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High-Pressure Effects on the Proteolytic Enzymes of Sea Bass (*Dicentrarchus labrax* L.) Fillets

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High-pressure processing is a nonthermal technique ensuring food product safety and enabling a longer shelf life. The purpose of this experiment was to evaluate the effect of high pressure on the main proteolytic enzymes involved in fish muscle degradation during storage. Enzymes were extracted with sarcoplasmic proteins from *Dicentrarchus labrax* sea bass white muscle. Activity of cathepsins B, D, H, and L was quantified in protein extract, whereas calpain activity was evaluated after isolation from its endogenous inhibitor. High-pressure processing up to 500 MPa enhanced the activity of cathepsin B, H, and L, whereas the activity of cathepsin D increased up to 300 MPa and decreased above 300 MPa. With regard to calpain activity, high-pressure processing led to a decrease of activity, which was zero above 400 MPa. We suggest a leading explanation based on simultaneous deactivation of enzymes and an increase of liberation from lysosomes for cathepsins and on dissociation of subunits for calpains.

KEYWORDS: High pressure; fish; protease; calpains; cathepsins

INTRODUCTION

High-pressure treatment is a nonthermal technology of growing interest for the processing and preservation of food in particular muscle-based foods. Some high-pressure treated products are already commercially available in Japanese (fruit juices, jam, soybeans, pasta, rice, and seaweeds), United States (avocado spread and oysters), and European (orange juice in France and ham in Spain) markets.

Generally, products are subjected to high pressure in the range of 100−1000 MPa. The main advantage of high pressure is to inactivate pathogenic microorganisms to extend the food shelf life even if it appears that complete microbial inactivation is currently not possible (*1*, *2*). The advantage of high-pressure treatment over traditional thermal processing rests on a better retention of nutritional and organoleptic characteristics. However, chemical bonds of molecules are affected and that may induce modifications in water, proteins, polysaccharides, and lipids. High pressure mainly causes changes in hydrophobic and electrostatic molecular interactions, with important consequences for the secondary, tertiary, and quaternary structures of proteins. In food muscle, high-pressure treatment affects enzymatic activities as well as proteins (mainly myofibrillar ones), resulting in structural modifications and texture changes (*3*). Pressure treatment of proteins can lead to significant conformational changes that influence functionality (*4*, *5*, and *6*).

The postmortem period is essential in the aging process of the muscle. In contrast with mammalian muscles, the fish muscle must keep a firm texture according to quality criteria, but tenderization softens the tissue and leads the muscle to be deteriorated. The mechanisms responsible for this biochemical phenomenon are complex and still not very clear. It is due to the ability of proteases to hydrolyze different proteins in the muscle. Thus, it is recognized that the proteolysis of myofibrillar proteins plays a key role in tenderization. Two characterized proteolytic systems are usually described to hydrolyze myofibrillar proteins, the calpains and cathepsins (*7*, *8*).

The calpains (EC 3.4.22.17), intracellular cysteine proteases, are calcium-dependent. These enzymes are further subclassified into μ-calpain and m-calpains, which differ in calcium ions concentration necessary for activation. They require respectively micromolar and millimolar concentrations of calcium. Moreover, a third isoform has been identified in sea bass muscle (*9*). Calpains are heterodimers composed of a large subunit and a small subunit that have molecular masses of about 80 and 28 kDa, respectively. Calpastatin is the endogenous inhibitor specific to calpains.

The cathepsins are acid proteases found packed in the vesicles called lysosomes (*10*). They are often inactive in living tissue and can be released into both the cytoplasm and the intracellular spaces as a consequence of lysosomal disruption, particularly

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during postmortem storage. Lysosomes are known to harbor about 13 types of cathepsins (*10*). Among these lysosomal proteases, four classes can be distinguished according to their active sites: aspartic, cysteine, metallo-, and serine proteases. The main cathepsins involved in muscle aging are cathepsins B (EC 3.4.23.1), L (EC 3.4.22.15), H (EC 3.4.22.16), and D (EC 3.4.22.5). Cathepsins B, H, and L (cysteine proteases) are regulated in vivo by a protease inhibitor called cystatin (*11*). Cathepsin D (an aspartic acid protease) is considered to be the most important enzyme in postmortem degradation of bovine muscle because of its optimum pH and the absence of a specific inhibitor in the muscle. Moreover, cathepsin D produces flavor-related compounds during conditioning.

