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Comparison of conventional heat treatment with selected non-thermal technologies for the inactivation of the commercial protease Protamex™

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ABSTRACT

Preparation of protein hydrolysates for food use requires the elimination of residual enzyme activity after the hydrolysis reaction, which is usually achieved by thermal inactivation. However, precise data on inactivation of commonly used enzymes is still not readily available. Sodium-caseinate hydrolysates were produced with the commercial protease Protamex™. A conventional thermal method to terminate enzymatic hydrolysis was evaluated and complete inactivation of Protamex™ was attained at 95 °C for 20 s. High intensity light pulses (HILP), Pulsed Electric Fields (PEF), and ultrasound (US) were evaluated as methods to terminate enzymatic hydrolysis. HILP was effective in inactivating Protamex™ in solutions with high optical transmittance particularly in the UV region. A 90% reduction in Protamex™ activity was achieved after a 1 min treatment with no significant temperature increase. A PEF treatment (18.2 kV/cm, 500 pulses) gave a 70% decrease in Protamex™ activity in a hydrolysate solution while sample temperatures remained below the range for thermal inactivation. Thermosonication did not decrease enzyme activity at temperatures <60 °C and at temperatures >60 °C any inactivation observed was most likely due to thermal effects. Despite the promising results, further optimisation would be required before these technologies could be considered as alternatives to conventional heat treatments.

1. Introduction

As well as fulfilling dietary requirements proteins are a source of bioactive peptides. These peptides are inactive within the native protein sequence but can be released by enzymatic or microbial proteolysis. Once these bioactive peptides are liberated they may have many beneficial physiological functions affecting the cardiovascular, endocrine, immune, and nervous systems (Korhonen and Pihlanto, 2003). During processing, proteins are hydrolysed to a certain degree to liberate bioactive peptides having the desired functional, nutritional and organoleptic properties. However, once the target degree of hydrolysis is reached enzyme inactivation is required to terminate the reaction as further hydrolysis can cause undesirable modifications such as degradation of the bioactive peptides, increased bitterness and reduced functionality. The most widely used method for enzyme inactivation is heat processing. However, excessive heat processes can adversely affect the functional, nutritional and biological properties of food proteins hydrolysates. Moreover, many enzymes are heat resistant or require relatively severe heat treatments for inactivation. For these reasons there has been increased interest in assessing the potential for alternative methods for enzyme inactivation (Ercan and Soysal, 2011). The so-called minimal processing technologies such as high-intensity ultrasound (US), Pulsed Electric Fields (PEF) and broad-spectrum pulsed-light (high intensity light pulses, HILP) are under research to explore whether they have the potential to terminate enzymatic hydrolysis as they may have a reduced impact on the sensory, nutritional, and bioactive quality of foods.

High-intensity US is a form of vibrational energy in the frequency range of 20–100 kHz with an intensity of 10–1000 W/cm^2 with multiple applications in the food industry (Chemat et al., 2011). The use of

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US has been widely investigated for microbial and enzyme inactivation in food and the precise mechanism whereby US affects enzyme activity remains to be elucidated. PEF treatment involves the application of very short electric pulses of high voltage to foods at moderate treatment temperatures generally below 60 °C. In general an increase in the electric field strength and treatment time induces a decrease in the proteolytic activity as observed for instance with a protease from *Bacillus subtilis* (Bendicho et al., 2003) or pepsin (Yang et al., 2004) and a decrease in activity of enzymes such as lipase, glucose oxidase, and α-amylase (Ho et al., 1997). However, little or no decrease in the activity of alkaline phosphatase and enhanced activity of pepsin following PEF treatment has been reported (Ho et al., 1997). HILP involves the application of intense and short duration light pulses of broad spectrum light (200–1100 nm) which includes wavelengths in the ultraviolet (UV), visible, and near infrared regions. The effect of HILP on the inactivation of microorganisms has been extensively reviewed (Gómez-López et al., 2007), however, there are very few studies on the effects of HILP on the reduction of the catalytic activity of different food enzymes. Dunn et al. (1989) demonstrated HILP to be effective for inactivating oxidoreductases, hydrolases, lipases, isomerases and proteinases in fruit, vegetables, meat, fish, and shellfish; and Manzocco et al. (2013) reported complete polyphenoloxidase inactivation in model solutions by pulsed light doses higher than 8.75 J/cm^2. Protamex™ is an enzyme preparation, sourced from *Bacillus* spp., which has been used in the manufacture of sodium caseinate hydrolysates (Kilcawley et al., 2002; Sindayikengera and Xia, 2006; O'Regan and Mulvihill, 2010). The preparation contains two principal activities belonging to the metallo- and serine- protease enzyme classes (E.C. 3.4.21.62/3.4.24.28). Protamex™ is known to produce less bitter hydrolysates than other enzyme preparations due to the debittering effect of the exo-peptidases activity and for this reason it is extensively used in the manufacture of protein hydrolysates (Liaset et al., 2002; Nguyen et al., 2011). The objective of this study was to compare the effect of heat, US, PEF, and HILP on the activity of Protamex™ in aqueous and sodium caseinate solutions.

