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Influence of high pressure treatment on microbial proteases of relevant spoilage organisms

Einfluss von Hochdruckbehandlungen auf mikrobielle Proteasen relevanter Verderbnisorganismen

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Summary

Alternative non-thermal preservation techniques are of increasing interest by producers and consumers to guarantee food safety and quality. Therefore the effect of high pressure (HP) treatment on extracellular proteases of nine relevant spoilage organism and α-chymotrypsin was tested. The enzymes were exposed at 20 °C for 5, 15, 30 and 60 min to pressures of 200, 400 and 600 MPa.

In contrast to vegetative forms of microorganisms, the microbial proteases tested could not be inactivated efficiently under the selected conditions. The proteases of *Pseudomonas (P.) aeruginosa, P. fluorescens, Proteus (Pr.) vulgaris, Serratia (S.) marcescens and Bacillus (B.) megaterium* were remarkably resistant during high pressure treatment, indicated by only a slight loss in activity of up to 15 % after treatment for 60 min at 600 Mpa. The proteases of *Aeromonas (A.) hydrophila* and *B. subtilis* can be classified as pressure resistant up to 400 MPa with a reduced activity of 10 %, whereas the proteases of *B. cereus* and α-chymotrypsin can be evaluated as pressure sensitive to only 200 MPa.

The relatively high residual activity of the studied enzymes may represent a source of spoilage and should be taken into consideration for shelf life determinations of HP-treated products.

Keywords: alternative food preservation, shelf life, food spoilage

Zusammenfassung

Alternative, sogenannte „kalte" Dekontaminationstechnologien rücken sowohl bei Produzenten und Verbrauchern hinsichtlich Sicherheit und Qualität von Lebensmitteln verstärkt in den Fokus. Deshalb wurde der Effekt von Hochdruckbehandlungen auf extrazelluläre Enzyme von neun relevanten Verderbniserregern sowie α-Chymotrypsin getestet. Die mikrobiellen Proteasen wurden bei 20 °C 5, 15, 30 und 60 min mit Drücken von 200, 400 und 600 MPa beaufschlagt.

Im Gegensatz zu den vegetativen Formen der Mikroorganismen konnten die mikrobiellen Proteasen unter den gewählten Parametern nicht effizient deaktiviert werden. Die bakterienfreien, proteasehaltigen Filtrate von *Pseudomonas (P.) aeruginosa, P. fluorescens, Proteus (Pr.) vulgaris, Serratia (S.) marcescens* und *Bacillus (B.) megaterium* wiesen eine bemerkenswerte Druckstabilität auf. Lediglich nach 60 min bei 600 MPa war ein Aktivitätsverlust von 15 % zu verzeichnen. Die Proteasen von *Aeromonas (A.) hydrophila* und *B. subtilis* erwiesen sich bei Drücken bis zu 400 MPa mit einem Aktivitätsverlust von 10 % ebenfalls als druckresistent, während die von *B. cereus* und α-Chymotrypsin bereits bei 200 MPa als drucksensibler einzuschätzen sind.

Die relativ hohen Restaktivitäten der geprüften Enzyme könnten sich in hochdruckbehandelten Lebensmitteln als verderbsnisauslösend erweisen und sollten bei der der Festlegung von Mindesthaltbarkeitszeiten Berücksichtigung finden.

Schlüsselwörter: hochdruckbehandelte Lebensmittel, Verderb, Haltbarkeit

Introduction

High pressure (HP) technology is known as an alternative food preservation method. The principle is that the packaged food is exposed to elevated pressures up to 800 MPa, with or without the addition of heat, for a specific time (for reviews see San Martin et al., 2002; Considine et al., 2008). This technology is being used in the United States, Europe and Japan for selected high-value liquid and solid foods, either to extend shelf life or to improve food safety. Some commercially produced goods such as ready-to-eat meats, avocado products, tomato salsa, applesauce, orange juice, and oysters have been introduced to the market. Compared to heated products, those items are superior due to their better texture, appearance and taste.

Extensive experiments have shown that high-pressure treatment satisfactorily eliminates vegetative forms of both pathogens and food spoilage organisms (Styles et al., 1991; Takahashi et al., 1993; Patterson et al., 1996; Patterson, 2005; Tahiri et al., 2006). However, knowledge on the efficacy of high pressure processing for destruction of microbial proteases of relevant spoilage organisms is still insufficient.

