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E. Dufour, Michèle Dalgalarrrondo, Guillaume Hervé, R. Goutefongea, Thomas Haertlé. Proteolysis of type III collagen by collagenase and cathepsin B under high hydrostatic pressure. Meat Science, 1996, 42 (3), pp.261-269. hal-02694822

HAL Id: hal-02694822

<https://hal.inrae.fr/hal-02694822>

Submitted on 1 Jun 2020

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Proteolysis of Type III Collagen by Collagenase and Cathepsin B Under High Hydrostatic Pressure

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(Received 25 August 1995)

ABSTRACT

The effects of high hydrostatic pressures on the kinetics of hydrolysis of type III collagen from calf skin by collagenase and cathepsin B were studied. Collagen hydrolysates sampled at different time intervals (0–90 min) and at different pressures (0.1–300 MPa) were analysed by reverse-phase high pressure liquid chromatography. The rate of collagen hydrolysis decreased up to 300 MPa for both enzymes. The rate of collagen hydrolysis with cathepsin B was drastically reduced between 0.1 and 100 MPa. Significant differences in the populations of the peptides in cathepsin B hydrolysates were observed in chromatograms corresponding to different pressures. This indicated that some amino acid side-chains were less exposed to cathepsin B recognition on the surface of collagen molecules at high pressures. In contrast, the chromatograms of collagenase hydrolysates showed similar patterns, varying only by the peak heights in chromatograms corresponding to the 0.1–300 MPa pressure range. Pressure-induced decreases of the enzyme-apparent activities suggested that the activation volumes for the reaction of both enzymes were positive.

INTRODUCTION

Tropocollagen, the basic collagen structure, consists of three helical polypeptide chains intertwining to form a right-handed superhelix. Collagen fibrils (types I, II and III) are formed by interaction between the telopeptides of one collagen monomer and the triple-helical domain of one or more adjacent collagen monomers (Light & Bailey, 1980). Telopeptides are small non-triple-helical extension peptides at the N- and C-terminal ends of collagen α -chains (Otter *et al.*, 1993). Collagen is the protein conferring resistance to mechanical stress on bones, cartilages, tendons and muscle connective tissues. It is predominantly responsible for the toughness of meat from aged animals, probably the most critical quality trait for the consumer. The meat quality of muscles with a high

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connective tissue content is, and could be still further, improved by specific proteolysis by endogenous or added proteases.

Collagenases are by definition the only proteases to efficiently digest the native collagen under physiological conditions. Bacterial collagenases generally cleave the triple-helical collagen at the X-Gly bond in X-Gly-Pro-Y sequences (Nagai, 1961; Steinbrink *et al.*, 1985). Although the triple-helical collagen molecule is extremely resistant to degradation by non-specific proteases, cathepsins B and L (Burleigh *et al.*, 1974; Kirschke *et al.*, 1982; Maciewicz & Etherington, 1988; Beltran *et al.*, 1992) have been shown to cleave native collagen, inducing the depolymerization of cross-linked fibres. Although the cleavage sites of cathepsins on collagen molecules have not yet been fully characterized, it has been suggested that these proteases act primarily on the telopeptides (Kirschke *et al.*, 1982). Based on differential scanning calorimetry studies, Beltran *et al.* (1992) suggested, however, that cathepsin B and bacterial collagenase may have similar mechanisms of action on insoluble collagen.

High hydrostatic pressures perturb protein structure (e.g., folding, specific volume, flexibility) and enzyme activity. In contrast to fibrous proteins, the interior of globular proteins is highly compressible (Gekko & Hasegawa, 1986). The thermal stability of collagen (Gekko & Koga, 1983) and gelatin gels (Gekko & Fukamizu, 1991) is enhanced under high pressure. These results indicate that the volume change following the helix to coil transition of collagen is positive in water. Protein-water interactions play an important role in the stability of collagen structure (Gekko & Koga, 1983). Most globular proteins are denatured in the 400–600 MPa pressure range and these conformational changes increase the solvent-accessible surface area, i.e., hydration of proteins (Heremans, 1982). The activity of several proteinases such as thermolysin (Fukuda & Kunugi, 1984), trypsin (Groß *et al.*, 1993) and cathepsin B (Kurth, 1986) is enhanced by moderate hydrostatic pressure (below 200 MPa). The rate of gelatin hydrolysis with pepsin is, however, decreased by high pressures (Laidler, 1951). In general, catalytic rate constants of enzymatic (and non-enzymatic) reactions are modified by high pressures. Pressure modifies both kinetic constants (depending on the catalyzed reaction), and protein structure—what is produced by pressure induced conformation changes of substrate and/or enzyme as, for example, altered enzyme flexibility (Groß *et al.*, 1993). High pressures in the 100–300 MPa range might be a way to increase proteolytic activities in meat and, as a consequence, the quality of aged meat (Homma *et al.*, 1994).

