulphate precipitation and affinity chromatography on pepstatin-A agarose bed the aspartyl proteases were purified and concentrated over 2600-fold from a marinating brine. Pepstatin-A completely inhibited the activity of the purified preparation. The preparation was active against fluorogenic substrates specific for cathepsin D and E and inactive against substrates specific for cysteine cathepsins. Depending on incubation time, the preparation showed pH-optimum at 2.0 or 4.5. The 2D SDS–PAGE separation demonstrated the presence of a few proteins with molecular weights and pI values typical of cathepsin D, E and pepsin.

1. Introduction

Fish marinades represent a high percentage of fish products in Europe. The ripening process of fish meat proceeds in a marinating brine (a solution of salt and acetic acid) and is principally based on proteolytic transformations. Fish marinating in brine takes at least one week; however, as early as after 2 days the pH value of the marinating brine increases from *ca.* 2.0 to 4.0, whereas that of meat decreases from over 6.5 to *ca.* 4.0. Hence, it is believed that mainly acidic proteases – the so-called cathepsins that occur naturally in muscle tissue of fish, are responsible for fish ripening (Shenderyuk & Bykowski, 1990).

Cathepsins are represented by more than 15 enzymes with various substrate specificity and cooperative action across a wide range of pH values and salt concentrations. The major and most frequently investigated aspartyl lysosomal protease is cathepsin D. Its activity is several times higher in muscles of fish than in muscles of mammals (Mukundan, Antony, & Nair, 1986). During hydrolysis, cathepsin D forms products that become substrates to exoproteases – cathepsin A and C (Kolodziejska & Sikorski, 1995; Makinodan, Toyohara, & Ikeda, 1983). In addition, An, Weerasinghe, Seymour, and Morrissey (1994) demonstrated that cathepsin D facilitates the release of other intracellular proteases.

Other proteases active at acidic pH may also be found in fish muscles, i.e.: cathepsin E, B, H and L, whereas pepsin occurs in the gastrointestinal tract of fish (Sriket, 2014). Literature addressing the effect of these proteases on the ripening of fish marinades is very scanty. For these reasons as well as due to low pH and profile of protein hydrolysis products in ripening marinades, cathepsin D is believed to initiate and predominate in the early stage of proteolysis in marinated fish (Levanidov, Ionas, & Sluckaja, 1987).

In our previous work, we described quantitative and qualitative losses of nitrogen fractions (protein, peptides and free amino acids) from muscle to marinating brine during herring marinating (Szymczak & Kołakowski, 2012). Probably, the process of diffusion concerns also the enzymes of herring. After the marinating process is complete, the content of salt and acid in the marinating brine usually reaches 2–5% and 2–3% (pH 3.6–4.2), respectively (Szymczak, Kołakowski, & Felisiak, 2012, 2015). These conditions facilitate spontaneous extraction of enzymes from the fish to the marinating brine. Results reported by other authors suggest that NaCl concentration in the marinating brine inhibits the activity of aspartyl proteases, including cathepsin D (Gomez-Guillen & Batista, 1997; Reddi, Constantinides, & Dymsza, 1972; Siebert, 1973).

Only a few works have so far demonstrated that fish processing may lead to diffusion of some enzymes (Gringer, Osman, Nielsen, Undeland, & Baron, 2014; Kilinc & Cakli, 2005; Stefánsson, Nielsen, & Gudmunsdóttir, 1995). To the best of our knowledge, apart from these studies, a thorough determination of the occurrence of acid aspartyl proteases, especially cathepsins, in marinating brines has never been performed.

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Considering the above, the aim of this study was to prove the presence of active acid aspartyl proteases in the brine remaining after herring marinating, by using isolation and determination of enzyme activity with methods specific for aspartyl proteases.

2. Materials and methods

2.1. Marinating brine

Marinating brine was obtained after marinating frozen-thawed Atlantic herring. Half kilogram of fillets with skin (mechanical filleting) was placed in glass jars, added (1.5:1) with the solution of brine including 6% NaCl and 5% acetic acid and marinated at 10 ± 1 °C for 7 days (Szymczak et al., 2012). Afterwards, the brine containing approximately 3% NaCl and 2.5% acetic acid (pH 4.0–4.1) was centrifuged at 4 °C and 10,000*g* for 10 min.

2.1.1. Purification

The supernatant of brine was subjected to ammonium sulphate fractionation. The precipitate in the saturation range of 0–80% was collected after centrifugation (4 °C, 10,000*g*, 10 min) and resuspended in Buffer **A** (50 mM citrate buffer: pH 3.3/0.6 M NaCl). After one-night dialysis against the same buffer, the solution was filtered through a 0.45-μm filter and the sample was introduced (0.5 mL/min) into a pepstatin-A agarose (P2032; Sigma–Aldrich, St Louis, MO) affinity column (1 × 8 cm). The column was washed with Buffer **A**, then with 25 mL of the same buffer containing 6 M urea, followed by 100 mL of Buffer **A** (1 mL/min). Aspartyl proteases were eluted with Buffer **B** (0.6 M NaCl prepared in 50 mM sodium bicarbonate buffer, pH 8.2) at a flow rate of 0.5 mL/min and collected in 5-mL fractions. The active fractions were pooled, dialysed overnight against 2 mM sodium phosphate buffer (pH 6.5) and freeze-dried. The entire purification procedure was performed at 4 °C. The dry snow-white residue was dissolved in 1 mL of water and analysed for cathepsin activity against fluorogenic substrate as described below.

