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Inactivation of bacterial proteases and foodborne pathogens in condensed globular proteins following application of high pressure

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ABSTRACT

The present investigation deals with the effectiveness of high pressure processing as a preservation technique for protein based materials. Increasing protein concentration up to 80% (w/w) in bovine serum albumin (BSA), soy glycinin and ovalbumin systems results in overall reduction in foodborne pathogens (i.e. *Staphylococcus aureus*, *Bacillus cereus* and *Escherichia coli*) for atmospheric samples due to reduced water activity. Application of high pressure (600 MPa for 15 min) reduces further microbial counts in these systems at comparable levels of solids. The effect of high hydrostatic pressure on proteolytic activity of proteases from *Pseudomonas fluorescens* strains 73 and 113 with globular proteins as substrates throughout the experimental range of protein concentration (10–80% w/w) has also been observed. Enzymatic activity in samples pressurized at 600 MPa for 15 min declined considerably, as compared to atmospheric counterparts. In addition, glycinin with the highest water holding capacity exhibits increased protease and microbiological activity at a given level of solids and treatment. Our work demonstrates that high pressure processing can act as an effective means of reducing microbial and enzymatic activity in condensed systems of globular proteins.

1. Introduction

The quality and safety of food products are the most important factors influencing consumer choice and considerations of market viability by manufacturers and distributors (Castro, Swanson, Barbosa-Canovas, & Zhang, 2001; Considine, Kelly, Fitzgerald, Hill, & Sleator, 2008). Thermal processing is used widely for food preservation but can alter the nutritional and sensory qualities of formulations. Increasing consumers' demand for fresh-like quality food, and the quest for energy conservation and reduction in carbon footprint by manufacturers have encouraged the development of innovative non-thermal technologies. High-pressure processing has been of interest recently, since it is considered as a promising technology within the perspective of industrial utilization for maintaining the sensory quality and nutritional profile in foods (Deliza, Rosenthal, Abadio, Silva, & Castillo, 2005).

High pressure treatment has been suggested as a food preservation technology for spore inactivation and sterilization processing in combination with elevated temperatures (Oey, Van der Plancken, Van Loey, & Hendrickx, 2008). In general, observations on the mechanism of microorganism inactivation by high pressure processing focused on the perturbation of the function of the cell membrane, the destruction of ribosome, and changes in cell morphology (Cheftel, 1995; Hoover, Metrick, Papineau, Farkas, & Knorr, 1989; Lee & Kaletunc, 2010; Mackey, Forestière, Isaacs, & Brooker, 1994; Ritz, Freulet, Orange, & Federighi, 2000; Smelt, 1998). These studies demonstrated that high pressure processing is able to inactivate some of the commonly found pathogens in low-solid food formulation, including *Salmonella*, *Shigella* and *Escherichia coli* (Yang et al., 2012).

Besides microbial kill, high hydrostatic pressure has a strong influence on the activity and stability of enzymes (Estrada-Giron, Swanson, & Barbosa-Canovas, 2005). Pressure inactivation of enzymes in food systems is different from that for pure components in buffer solutions (Bang & Chung, 2010), and can be divided into two classes. Pressures up to 100 MPa have shown a positive effect where the enzyme activity is enhanced, and this stimulating effect is mainly observed in monomeric enzymes with one polypeptide chain. Secondly, pressures of higher magnitude, i.e. above 600 MPa, have shown an inactivation effect where the enzyme activity is retarded (Curl & Jansen, 1950), and both activation and inactivation processes are important in food quality. Enzymes become inactivated at a threshold pressure value, which is based on the structure and source of enzyme, pH, temperature of pressure treatment, etc.

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Efficiency in retarding enzymatic activity is further enhanced by utilizing processing cycles in successive application of high pressure to inactivate trypsin, chymotrypsin and pepsin (Curl & Jansen, 1950; Ludikhuyze, Van den Broeck, Weemaes, & Hendrickx, 1997).

Literature has dealt extensively with the effect of high pressure on the stability of enzymes and microorganisms in protein systems up to about 20% (w/w) total solids content. In condensed systems finding application in breakfast cereals, snacks, sport supplements, etc. research using small-deformation oscillatory, denaturation enthalpy and infrared spectroscopy measurements argues that proteins subjected to high hydrostatic pressure retain the secondary conformation leading to preservation of bioactivity (Aimutis, 2004; Dissanayake et al., 2012). There is no corresponding microbiological or biochemical work in high-solid systems, where physicochemical properties are distinct from those in the low-solid counterparts. Therefore, the aim of the present study is to assess the residual activity of bacterial proteases and food pathogens in globular protein preparations of intermediate and high levels of solids following application of static pressure.

2. Material and methods

2.1. Preparation of globular protein samples

Bovine serum albumin was lyophilized powder from Sigma–Aldrich, Castle Hill, NSW, Australia. It had purity of more than 98% based on agarose gel electrophoresis, the molecular weight was ~66 kDa, and the pH of 1% (w/w) solution in 0.15 M NaCl was 6.8–7.2.