In the present study, we have examined the influence of pressures in the range 0.1–300 MPa on collagenase and cathepsin B hydrolysis of type III collagen. Aliquots withdrawn at various time intervals have been analysed by reverse-phase HPLC. Pressure-dependent kinetics of the hydrolysis of type III collagen by collagenase and cathepsin B are reported.

MATERIALS AND METHODS

Trifluoroacetic acid (TFA) was obtained from Pierce Chemicals (Rockford, IL, USA). Collagen (type III, acid soluble, from calf skin), cathepsin B (from bovine spleen) and collagenase (type F from *Clostridium histoliticum*) were from Sigma (St Quentin Fallavier, France) and were used without further purification. All other reagents were of analytical grade. Collagen stock solution (2 mg/ml) was prepared in 10 mM HCl.

The HPLC equipment consisted of an auto-sampling injector (Gilson model 231), assisted by a chromatography work station (Maxima 820, Waters), two solvent delivery systems (Waters model 510), a temperature control system and a variable wavelength U.V. monitor (Waters model 455).

High pressure apparatus

The pressure bomb described by Hui Bon Hoa *et al.* (1990) was used in this study. It allows intermittent sampling without release of pressure in the reaction chamber. Its maximum operating pressure is 400 MPa and the dead time of sampling is 10–15 s. The reaction medium (volume = 17 ml) was stirred and thermostated in the high pressure chamber.

Hydrolysis of collagen by collagenase and cathepsin B

Collagenase hydrolysis

The collagen stock solution was diluted to 1 mg/ml with 0.2 M Tris buffer pH 7.5 containing 20 mM CaCl₂. Collagenase (0.5 mg/ml) was dissolved in 0.1 M Tris buffer, 10 mM CaCl₂, pH 7.5 and added to the incubation medium at an E/S ratio (enzyme/substrate ratio) of 0.5% (w/w). The reaction mixture was incubated at 27°C at pressures ranging between 0.1 and 300 MPa. Aliquots (0.8 ml) were withdrawn at intervals of 0, 20, 40, 60 and 90 min. The reaction was stopped by the addition of 80 µl of 2 M HCl.

Cathepsin B hydrolysis

The collagen stock solution was diluted to 1 mg/ml by adding 0.1 M Tris/acetate buffer, 4 mM EDTA, 10 mM DTT, pH 6.5. Cathepsin B (1 mg/ml) was dissolved in 50 mM Tris/acetate buffer, 2 mM EDTA, pH 6.5 and added to the reaction mixture at an E/S ratio of 1.3% (w/w). During incubation at 35°C at different pressures (0.1, 100, 200 and 300 MPa), aliquots (0.8 ml) were withdrawn at intervals of 0, 20, 40, 60 and 90 min. The reaction was stopped by the addition of 0.2 M iodoacetic acid (0.8 ml).

All the pH values reported are those measured at 0.1 MPa. As described previously, the pH of Tris buffer increases by 0.02 units per 100 MPa (Neuman *et al.*, 1973).

HPLC analysis of collagen hydrolysates

Collagen hydrolysates were analysed by reversed phase HPLC using a Nucleosil C18 column (4.6 mm diameter × 25 cm, SFCC, Gagny, France). The column was equilibrated with 100% solvent A (0.11% TFA in H₂O). In the case of collagenase hydrolysates, elution was performed with a linear gradient from 0 to 60% solvent B (H₂O 40%, CH₃CN 60%, 0.09% TFA) in 39 min. In the case of cathepsin B hydrolysates, isocratic elution in 100% solvent A was performed during the first 15 min to remove salts, then elution was continued with a linear gradient to 60% solvent B in 39 min. In both cases, the temperature of column was maintained at 30°C and absorbance was measured at 214 nm. The data were acquired on a NEC APC-IV microcomputer.

For all chromatograms, collagen and peptide yields were determined by peak integration using Maxima 820 software (Waters).