The effects of each protease on muscle proteins during postmortem tenderization are not yet fully understood. However, most of the results report on a synergistic proteolytic action of calpains and cathepsins on key myofibrillar proteins. This may be of great importance when high-pressure treatment of fish flesh and behavior of proteolytic enzymes are considered. Thus, the aim of our study was to understand how the proteases behave after a high-pressure treatment of the fish muscle or of the enzymatic extract, to gain insight into high-pressure effects on muscle proteolysis.

MATERIALS AND METHODS

1. Materials. Unless specified, chemicals were purchased from Sigma−Aldrich (Saint-Quentin Fallavier, France). Chromatographic gels were from Amersham Biosciences (Uppsala, Sweden).

2. Preparation of the Fish Samples. Twenty farmed fresh sea bass (*Dicentrarchus labrax L.*), 4 years old, average weight of 325 g and total length of 30 cm, were collected from a local aquaculture farm (Les Viviers du Gois, Beauvoir-sur-Mer, France) and brought back alive to laboratory. Fish were killed by decapitation and filleted and skinned in prerigor conditions. The white muscles were minced together, vacuum-packed in 30 g portions, and frozen at −80 °C until use.

3. Preparation of Sarcoplasmic Proteins from Sea Bass Muscle. A 30 g portion of frozen minced muscle was homogenized twice for 30 s with Ultra Turrax (T25, IKA, Labortechnik, Staufen, Germany) equipped with an 18 mm diameter head (S 25−18 G) in 90 mL of buffer A containing 50 mM Tris-HCl (pH 7.5), 10 mM β-mercaptoethanol, and 1 mM ethylenediaminetetraacetic acid (EDTA). After centrifugation at 10000*g* (GR 20.22, Jouan, France) for 40 min at 10 °C, the supernatant was filtered through a 0.45 μm filter. Three sarcoplasmic extracts were prepared for each pressure value.

4. Purification of Calpains from Muscle. The entire procedure was carried out at 4 °C. The chromatographic column (phenyl-Sepharose, ∅ 26 mm, length 10.5 cm) was balanced with equilibration buffer composed of 50% buffer A and 50% buffer B (buffer A with 1 M NaCl).

Fifty milliliters of sarcoplasmic extract with added 0.5 M NaCl to the desired final concentration was directly run into the chromatographic column. The nonabsorbed proteins, including calpastatin, the endogenous inhibitor of calpains, were washed through with the equilibration buffer. The calpain active fraction was then eluted in batch with buffer A. These different protein peaks were collected on ice.

5. High-Pressure Processing. High-pressure processing was carried out in a 3.5 L reactor unit (ACB Pressure Systems, Nantes, France) equipped with a temperature and pressure regulator device (**Figure 1**). Prior to pressure processing, sea bass muscle, sarcoplasmic extracts, and calpain extracts previously prepared were vacuum-packed individually in polyethylene bags (La Bovida, France). Samples were subjected to high-pressure treatment between 50 and 500 MPa (±7 MPa) for 5 min. The expected high pressure was reached at 3 MPa/s, and after 5 min was then quickly released (<1.5 s). Temperature of transmitting medium in the vessel was settled at 10 °C (±5 °C) during pressure treatment. Temperature of the cooling jacket that surrounded the pressure vessel was also controlled at 10 °C during pressure treatment.

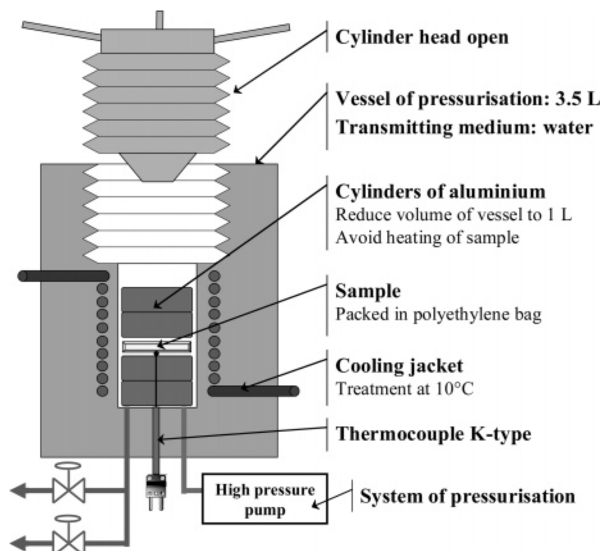

Figure 1. High-pressure vessel from ACB pressure systems.