Pressure inactivation of enzymes in general depends on the type and source of enzymes. Proteolytic enzymes (Hydrolases acting on peptide bonds (peptidases; Enzyme category E.C. 3.4.) hydrolyse peptide bonds and therefore lead to the disassembly of proteins. The breakdown into smaller peptide molecules and eventually into their constituent amino acids and further to ammonium, alcohol, carbon dioxide, amines and hydrosulfide leads either to desirable sensory properties (e. g. ripening processes of food) or to undesirable bitterness, gelation, putrid flavour, etc. According to EC regulation 178/2002, article 14, spoiled products have to be considered as unfit for human consumption and as unsafe. In that case food shall not be placed on the market.

High pressure treatment for enzyme denaturation is based on the Le Chatelier principle, i. e. any reaction accompanied by volume decrease is accelerated by elevated pressures (Cano et al., 2006). Small changes to the active site result in a loss of enzyme activity (Tsou, 1986; Riahi and Ramaswamy, 2004). Additionally, the nature of the medium in which the enzyme is dispersed, pressure, temperature and exposure time are important parameters (Cheftel, 1992; Kunugi, 1992; Seyderhelm et al., 1996).

Literature data for several enzymes (for review see Eisenmenger and Reyes-De-Corcuera, 2009) demonstrate inconsistent results; but confirm, in many cases, considerable pressure stability, e. g. pressures of 600 MPa are necessary to partially inactivate pectinmethylesterase (PME) in orange juice up to 90 % (Ogawa et al., 1990; Irwe and Olsson, 1994), 800 MPa to inactivate alkaline phosphatase (Rademacher et al., 1999) and 980 MPa to inactivate *B. subtilis*-proteases (Kitamura, 1966).

Other studies even document increased catalytic activity after high pressure treatment; e. g. a 6.5 fold increase for Chymotrypsin at 4.7 kbar (470 MPa) (Mozhaev et al., 1996) or a higher activity for a metalloprotease using a pressure of about 220 MPa (Kunugi and Tanaka, 1998). In our own studies, we could show that a microbial transglutaminase from *Streptomyces mobaraensae* is highly effective during high pressure treatment and able to crosslink globular proteins which are not used as a substrate by the enzyme under ambient pressure (Lauber et al., 2001; Lauber et al., 2003). Therefore, the objective of our investigations was to determine the degrees of inactivation resulting from exposure of proteases of selected spoilage organisms to pressures ranging from 200–600 MPa for 5 until 60 minutes at 20 °C. Data should deliver information about the susceptibility of high pressure treated products to proteolysis.

Material and methods

Strains

Nine strains of bacteria, provided by DSM (Deutsche Sammlung von Mikroorganismen) or isolated from food of animal origin, were used in all of the experiments and are summarised in Table 1. Additionally, a purified enzyme, α-chymotrypsin (Sigma Aldrich C-4129), was tested for pressure sensitivity.

Cultivation and enzyme production

Master cultures were maintained on cryobeads (Simport) at –85 °C and working cultures on Tryptone Soya Agar (TSA, Oxoid CM 131) at 4 °C, with subculture every month.

For maximal enzyme synthesis, strains were incubated in Nutrient Broth I (Sifin, TN 1172) for 72 h at 30 or 37 °C. Subsequently, proteases were extracted by filtration (Sartorius, 0.20 µm). Activities of proteases were determined by fluorescence measurement before and after high-pressure treatment, using the EnzChek®Protease Assay (Invitrogen), which is based on the protease-specific cleavage of casein labelled with a fluorescent dye. Protease-catalyzed hydrolysis releases highly fluorescent dye-labelled peptides and the accompanying increase in fluorescence is proportional to protease activity. Activity is expressed in fluorescence units.