RESULTS

The disappearance of type III collagen hydrolysed with collagenase and cathepsin B at various pressures is shown in Fig. 1A and B, respectively. The area of the two collagen peaks (see Discussion and Fig. 2A) was used to measure the disappearance of collagen by proteolysis. The rate of collagen hydrolysis decreased with increasing pressure up to 300 MPa for both enzymes. However, the trends are different for the two enzymes. It was

Fig. 1. Disappearance over time of collagen (expressed as its normalized HPLC peak area) after hydrolysis by collagenase (A) and cathepsin B (B) as a function of pressure. (○): 0.1 MPa; (●): 100 MPa; (▽): 200 MPa; (▼): 300 MPa.

observed that the rate of collagen hydrolysis with collagenase decreased linearly in the 0.1–200 MPa pressure range. In contrast, collagen hydrolysis with cathepsin B showed the greatest reduction between 0.1 and 100 MPa. After 90 min, 85, 35 and 25% of the collagen had been hydrolysed at 0.1, 100 and 200 MPa, respectively.

Figure 2 shows the chromatographic profiles of collagen and collagen hydrolysates obtained with collagenase at 0.1, 100 and 200 MPa after a 60 min incubation. Collagen was eluted as two peaks (retention times = 33.0 and 35.3 min) under the conditions used (Fig. 2A). The chromatograms of the collagenase hydrolysates in the 100–300 MPa pressure range resemble one another, with some variation in peak heights (Fig. 3). The yield of peak at 10.18 min was decreased significantly only at 300 MPa; but the amounts of peaks at 22.3 min and 27.2 min were reduced drastically at high pressure (Fig. 3). Due to the occurrence of glycine and proline residues in the $\alpha 1$ -chains, collagen has a very large number of potential cleavage sites for collagenase. It appears that most of them remain capable of being hydrolysed by collagenase under high pressure, but the rate of release is decreased. In contrast with collagenase, cathepsin B hydrolysates of collagen showed different chromatographic profiles as a function of pressure (Fig. 4). As shown in

Fig. 4A, peaks corresponding to dithiothreitol derivatives were eluted at 25.5 and 30.2 min. At atmospheric pressure, most of the initial collagen disappeared after 90 min. Numerous products of collagen hydrolysis were eluted between 35 and 46 min with the highest peak at 45.6 min. However, at higher pressures, the peptides eluted between 35

Fig. 2. Reverse-phase HPLC analysis of collagen hydrolysates obtained after 60 min hydrolysis by collagenase at different pressures. (A) collagen; (B) 0.1 MPa; (C) 100 MPa; (D) 200 MPa. Chromatography conditions are as described in Materials and Methods.

Fig. 3. Time course of the evolution of four peptides as a function of pressure: (A) peak at 10.18 min; (B) peak at 17.20 min; (C) peak at 22.30 min; and (D) peak at 27.2 min. For peptide numbering see Fig. 2. Pressures are: (○): 0.1 MPa; (●): 100 MPa; (▽): 200 MPa; (▼): 300 MPa.

and 43 min were not observed, even after 90 min incubation (Fig. 4C and D). After 90 min, only the peak at 45.6 min was still observed. This suggests that in the tropo-collagen molecule, only a few proteolytic sites are accessible to cathepsin B under high pressure.

DISCUSSION

As reported by Stoltz *et al.* (1972), the observed heterogeneity of the collagen used (Fig. 2A) could result from the hydrolysis of a small portion of the telopeptide during purification. Previous studies (Burleigh *et al.*, 1974; Kirschke *et al.*, 1982; Maciewicz & Etherington, 1988; Beltran *et al.*, 1992) have shown that cathepsins B and L hydrolyse collagen in solution, as well as collagen fibrils and insoluble collagen. The β -components of collagen fibrils are degraded by cathepsin B giving α -chains (Burleigh *et al.*, 1974). This has been interpreted as an attack on the non-helical peptide regions eliminating cross-linkages. The major peak observed at 45.6 min (and eluted just before collagen) could result from the removal of collagen telopeptides by cathepsin B. This suggests that the enzyme is almost unable to attack the collagen triple helix.

The patterns of collagenase hydrolysates obtained at 0.1 MPa are different from the patterns seen with cathepsin B. No high molecular weight component is left after

Fig. 4. Reverse-phase HPLC analysis of collagen hydrolysates obtained after 90 min hydrolysis by cathepsin B at different pressures. (A) collagen + DTT; (B) 0.1 MPa; (C) 100 MPa; (D) 200 MPa. Chromatography conditions are as described in Materials and Methods.

hydrolysis with collagenase. The collagen $\alpha 1$ -chain is rapidly degraded by collagenase to lower molecular weight peptides than the peak with 45.6 min retention time (cathepsin B hydrolysates; Fig. 4). These observations imply that cathepsin B and collagenase have different mechanisms of collagen breakdown. Cathepsins are able to degrade collagens within their non-helical domains (Evans & Etherington, 1978; Maciewicz *et al.*, 1990). Collagenases are the only proteases to be able to cleave the triple helical collagen.