One thermocouple K-type (0.3 mm diameter, Omega, Stamford, CT) positioned close to the sample allowed us to follow variation during treatment.

6. Determination of Proteins. The amount of proteins was evaluated by two methods: (i) the quantity of proteins was assessed by measurement of the optical density at 280 nm (absorbance method); (ii) alternatively, total protein was assayed by the Biuret method according to Gornall et al. (*12*). Calibration of the assay was performed with standard bovine serum albumin solution. The values were the means of three measurements for each sample.

7. Calpain Activity Measurement. Calpain activity was determined in triplicate at 30 °C in a 303 μL reaction mixture containing 3 μL of 0.5 M $CaCl_2$, 6 μL of 5% CHAPS {3-[3-(cholamidopropyl)dimethylammonio]-1-propanesulfonate}, and 5 μL of 20 mM synthetic fluorogenic substrate SucLT (*N*-succinyl-Leu-Tyr-7-amido-4-methylcoumarin) prepared in methanol. The reaction was initiated by adding 255 μL of enzymatic sample. During the 2 h reaction, fluorescence was monitored in microplate wells by use of the spectrophotofluorometer FLUOstar Optima POLARstar Optima reader (BMG Labtech, Champigny sur Marne, France) with an excitation wavelength set at 355 nm and emission wavelength set at 460 nm. A control in which 3 μL of 0.5 M $CaCl_2$ is replaced by 3 μL of 0.5 M EDTA was also performed. The activity was expressed in FU (units of fluorescence) per minute per gram of protein in extract. The values were the means of three measurements for each sample.

8. Activity Measurement of Lysosomal Enzymes. *8.1. Cathepsin D.* Cathepsin D activity was determined with hemoglobin as a substrate according to Anson's method (*13*). Activity was determined at 37 °C on a 1000 μL reaction mixture containing 250 μL of 0.2 M acetate/acetic acid (pH 4) buffer, 10 mM β-mercaptoethanol, 1 mM EDTA, and 250 μL of 2% (w/v) denatured hemoglobin. The reaction was initiated by adding 500 μL of sarcoplasmic protein extract and later stopped by adding 125 μL of 10% trichloroacetic acid (TCA) to 125 μL of mixture reaction sampled at different interval times. After an overnight incubation at 4 °C, the sample was centrifuged at 18000*g* for 15 min at 10 °C. An aliquot (150 μL) of supernatant was reacted with 150 μL of Bio-Rad protein assay reagent (Bio-Rad Laboratories GmbH, München, Germany) for the quantification of TCA-soluble peptides released by digestion. The absorbance was measured spectrophotometrically at 595 nm. The activity was expressed as absorbance units at 595 nm per minute per gram of protein in extract. The values were the means of three measurements for each sample.

8.2. Cathepsins B, L, and H. B, H, and L cathepsin activities were determined at 30 °C in a 298 μL reaction mixture containing 70 μL of 0.4 mM acetate/acetic acid (pH 4) buffer, 10 mM β-mercaptoethanol, 1 mM EDTA, 6 μL of 5% CHAPS, 1 μL of 1.40 M 2-mercaptoethanol, 16 μL of 5% (w/v) Brij 35, and 5 μL of synthetic fluorogenic substrate prepared in methanol at 20 mM. Z-Arg-Arg-7-amido-4-methylcoumarin

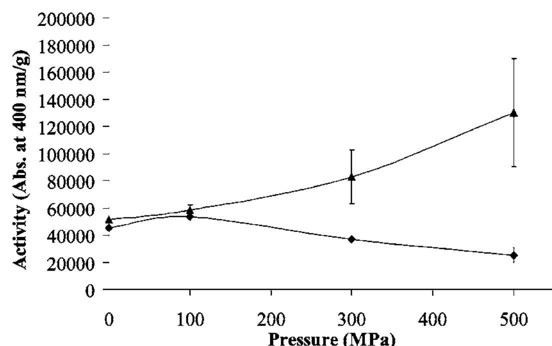

Figure 2. Evolution of phosphatase acid activities when the sarcoplasmic extract is pressurized (◆) and when the white muscle is pressurized (▲) during 5 min. Results are means of three samples; vertical bars represent the standard deviation.

