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Bovine cathepsin D activity under high pressure

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ABSTRACT

The stability and catalytic activity of bovine cathepsin D in Bis-Tris buffer (pH 6.0) in different pressure–temperature domains (0.1–650 MPa, 20–75 °C) were investigated and described with mathematical models. Cathepsin D inactivation followed first-order kinetics at all pressure–temperature conditions tested. The protease was largely pressure stable at room temperature and heat stable at ambient pressure up to 300 MPa and 55 °C, respectively, causing less than 10% inactivation after 10 min treatment. Pressure and temperature act synergistically on the enzyme inactivation under most conditions. However, at 100 MPa a significant stabilisation of the enzyme against temperature-induced inactivation was observed. Pressure drastically inhibited the cleavage of a synthetic substrate by cathepsin D in Bis-Tris buffer (pH 6.0) causing a reduction of the catalytic rate of more than 50% at 100–400 MPa. Maximal substrate cleavage by cathepsin D was identified at 60 °C and ambient pressure conditions after 20 min treatment.

1. Introduction

The application of high hydrostatic pressure offers some interesting opportunities for the processing of muscle based food products. It is well known that high pressure processing can prolong the shelf life of raw meat in combination with chilling (Carlez, Rosec, Richard, & Cheftel, 1994), but the pressure labile nature of some meat protein systems such as myosin or myoglobulin often limits the range of attractive commercial applications to pre-fermented and cooked meat products (Garriga, Grebol, Aymerich, Monfort, & Hugas, 2004). Alternatively, high pressure has been reported to affect the texture and gel-forming properties of myofibrillar proteins and hence, it has been suggested as a physical and additive-free alternative to tenderise and soften meat products (Cheftel & Culioli, 1997; Ichinoseki, Nishiumi, & Suzuki, 2006; Jung, De Lamballerie-Anton, & Ghoul, 2000a). However, the rate and magnitude at which pressure and temperature effects take place in muscles are variable and depend on a number of factors which are still not fully known and understood.

Tenderness is often considered to be the most important organoleptic characteristic of meat. However, meat tenderness is biologically complex and difficult to control during production, handling and processing (Maltin, Balcerzak, Tilley, & Delday, 2003). The time required for effective meat ageing is highly variable depending on many biological factors such as age, sex, muscle type as well as processing conditions (Tornberg, 1996). With beef, the suitable ageing time can extend to 10–15 days. This could lead to lower quality in microbiological and chemical aspects and is not economically attractive. Therefore, much research has been conducted to determine the mechanisms of meat tenderisation with the aim of improving meat tenderness and reducing the required duration for meat ageing.

It has been reported frequently in the literature that the application of high pressure may have a favourable effect on meat tenderness (Homma, Ikeuchi, & Suzuki, 1994; Ichinoseki et al., 2006; Jung, De Lamballerie-Anton, & Ghoul, 2000b; MacFarlane, 1985), but often conflicting results are obtained (Cheftel & Culioli, 1997; Cofrades, Banon, Carballo, & Jimenez Colmenero, 2003; Jung et al., 2000a, 2000b). Despite many years of studies, the underlying mechanisms responsible for post-mortem tenderizing of meat during ageing are still not fully understood (Bowker, Fahrenholz, Paroczay, & Solomon, 2008; Sancho, Jaime, Beltran, & Roncales, 1997; Sentandreu, Coulis, & Ouali, 2002). Regarding meat tenderisation under pressure it has been suggested that the synergistic activity of proteolytic systems, particularly cathepsins, could be responsible (Homma et al., 1994; Ichinoseki et al., 2006; Jung et al., 2000b). In this context, the role of cathepsins, and particular cathepsin D, in regular meat tenderisation without high pressure is still controversial and debated in literature. For example, cathepsin activities failed to explain differences in tenderness of meat samples (Whipple, Koohmaraie, Dikeman, & Crouse, 1990); actin and myosin, two proteins highly sensitive to the action of cathepsins, showed little or no degradation during meat ageing and cathepsin D inhibitors did not influence the ageing process (Uytterhaegen, Claeys, & Demeyer, 1994). In contrast, the general cysteine

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peptidase inhibitor, which inhibits both calpains and cathepsins, effectively prevented post-mortem proteolysis in rabbit meat and, thus, the major role in meat tenderisation was assigned to calpains (Mestre-Prates, Ribeiro, & Dias Correia, 2001).

However, the research efforts in meat science and the application of new methods for improving meat tenderness by accelerating the activity of cathepsins have partly clarified this controversy in more recent years. Much of the research on the effects of high pressure on meat tenderisation suggests that high pressure can improve the activity of cathepsins by both liberating them from lysosomes and/or accelerating its activity (Dufour, Dalgalarrondo, Herve, Goutefongea, & Haertle, 1995; Homma et al., 1994; Jung, De Lamballerie-Anton, Taylor, & Ghoul, 2000c). Furthermore, cathepsins are suggested to have maximum activity in particular combinations of temperature and pressure (Cofrades et al., 2003; Kurth, 1986).

Therefore, the objective of this study was to investigate the stability and catalytic activity of bovine cathepsin D (EC 3.4.23.5) in a model system at various high pressure–temperature combinations. The kinetic information of this study is then used to identify pressure–temperature combinations that maximise the activity of this protease and possibly provide an explanation for the observed tenderising effect of high pressure-heat treatments on post-rigor beef.