Soy protein isolate (SPI) was obtained from Oppenheimer, Sydney, NSW, Australia. The composition of the defatted SPI was reported by the supplier as 90.0% protein, 1.5% fat, 7% moisture, 7% ash and 1% carbohydrate. The material was used in this investigation to extract the glycinin (11S) fraction *via* the method of Wu, Murphy, Johnson, Fratzke, and Reuber (1999) and Kasapis and Tay (2009) with minor modifications. In doing so, sodium metabisulphite (0.98 g/L) was added to 10% (w/w) SPI dispersion in deionized water with pH adjustments to 6.4 using 1 M HCl, and the preparation was kept in an ice bath overnight. This was then centrifuged at 6500 *g* for 20 min at 4 °C using Sorvall RC5B Refrigerated Superspeed Centrifuge (Bad Homburg, Germany). The pellet was dissolved in deionized water with pH adjustment to 7.5 and the solution was dialysed in semi-permeable membrane against deionized water for 24 h at 4 °C. The resultant salt-free dialysate was freeze dried and stored at 5 °C for further experimentation.

Ovalbumin was extracted from chicken eggs using a four-stage crystallization technique according to the method of Kekwick and Cannan (1935). A solution of Na_2SO_4 (40% w/w) (Sigma Aldrich, Australia) was prepared by dissolving anhydrous salt in warm water. Fresh chicken eggs were collected from a local market in Melbourne, Australia, their volume was measured and an equal volume of the salt solution was added. The mixture was stirred for 2 h and the precipitate was discarded using centrifugation at 6500 *g* for 10 min at 25 °C. The liquid phase was filtered and a solution of 0.2 N H_2SO_4 was slowly added to the filtrate, with the latter being stirred mechanically until the pH reached 4.6–4.8. Stirring was continued and anhydrous Na_2SO_4 was added slowly until a permanent opalescence developed. Once the crystallization of protein became evident, the mixture was kept at room temperature overnight, and the crystalline material was separated using centrifugation at 6500 *g* for 10 min at 25 °C. The resultant was redissolved in a volume of water, which was approximately equal to the original volume of egg white. Recrystallization was then effected by addition of anhydrous Na_2SO_4 accompanied with stirring. After two stages of further recrystallization, the final product was brought into solution and dialysed in deionized water for 24 h at room temperature. The dialysate was freeze dried and stored at 5 °C for further experimentation. Three replicates of the freeze dried material were analysed using the Bradford method to identify the protein content, which was found to be 91.5%.

2.2. Sodium dodecylsulphate polyacrylamide gel electrophoresis

SDS-PAGE was performed according to the method of Laemmli (1970) in a Bio-Rad mini-Protein electrophoresis cell at a constant voltage (100 V) with a gel concentration of 6%. Protein and protease samples for SDS-PAGE were prepared by mixing with sample buffer (60 mM Tris–HCl, 2% SDS, 14.4 mM β-mercaptoethanol, 25% glycerol, 0.1% bromophenol blue, pH 6.8). Proteins were resolved on a 12.5 or 10% separation gel and stained with Coomassie Brilliant Blue for 30 min and were stored overnight. The solution was homogenized by 30 s sonication cycles for 3 min. Two different concentrations of denatured glycinin and ovalbumin samples (100 and 50%) and five different concentrations of non-denatured protein samples (100, 90, 70, 40, and 10%) were prepared from the stock solution. Following migration, the protein and protease fractions were stained with 0.1% Coomassie blue.

2.3. Cultures and growth conditions of foodborne pathogens

The bacterial cultures used were *Bacillus cereus* (strain 3012), *Escherichia coli* (strain 103/1-1) and *Staphylococcus aureus* (strain ATCC 25923). They were obtained from the culture collection of the Discipline of Biosciences in the School of Applied Sciences at RMIT University. Prior to inoculation and dilution into the protein samples, the cultures were grown individually to a concentration range of about 10^7 cfu/mL (typical population during the late exponential phase of growth) in Tryptic Soy Broth Yeast Extract (Oxoid; Thebarton, SA) over a period of 20 ± 2 h at 37 ± 1 °C.

A 1:100 dilution in 150 mM sodium chloride (Merck; Kilsyth, VIC) of each culture was made and added to the protein samples, which were prepared as a 40% (w/w) solution in deionized water. This inoculation step resulted in a further 1:10 dilution of the microbial cultures. Part of the diluted protein samples with microbial cultures was removed at this stage for enumeration and the remaining material was concentrated up to 80% with a model R-200 Rotavapor rotary evaporator (Flawil, Switzerland) coupled to a model N 840.3 FT.18 LABOPORT diaphragm vacuum pump (Munzingen, Germany). The temperature during the evaporation process was maintained at 39 ± 1 °C using a model B-491 Heating Bath (Flawil, Switzerland).

2.4. Cultures and preparation of crude proteases

Isolates of *Pseudomonas fluorescens* 79 and 113 were obtained from the dairy culture collection at Food Sciences, RMIT University. The cultures were stored at −80 °C in nutrient broth containing 20% glycerol. Glycerol helps in reducing dehydration of the bacterial cells during the freezing process. A small portion of the frozen bacterial culture was inoculated from the cryotube into 10 ml of nutrient broth and incubated at 25 °C for 24 h. The incubated culture was centrifuged at 3020 *g* for 10 min and the supernatant was discarded. The obtained pellet was washed in sterile 145 mM NaCl followed by centrifugation at 3020 *g* for 10 min. The pellet containing bacterial cells were resuspended in 145 mM NaCl solution. This suspension containing bacterial cells was then introduced to UHT skim milk to achieve a cell count of 10^5–10^6 cfu/ml. The UHT skim milk culture was incubated with circular agitation (150 rpm) at 4 °C for 8 days before being centrifuged at 28000 *g* for 40 min at

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