High pressure treatment

All experiments were carried out using a pressure apparatus with two autoclaves (volume 25 ml; Bernd Dieckers GmbH Mess- und Prüfanlagentechnik). Bacteria free, enzyme containing samples (0.5 ml) filled in flexible micro tubes (APEX microtubes) were placed in a high pressure vessel. The pressure transmission medium was ethylene glycol. High pressure treatments of 200, 400 and 600 MPa at 20 °C were applied for 5, 15, 30 and 60 min. Times required to attain these pressures were about 23, 41 and

TABLE 1: *Bacteria used for high pressure treatment*

Species	Incubation temperature (°C)	Origin and number
Pseudomonas(P.) aeruginosa	37	CCIBML
Pseudomonas (P.) fluorescens	30	CCILL 390
Proteus (Pr.) vulgaris	37	DSM 2140
Aeromonas (A.) hydrophila	30	CCILL B1
Serratia (S.) marcescens	30	DSM 30126
Bacillus (B.) cereus	37	CCILL
Bacillus (B.) subtilis	37	CCILL Su95
Bacillus (B.) cereus	37	CCILL Pu4
Bacillus (B.) megaterium	37	CCILL Me1
α-chymotrypsin	–	Sigma Aldrich

DSM = Deutsche Stammsammlung; CCILL = strain collection of the Institute of Food Hygiene

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TABLE 2: *Influence of high pressure treatment on proteases of A. hydrophila and Bacillus spp.*

Species	Exposure time (min)	Before treatment	Residual activity in fluorescence 200 MPa	400 MPa	600 MPa
A. hydrophila		29908.8			
	5		28347.3	25654.3	17036.8
	15		27920.3	24696.0	15538.0
	30		27681.0	23696.8	13693.8
	60		27894.0	23101.5	11712.3
B. cereus		34580.5			
	5		33383.8	18944.5	12174.3
	15		32929.8	17687.5	11268.5
	30		31721.3	16348.8	10125.5
	60		29604.5	15895.5	8574.8
B. megaterium		33178.5			
	5		32127.3	30724.3	29082.0
	15		32958.0	30648.0	29133.5
	30		32708.3	30788.8	29228.0
	60		31742.8	30826.5	27933.5
B. pumilus		23697.3			
	5		22869.5	18702.0	17094.5
	15		22860.0	18371.5	17265.5
	30		22748.8	16299.3	16993.5
	60		22178.8	18067.5	16086.5
B. subtilis		49505.0			
	5		49147.5	48394.0	41352.3
	15		50945.8	47326.0	41469.8
	30		50024.5	46189.5	40982.8
	60		48719.8	44890.0	37742.8

75 seconds, respectively. The depressurisation time to atmospheric pressure was within one second. Following high-pressure treatment, remaining enzyme activity was determined in duplicates as described above. Experiments were repeated twice.

Results and discussion

The data (arithmetic mean) in Table 2 and in Figures 1 through 3 were obtained from a duplicate series of experiments.