2. Materials and methods

2.1. Cathepsin D

Purified dried cathepsin D from bovine spleen was purchased from Sigma (#C-3138) and dissolved in pressure insensitive (Kitamura & Itoh, 1987) 10 mM Bis-Tris buffer (#B-4429, Sigma–Aldrich, St. Louis, MO, USA) at pH 6.0 to a concentration of 0.1 units/ml. This cathepsin D solution was then stored frozen at −18 °C until further use.

2.2. Enzyme assay

The cathepsin D assay used is based on Anson's (1938) and Rico, Toldra & Flores's (1991) procedures with slight modifications. A quantity of 0.5 ml enzyme solution was mixed with 1.5 ml citrate buffer (0.2 M, pH 3.7) containing 2% (w/v) denatured hemoglobin (#H-2625 Sigma–Aldrich, St. Louis, MO, USA). After 3 h incubation at 40 °C the reaction was terminated by the addition of 1.5 ml trichloroacetic acid (TCA, 10% w/v). After vigorously stirring, the precipitate was removed by filtration (Whatman 40, #1440070) and TCA-soluble peptides of the filtrate were measured spectrophotometrically at 280 nm (#UV-1700, Shimadzu, Kyoto, JP). The blank sample was prepared similarly but contained 0.5 ml 10 mM Bis-Tris buffer (pH 6.0) instead of the enzyme solution. The activity of cathepsin D was calculated by the difference in absorbance of the enzyme sample and the blank. All analyses were carried out in triplicate.

2.3. Isothermal and isobaric inactivation kinetics

The stability of bovine cathepsin D in Bis-Tris buffer (10 mM, pH 6.0) was investigated at isobaric/isothermal conditions in the range of 0.1–650 MPa and 20–75 °C. Isothermal treatments at ambient pressure were performed by immersing 5 ml glass test tubes containing approximately 1 ml enzyme solution in a temperature controlled water bath. Samples were withdrawn and immediately cooled in ice water after preset time intervals. Residual enzyme activity was measured within 120 min storage on ice. In order to obtain kinetics at isothermal conditions the initial enzyme activity (A_0) at time = 0 was defined as the activity detected after heating a sample to target temperature (in approximately 90 s) followed by immediate cooling in ice water.

Cathepsin D inactivation kinetics of combined pressure–temperature treatments were obtained from isothermal/isobaric experiments as described previously (Buckow, Isbarn, Knorr, Heinz, & Lehmacher, 2008). A quantity of 1.5 ml of enzyme solution was transferred into cryo vials (#5000-1012, Nalgene, Rochester, NY, USA) and stored on ice. The cooled samples were inserted in the pressure vessel preheated to target temperature (#U111, Unipress, Warsaw, PL). Compression was commenced when the temperature of the sample reached the target temperature (±1 °C) due to the compression heating during the pressure come-up time. Temperature measurement of the sample was conducted with a thermocouple placed directly in the middle of the cryo tube. The compression rate was standardised at approximately 20 MPa/s to minimise the loss of enzyme activity during the pressure build-up. Dwell time was started immediately after the desired pressure–temperature conditions were reached. The initial enzyme activity (A_0) at time = 0 was defined as the activity detected after the pressure was increased to the target pressure followed by immediate pressure release. Pressure release was performed at a rate of approximately 150 MPa/s. After the pressure treatment, samples were immediately cooled and stored for up to 4 h on ice. No reactivation of bovine cathepsin D was observed in pressure or heat treated samples after cooling on ice for 4 h and within 24 h of subsequent storage under refrigerated conditions (4 °C).

2.4. Isothermal and isobaric kinetics of substrate conversion

Myoglobulin was considered to be impractical for measurement of cathespin D catalytic activity under pressure as the hydrolysis rate is reported to be fairly slow, particularly at pH 6.0 (Anson, 1938). Thus, a biotinylated fluorescent peptide substrate with high affinity and sensitivity to cathepsin D (Baechle et al., 2005) was obtained (SensoLyte™ 520 Cathepsin D Assay Kit, AnaSpec, San Jose, CA, USA) and used in this part of the study. Hydrolysis of this fluorescence resonance energy transfer (FRET) substrate by cathespin D at ambient pressure was performed by adding 100 µl cathespin D solution (0.2 U/ml in 10 mM Bis-Tris buffer, pH 6.0) to 1.42 ml substrate solution (0.5 mM DTT + 0.0125 mM FRET substrate in 10 mM Bis-Tris buffer, pH 6.0), which was heated to the desired temperature as described in the previous section. Samples were kept at different temperatures (20–60 °C) for up to 30 min. After different times, tubes were removed and the reaction was terminated by addition of 1 ml 10% TCA. The samples were then stored on ice until the fluorescence was measured in a spectrophotometer (Cary Eclipse Fluorescence spectrophotometer, Varian, Palo Alto, CA, USA). FRET substrate cleavage by cathepsin D results in an increase of 5-carboxyfluorescein (5-FAM) fluorescence, which was monitored at room temperature and at an excitation/emission of 490 nm/520 nm.

For the measurement of the catalytic activity of cathepsin D under high pressure conditions, cooled enzyme and substrate was mixed in cryo vials (#5000-1012, Nalgene, Rochester, NY, USA), which where then quickly placed into the heated high pressure rig (#U111, Unipress, Warsaw, PL). A thermocouple was placed inside the tubes to monitor the temperature. Pressurisation was initialised when the sample reached a temperature which resulted in the desired target temperature after compression. Samples were kept at different p–T conditions (100–400 MPa, 20–60 °C) for up to 30 min. The compression and decompression rates were approximately 20 and 150 MPa/s, respectively. After pressure release, the samples were removed from the vessels and the reaction was terminated immediately by addition of 1 ml 10% TCA. Subsequently, the fluorescence of the samples was measured as described above.