hydrochloride, Z-Phe-Arg-7-amido-4-methylcoumarin hydrochloride, and L-Arg-7-amido-4-methylcoumarin hydrochloride are used as the substrates for cathepsins B, B + L, and H, respectively. The reaction was initiated by adding 200 μL of protein extract. A control with buffer A instead of enzymes was run in parallel. The activity was expressed in FU (units of fluorescence) per minute per gram of protein in extract. The values were the means of three measurements for each sample.

8.3. Acid Phosphatase. Acid phosphatase activity was measured with *p*-nitrophenyl phosphate (Sigma, N22002) as a substrate. The method outlined by Ohmori et al. (*14*) was used with a slight modification. Sarcoplasmic extract (100 μL) was added to 450 μL of 0.1 mM sodium acetate buffer, pH 5.5, containing 4 mM *p*-nitrophenyl phosphate and 1 mM EDTA. After 20 min at 37 °C, the reaction was stopped by adding 2 mL of 0.1 M NaOH and the absorbance was determined spectrophotometrically at 400 nm. The activities of acid phosphatase were expressed as absorbance units at 400 nm per gram of protein in extract. The values were the means of three measurements for each sample.

9. Casein Zymography. Casein (4% w/v) was copolymerized with 10% (w/v) polacrylamide as the separating gel and 5% (w/v) as the stacking gel. Ammonium persulfate and TEMED (*N*,*N*,*N*′,*N*′-tetramethylethylenediamine) were used to catalyze the polymerization.

The casein gel was prerun with the electrophoretic buffer composed of 50 mM Trizma base, 40 mM glycine, 1 mM EDTA, and 5 mM β-mercaptoethanol. The sarcoplasmic extracts were pressurized between 0 and 300 MPa. Sarcoplasmic protein samples (30 μL) were loaded into the wells. Electrophoresis was run at 15 mA/gel at 4 °C for 10 min and then 30 mA/gel at 4 °C until the dye front had reached the end of the gel.

The gel was incubated overnight at ambient temperature in a buffer (100 mL) composed of 200 mM Tris-HCl (pH 7.5), 1 mL of 0.5 M $CaCl_2$, 1 mL of 0.5 M cysteine, and 35 μL of 1.4 M β-mercaptoethanol. Finally, the gel was stained with Coomassie blue. Clearer bands indicated the calpain activities.

RESULTS

1. Effect of Pressurization on the Effect of the Lysosomal Proteases. In this study, acid phosphatase was used as an indicator of the level of lysosome disruption according to Ohsumi et al. (*15*). **Figure 2** shows the evolution of acid phosphatase activities after high-pressure treatment. The acid phosphatase exhibits two different behaviors depending on whether the muscle or the sarcoplasmic extract is pressurized. When the fish muscle is pressurized, the activity increases, indicating a release of the enzyme from the lysosomes, whereas when the sarcoplasmic extract (containing no intact lysosome) is pressurized, the activity decreases, probably due to inactivation by high pressure. These results are in accordance with previous studies in meat (*14*, *16*, *17*), which established that high-pressure treatments destroy the lysosomal membrane and therefore let enzymes release from inside.

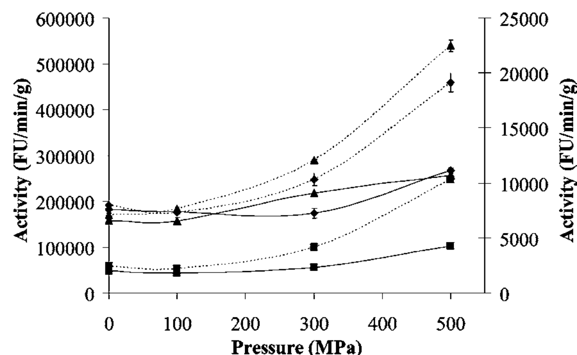

Figure 3. Evolution of cathepsins B (■), B + L (▲), and H (◆) activities when the sarcoplasmic extract is pressurized (—) and when the white muscle is pressurized (---). The values of the cathepsins B and B + L activities are read on the left axis, and those of cathepsin H activity are read on the right axis. Results are means of three samples; vertical bars represent the standard deviation.