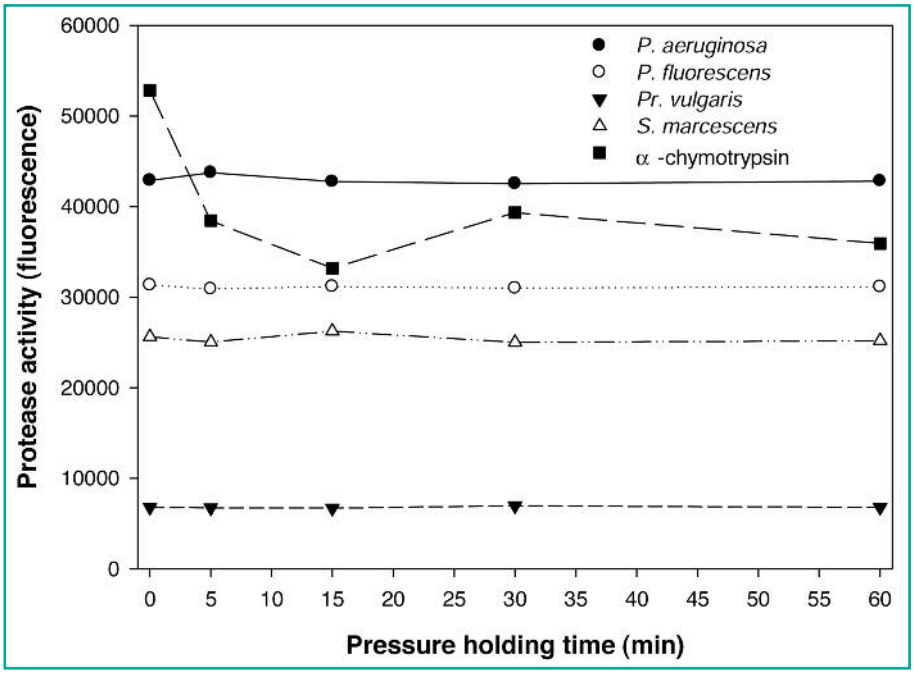

FIGURE 1: *Effect of 200 MPa on microbial proteases*

The data for untreated proteases (Tab. 2, Fig. 1–3) demonstrate that the proteolytic organisms synthesised different amounts of enzymes confirming data of Klug (1998). Whereas most of the proteases of *Bacillus* spp. and *Pseudomonas* spp. showed activities between 30.000 and 50.000, the proteases of *B. pumilus, S. marcescens* and *A. hydrophila* demonstrated activity between 20.000 and 30.000 and proteases of *Pr. vulgaris* had an activity less than 10.000 fluorescence.

According to the activity data obtained after high pressure treatment, most of the tested enzymes seem to be pressure-stable up to 600 MPa, confirming studies of Irwe and Olsson (1994), Ogawa et al. (1990) or Kitamura (1966). Exposing enzymes for 5 min to 600 MPa resulted in a maximal loss in activity of 2–5 % for proteases of *Pseudomonas, Serratia, Proteus* and 12–28 % for proteases of *Bacillus* spp. *(B. megaterium, B. pumilus, B. subtilis).*

In contrast, pressure-induced inactivation of 43 % was observed using 600 MPa/5 min for proteases of *A. hydrophila;* and 45 % for *B. cereus* proteases held at 400 MPa/5 min. α-chymotrypsin was inactivated at 200, 400, 600 MPa within 5 min and was the most pressure sensitive of all enzymes tested, which differs from the observations of Mozhaev et al. (1996) who observed partially an increasing activity. However, Mozhaev et al. (1996) used a different catalytic activity assay and a buffered system or organic media for α-chymotrypsin which may influence the pressure effect.

Considering the exposure times, it was concluded that prolonged exposure to high pressure of up to 60 min resulted in a higher inactivation rate of up to 20 %; in particular for the proteases of *B. cereus, A. hydrophila, P. fluorescens* and *P. aeruginosa.* This effect was also described for native milk enzymes such as alkaline phosphatase (Rademacher et al., 1999). For all other enzymes tested, prolonged exposure times had only relatively minor effects. As reasons for the different pressure stability their molecular structure, the tertiary and quarternary structure in particular is discussed, i. e. monomeric molecules result in high pressure stabilities while the activity of multimeric enzymes were inhibited by application of pressure (Penniston, 1971; Rademacher et al., 1999).