This work was designed to address two questions: could high pressure increase the rate of collagen proteolysis? What are the effects of high pressure induced conformational changes and stability of collagen structure on the outcome of proteolysis?

The results indicate that the rates of collagen hydrolysis decrease with increasing pressure. It is well known that high pressures induce conformational changes on both substrates and enzymes. Pressure also modifies the flexibility of enzymes. Globular proteins (e.g., enzymes) exhibiting a positive compressibility are more subject to a decrease of their flexibility than fibrous proteins (Gekko & Hasegawa, 1986). As suggested by Groß *et al.* (1993), pressure induced reduction in partial specific volume may reduce flexibility and hence inhibit enzymatic activity. However, the (apparent) activity of cathepsin B is increased by high pressure (up to 150 MPa) using synthetic substrates (Kurth, 1986) suggesting that the enzyme is not inhibited by high pressures. Despite that to our knowledge there are no kinetic data for collagenase under high pressure, pressure-dependent increasing activity has been reported for thermolysin (Fukuda & Kunugi, 1984; Groß *et al.*, 1993), a zinc protease like collagenase. In addition, it has been shown that the rates of β -lactoglobulin hydrolysis by thermolysin (Dufour *et al.*, 1992) and pepsin (Dufour *et al.*, 1995) increase with pressure up to 200 MPa. In contrast, Laidler (1951) has reported that the rate of gelatin hydrolysis by pepsin decreases with increasing pressure.

Globular and fibrous proteins display different compressibilities under high pressure. The partial specific adiabatic compressibility of gelatin, but not of globular proteins, is negative due to the predominance of hydration effects (Gekko & Hasegawa, 1986). Furthermore, the enhanced stability of collagen under high pressure indicates that the volume change following the helix to coil transition of the protein is positive in water (Gekko & Koga, 1983).

It is known that pressure modifies also the kinetic constants of chemical reactions. The logarithm of the rate of reaction changes linearly with pressure and the slope of the line corresponds to the activation volume (Groß *et al.*, 1993). Decreasing activity with increasing pressure implies a positive volume of activation, and vice versa. Activation volumes measured for thermolysin using dipeptides as substrates are negative (Fukuda & Kunugi, 1984; Groß *et al.*, 1993). Similarly, the enhanced activity of cathepsin B toward *N*-benzoyl-(D,L)-arginine-2 naphthylamide substrate under high pressure (Kurth, 1986) implies a negative volume of activation. In the case studied of collagen proteolysis, it is impossible to evaluate activation volume since many peptides' bonds are cleaved. The effect of pressure on the kinetics of each cleavage site cannot be distinguished, but the volume change of the reaction can be calculated according to Laidler (1951). Assuming that the state in question is that of an ideal gas, the slope of the logarithmic plot of hydrolysis rate vs pressure allows derivation of the volume change of the reaction. Studies of β -lactoglobulin hydrolysis by thermolysin under high pressure also indicate that the activation volume is negative (Dufour *et al.*, 1992). In contrast, the apparent activities of cathepsin B and collagenase, using collagen as substrate are decreased under high pressure. Our results, as well as the study of gelatin hydrolysis by pepsin under high pressure (Laidler, 1951), reveal a positive activation volume for these reactions. Considering this thermodynamic property of collagen, and of fibrillar proteins in general, under high pressure, it may seem unlikely that high pressure could be used to increase specific proteolysis of collagen in meat. This speculation is in good agreement with the

results obtained by Suzuki *et al.* (1993). In their report, no significant differences in the ultrastructure, electrophoretic pattern, thermal solubility and thermogram of differential scanning calorimetric analysis of the intramuscular collagen were observed among the pressurized and untreated muscles. Nevertheless, high pressure is a valuable tool used to perturb the structure of proteins and to modify the outcome of globular protein hydrolysis with various proteases (Dufour *et al.*, 1995; Homma *et al.*, 1994).

ACKNOWLEDGEMENTS

This work was supported by the Ministère de la Recherche et de la Technologie (M.R.T. 91 G 0574).

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