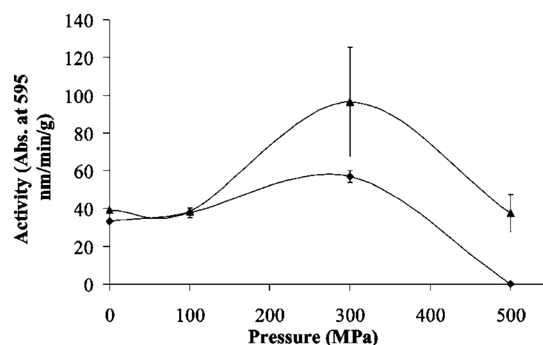

Figure 4. Evolution of cathepsin D activities when the sarcoplasmic extract is pressurized (◆) and when the white muscle is pressurized (▲). Results are means of three samples; vertical bars represent the standard deviation.

The effects of high-pressure treatment on the cysteine proteases (cathepsins B, H, and L) are summed up in **Figure 3**. From a quantitative point of view, it can be noticed that cathepsin H is present in very small amount in comparison with the two others and that cathepsin B is more active in the white muscle of sea bass than cathepsin L. These results are in accordance to those already observed by Aoki et al. (*18*). When the sarcoplasmic extract is pressurized, the activity weakly increases in relation to the pressure treatment. The proteases seem to be slightly activated by the high-pressure treatment. When the muscle was pressurized, the activity is increased in higher proportion. The results could be explained by the liberation of the enzymes from the lysosomes, which disruption is indicated by acid phosphatase activity. These results are in agreement with those found previously in meat, showing a release of lysosomal proteases thanks to the treatment (*17*).

The aspartic protease cathepsin D activities in pressurized fish muscle or sarcoplasmic extract are presented in **Figure 4**. Application of pressure on sarcoplasmic extract and on fish muscle induced a biphasic behavior of cathepsin D activity. Up to 300 MPa, measured activity is increasing, probably due to a slight activation as indicated in sarcoplasmic extract as well as a higher release from the lysosomes in the case of pressurized muscle. Above 300 MPa, this activity is decreasing. At 500 MPa, no cathepsin D activity is detected in pressurized sarcoplasmic extracts due to complete inactivation, while the amount of activity in pressurized muscle is equal to that measured after a treatment at 0 and 100 MPa, suggesting a protection by cytosolic environment as proposed by Bessière et al. (*19*).

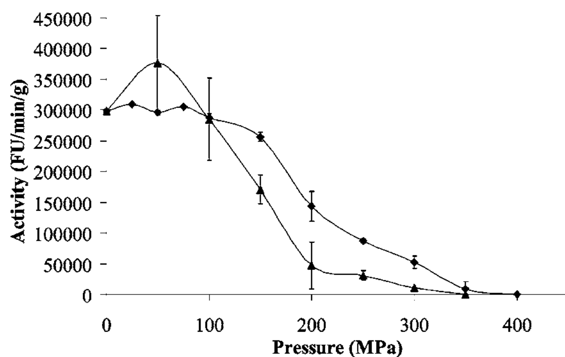

Figure 5. Evolution of calpain activities when the calpain extract is pressurized (◆) and when the white muscle is pressurized (▲). Results are means of three samples; vertical bars represent the standard deviation.

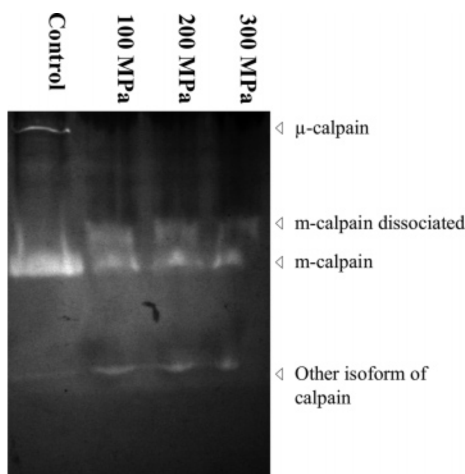

Figure 6. Casein zymogram of sarcoplasmic proteins. Arrowheads indicate the positions of the different calpain isoforms.