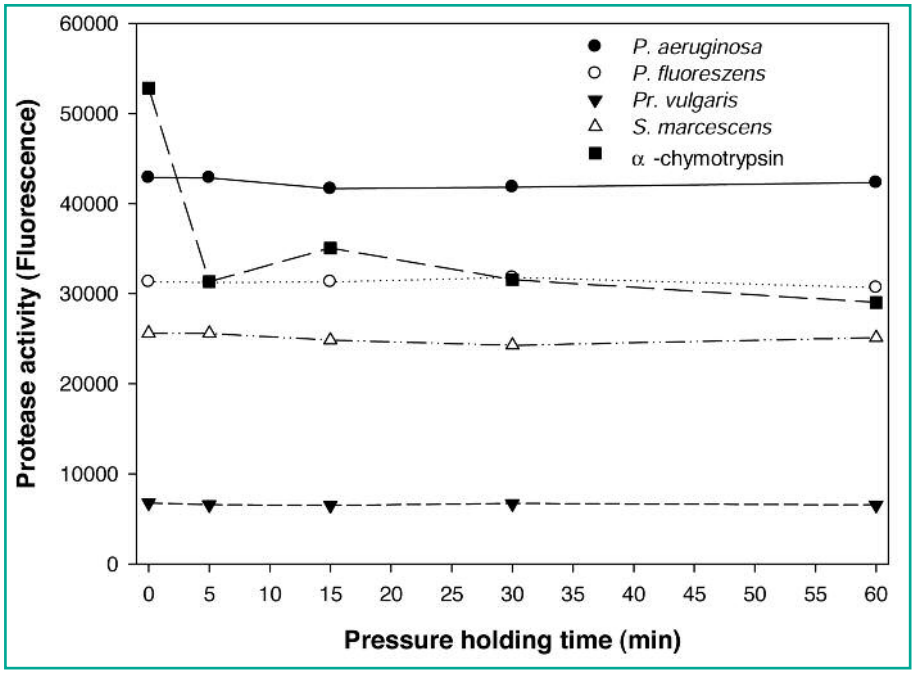

FIGURE 2: *Effect of 400 MPa on microbial proteases*

Conclusion

The preliminary data deliver more information about the response of microbial proteases of relevant spoilage organisms to high pressure. The enzymes of *P. aeruginosa, P. fluorescens, Pr. vulgaris, S. marcescens* and *B. megaterium,* can be regarded as pressure-resistant at 600 MPa for up to 60 min, with an activity loss of up to 15 %. The proteases of *A. hydrophila* and *B. subtilis* can be classified as relatively pressure resistant up to 400 MPa with a reduced activity of 10 %, whereas the proteases of *B. cereus* and α-chymotrypsin can be evaluated as pressure sensitive, showing an activity loss of up to 15 or 32 % at only 200 MPa.

It can be concluded that, in contrast to vegetative cells of microorganisms, the proteases tested could not be inactivated completely under the selected conditions. Residual activity of these enzymes, even after high pressure treatment up to 600 MPa, could still be a reason for proteolysis of stored food and should be considered during estimation of best before dates for high pressure-processed products. Further studies are necessary in order to clarify molecular aspects, which may explain the observed pressure-resistance. Furthermore, practical consequences with respect to possible deteriorating effects caused by pressure-resistant proteases during storage of high-pressure pasteurized food items must be elucidated.

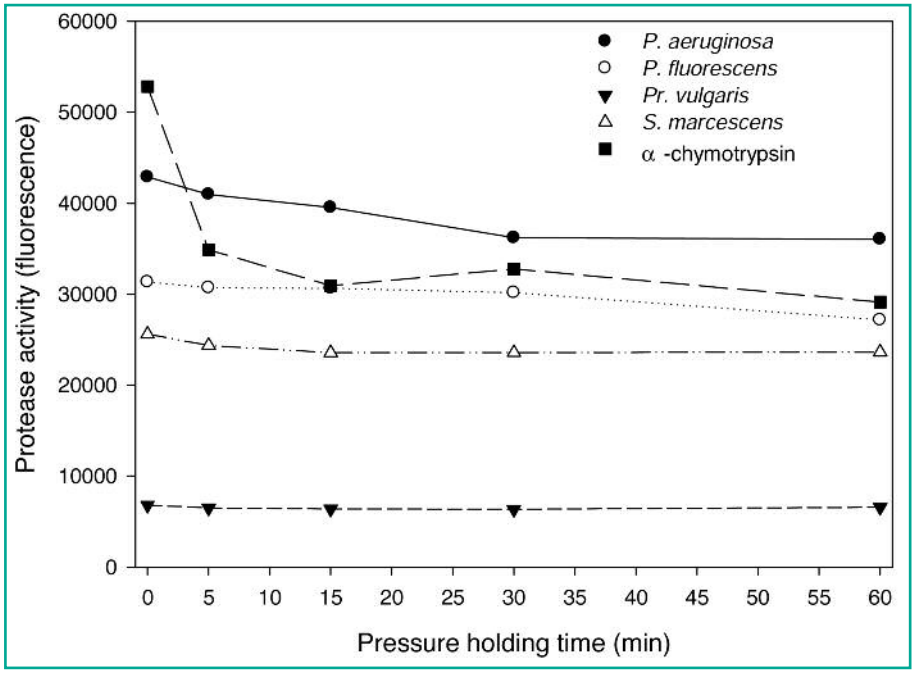

FIGURE 3: *Effect of 600 MPa on microbial proteases*

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