2. Materials and methods

2.1. Chemicals

All chemicals were of reagent grade or better and were obtained from Sigma Aldrich (Arklow, Ireland) and Fisher Scientific (Ballycoolin, Ireland). The enzyme Protamex™ (Novozymes A/S, Denmark) was supplied as a dry powder with an activity of 1.5 Anson units per gram and was stored at 4 °C. Sodium caseinate powder (88.6% w/w protein) was obtained from Kerry Ingredients (Ireland).

2.2. Enzymatic hydrolysis of sodium caseinate

A 10% (w/v) sodium caseinate solution (1.4 L) was prepared by dispersing the sodium caseinate powder in distilled water using a laboratory mixer (Model L4RT, Silverson Machines Ltd, England) at 4000 rpm for 1 h at room temperature. The dispersion was allowed to hydrate overnight at 4 °C which also allowed for any foam to collapse. The solution was subsequently allowed to equilibrate to 50 °C in a water bath and then transferred to a 2-L temperature- and pH-controlled reaction vessel (Fermac 230, Electrolab, UK). Once the pH was adjusted to 7.0 with 1 mol/L NaOH, 100 mL of an aqueous Protamex™ enzyme solution was added (0.67% w/v) to give an enzyme/protein substrate concentration of 0.446 g/100 g protein. Hydrolysis was carried out with continuous agitation (300 rpm) and temperature and pH monitored using a data acquisition system and a recorder software (Picotechnology Ltd., UK). The degree of hydrolysis (DH) was determined using the pH-stat method (Adler-Nissen, 1986). Briefly, NaOH was added to the reaction vessel continuously to maintain a constant pH at 7.0 and monitored using an electronic balance (Model TE153S, Sartorius, Germany) and a data acquisition software (SartoCollect V.1.0, Sartorius). The amount of NaOH consumed (mL) during the reaction, which is proportional to the degree of hydrolysis, gives a direct measurement of the percentage of hydrolysed peptide bonds. The DH (%) was calculated using the following equation (Adler-Nissen, 1986) (Eq. (1)):

$$DH\,(\%) = B \times M_b \times \frac{1}{MP} \times \frac{1}{h_{tot}} \times \frac{1}{\alpha} \times 100 \quad (1)$$

where B is the base (NaOH) consumption in mL, M_b is the molarity of the base used (1 M), MP is the mass of the protein being hydrolysed in grams (150 g), h_{tot} is the total number of peptide bonds in the protein substrate (8.2 meqv/g protein) and α is the average degree of dissociation of the α-amino groups (dimensionless) which was calculated using the following equation (Adler-Nissen, 1986) (Eq. (2)):

$$\alpha = \frac{10^{(pH-pK)}}{1 + 10^{(pH-pK)}} \quad (2)$$

where pH is the hydrolysis reaction pH (7.0) and pK is the average dissociation value for the α-amino groups liberated during hydrolysis and is dependent on temperature, peptide chain length and the nature of the terminal amino acid. In this study the average pK value was taken as 7.1 (Adler-Nissen, 1986). Therefore α can be calculated as 0.44 (Adler-Nissen, 1986).

Once a DH of 7% was achieved, the addition of base was stopped and the hydrolysate was immediately frozen (−18 °C).

2.3. Determination of Protamex™ endoproteinase activity

The endoproteinase activity of Protamex™ was determined spectrophotometrically using a modified version of the azocasein method of Charney and Tomarelli (1947). Briefly, four reaction solutions were first prepared: solution A or substrate solution, which is an azocasein substrate (0.4% w/v) dispersed in 0.05 M Tris–HCl pH 7.0; solution B or enzyme solution, which contains Protamex™ enzyme powder (75 mg) dissolved and diluted to 100 mL in distilled water; solution C or sodium caseinate hydrolysate prepared with Protamex™ enzyme to a DH of 7% as outlined in Section 2.2; and solution D containing trichloracetic acid (TCA) in distilled water (4% w/v). For the assay method, 3 mL aliquots of solution A were dispensed into clean test tubes to which 0.1 mL of solution B or solution C were subsequently added. The reaction blank was prepared by substituting Solution B with distilled water and solution C with boiled hydrolysate of equivalent volume. The test tubes containing solution B were then incubated at 50 °C for 30 min while those containing solution C were incubated at the same temperature for 100 min due to the lower enzyme activity in the hydrolysate. Following incubation, the tubes were immersed in ice-cold water and the reaction was ended by the addition of 3 mL of solution D. The resultant solutions were filtered through a Whatman no. 42 filter paper and the absorbance of the filtrate measured at 400 nm. The absorbance values (A_{400}) of each sample was corrected by subtracting the appropriate substrate blanks and the