2.2. Assay of proteases

2.2.1. Method with haemoglobin

The acidic protease activity was assayed with haemoglobin according to Anson (1938). Briefly, 2 mL of 2% acid-denatured haemoglobin (Hb) were added to the pre-incubated (10 min at 37 °C) 0.5 mL of brine with and without pepstatin-A (1 μM final concentration) and the mixture was incubated at 37 °C and pH 3.7–3.8 for 2 h. The reaction was stopped by the addition of 2.5 mL of 10% TCA, and the mixture was centrifuged after 30 min. Released tyrosine was measured with Lowry's assay (Lowry, Rosebrough, Farr, & Randall, 1951). In the control sample, TCA was added before Hb. One unit of enzyme activity (U_{Hb}) was defined as mg of tyrosine released per 1 L of brine per 2 h at 37 °C. All analyses were performed in three replications.

2.2.2. Methods with synthetic substrates

The activity of aspartyl proteases in the samples was measured against (***a***) Mca-GKPILFFRLK(Dnp)-r-NH_2 (Cathepsin D Assay Kit, CS0800; Sigma–Aldrich) and against (***b***) Mca-GSPAFLAK(Dnp)-r-NH_2 (PeptaNova, Concord, CA) using the instruction recommended by Sigma–Aldrich. An assay buffer (50 mM sodium acetate, pH 4.0) and brine (30 μL) or enzyme preparation (10 μL) were incubated at 37 °C for 10 min, with and without pepstatin-A. The reaction (100 μL) was started with the addition of 2 μL of substrate (final concentration 20 μM), and the release of MCA was followed at 37 °C for 5 min. The activities of cysteine proteases in the samples were measured against (***c***) Z-RR-AMC for cathepsin B and against (***d***) Z-FR-AMC for cathepsin B + L with the method of Barrett and Kirschke (1981). Fluorescence was measured with a microplate spectrofluorometer (Infinity M200Pro; Tecan, Männedorf, Switzerland) with excitation and emission wavelengths at 328 and 393 nm for aspartyl proteases, and at 322 and 460 nm for cathepsin B and L. One unit of enzyme activity (U_{MCA}) was defined as 1 nmol of MCA released from 1 mL of sample per minute at 37 °C.

The activity of pepsin (77160; Sigma–Aldrich) was measured after dissolving 5 μg of the preparation in 1 mL of water.

2.3. Estimation of optimum pH

For optimum pH estimation the enzyme preparation was incubated in McIlvaine buffer at pH values of 2.0–8.0. The activity was determined against fluorogenic substrate ***a*** for cathepsin D. All values presented are means of two independent determinations.

2.4. Determination of protein content

Protein content was determined as described by Lowry et al. (1951), with crystalline bovine serum albumin as a standard.

2.5. Electrophoresis

The lyophilised enzyme preparation (50 μg) was dissolved in the lysis buffer [5 M urea, 2 M thiourea, 4% w/v 3-[(3-cholamido propyl)dimethylammonio]-1-propanesulfonate (CHAPS), 40 mM Tris, 0.2% w/v 3–10 ampholytes and 100 mM dithiothreitol (DTT)] and applied onto 11-cm ReadyStrip™ IPG Strips (Bio-Rad, Hercules, CA) with non-linear pH gradient 3–10. The first dimension was run using Protean i12®IEF Cell (Bio-Rad) in total 90,000 V h. Prior to the second dimension of sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), focused IPG strips were reduced with DTT (Sigma–Aldrich) in the equilibration buffer (6 M urea, 0.5 M Tris/HCl, pH 6.8, 2% *w*/*v* SDS, 30% *w*/*v* glycerol and 1% *w*/*v* DTT) and alkylated with iodoacetamide (2.5% *w*/*v*). SDS–PAGE was run in 12% polyacrylamide gels in a Protean Plus™ Dodeca Cell™ electrophoretic chamber (Bio-Rad) at 40 V for 1 h and subsequently at 90 V for 15 h at 10 °C. After 2-DE separation, the gels were visualised with colloidal CBB G-250 according to Pink, Verma, Rettenmeier, and Schmitz-Spanke (2010). Image acquisition was performed using a GS-800 calibrated densitometer (Bio-Rad). Molecular weight of the analysed protein was determined using PDQuest 8.01 advanced bioinformatic software (Bio-Rad).

2.6. Statistical analysis

Results were analysed using 1-way analysis of variance (ANOVA) with StatSoft Statistica 10.0 (Statsoft, Tulsa, OK). The ANOVA *p*-value was set at 0.05, and the differences between treatments were examined using the post hoc Tukey's honestly significant difference test ($p < 0.05$).

3. Results and discussion

After 7 days of herring marinating, the brine was centrifuged as a result of which only dissolved enzymes were left in the supernatant. The enzyme activity was determined in the brine supernatant diluted 4–6 times with water/reagents decreasing NaCl concentration to 0.5–1.0%, sufficient to reduce the concentration of sodium ions that inhibit aspartyl proteases (own preliminary studies). The proteolytic activity was assayed against haemoglobin and synthetic substrates specific for cathepsins that are commonly