2. Effect of Pressurization on the Activities of the Calpains. Calpains are cytosolic enzymes highly regulated by their endogenous inhibitor, calpastatin, within the muscle. To study the calpain activity, the proteases have been separated from their inhibitor by hydrophobic interaction chromatography before (calpain extract was treated by high pressure) and after pressurization; in this latter case, the whole muscle was pressurized. **Figure 5** shows that the activities of calpains decrease in a significant way when pressure increases. The activities decrease from 100 MPa for the pressurized muscle and for the pressurized calpain fraction. The application of high-pressure processing induces a total loss of calpain activities at 350 MPa for the muscle pressurized and for the calpain fraction pressurized. Between 100 and 350 MPa, the residual activity in treated calpain extract is higher than in treated muscle. These results do not speak in favor of protection of calpain by cytosol as proposed by Bessière et al. (*19*).

Bessière et al. (*20*) have observed that the high-pressure treatment involved a dissociation of the two subunits of calpain. The dissociation curve according to pressure level can be almost superimposed with the curves obtained in this experiment. Thus, the high-pressure treatment dissociated the calpains and decreased the activities since the dissociated form is unstable (*21*).

Casein is usually used as an in vitro substrate for calpains and it has been used in zymogram electrophoretic gels to visualize the calpains. Upon a stained casein gel, calpain bands appear as clearer bands (**Figure 6**). Three bands that correspond to the three isoforms of calpains (respectively μ, m, and major m) are visible for the control sample (not pressurized sample). Indeed, Ladrat et al. (*9*) showed that three isoforms of calpains are present in sea bass muscle. Elution from an anion-exchange column by an ascending NaCl gradient gave μ-calpain, m-calpain, and then a major m-calpain, a result that is consistent with a higher electrophoretic mobility for the major m-calpain. After pressurization, the band corresponding to the μ-calpain has disappeared. In the pressurized samples, the major m-calpain is present as two forms: probably the native calpain and the dissociated active calpain (80 kDa catalytic subunit). Thus, high-pressure treatment would promote calpain subunit dissociation, as already shown by Bessière et al. (*20*). This can be related to the decrease in calpain activity observed after high-pressure treatment since dissociated calpain is rapidly further autolyzed (*21*).

DISCUSSION

In this study, we can notice that the cathepsin H is present in small quantity. During the high-pressure treatment, the cathepsins are released from the lysosomes thanks to a disruption. That explains the increase in their activities. Thus, depending on the family of cathepsins (cysteine or aspartic acid proteases) taken into consideration as well as whether the muscle or the extract is pressurized, the effect of the high pressure on their activity is different. High-pressure treatment can affect enzymatic activity by changing specific enzyme activity (substrate binding, substrate hydrolysis, or inactivation by denaturation) as well as by changing its concentration, resulting from its release from lysosomes into the medium. In muscle, cathepsin activities are increased or stable upon high-pressure treatment, probably due to activation but also due to release into the intracellular medium where they can interact with their myofibrillar substrates, speeding up the softening; thus, in the hypothesis that cathepsins are involved in muscle degradation, high-pressure treatment of fish muscle could be of less interest when cathepsin activity and final muscle firmness are considered.

Regarding the calpains, μ-calpain is degraded with high-pressure treatment from 100 MPa as shown on the casein gel, whereas for treatment at 100 MPa, the same level of calpain activity is present due to the major m-calpain, suggesting that μ-calpain activity is negligible in muscle. We can then suggest that the major m-calpain activity plays an essential role in the total calpain activity in vitro. This calpain is dissociated by high pressure and further loses activity. The other calpain isoform (m-) seems not to be affected by high pressure. The interest in high-pressure treatment to maintain fish muscle textural features during the postmortem storage should be then modulated as a function of the calpain isoform considered and responsible for the muscle degradation. In future studies, high-pressure effects on calpastatin should also be characterized in order to gain a better insight into the proteolytic global activity present within the muscle cells.

Finally, the fish's appearance becomes opaque with high-pressure treatment. This difference vanishes after cooking. Therefore, since fish is mainly eaten after cooking, this drawback could be minor in comparison with the possible enhancement of textural quality linked with protease changes.

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