<table>
<thead>
<tr>
<th>Title</th>
<th>The effects of selected non-thermal interventions on various aspects of the hydrolysis of sodium caseinate by Protamex®</th>
</tr>
</thead>
<tbody>
<tr>
<td>Authors(s)</td>
<td>Holton, Mairead</td>
</tr>
<tr>
<td>Publication date</td>
<td>2014-06</td>
</tr>
<tr>
<td>Publisher</td>
<td>University College Dublin. School of Agriculture and Food Science</td>
</tr>
<tr>
<td>Link to online version</td>
<td><a href="http://dissertations.umi.com/ucd:10024">http://dissertations.umi.com/ucd:10024</a></td>
</tr>
<tr>
<td>Item record/more information</td>
<td><a href="http://hdl.handle.net/10197/7899">http://hdl.handle.net/10197/7899</a></td>
</tr>
<tr>
<td>Notes</td>
<td>A hard copy of this thesis is available in UCD Library, thesis 12619</td>
</tr>
</tbody>
</table>
The effects of selected non-thermal interventions on various aspects of the hydrolysis of sodium caseinate by Protamex®

School of Agriculture and Food Science,
Head of School,
Professor A. Evans

The thesis is submitted to University College Dublin, in fulfilment of the degree of Master of Science in the College of Agriculture, Food Science and Veterinary Medicine

By

Mairead Holton, B.Sc.

Student number: 11293390

Research Supervisors:

Dr. James Lyng, School of Agriculture and Food Science, UCD

Mr. Michael O’Sullivan, School of Agriculture and Food Science, UCD

June 2014
Declaration:

I hereby declare that the work submitted in this thesis is the result of my own Investigation, except where reference is made to published literature. I also certify that this thesis or any part of it has not been previously submitted for any other degree to the National University of Ireland, or any other university.

____________________
Mairead Holton
Acknowledgements

I am very grateful to all of the people who contributed to this thesis. First and foremost I would like to thank my supervisors; Dr. James Lyng and Mr. Michael O'Sullivan, without your guidance, expertise and enthusiasm this project would not have been possible. I would also like to thank Enterprise Ireland who provided funding for this project.

I would like to mention Mr. Michael Cooney, thanks for all the technical help throughout this project. Professor Alan Kelly, Mr. Jim McNamara and Dr. Malco Cruz in UCC I'm very grateful for all the assistance with the high pressure processing, it was very much appreciated. Ania, Tara, Vani and Nikolina thank you for all of your advice and support during my time in UCD.

A huge thank you to my family for all of the support, encouragement and understanding each of you provided during this project. Thanks to my parents, Liam and Mary, and to my sisters, Claire, Therese, Eilish and Maeve, for everything. I would also like to thank all of my friends who helped me through this project and were always available to cheer me up.

Finally, I would like to extend my deepest gratitude to my boyfriend Ross for everything in the last two years, you’re always there to help and support me through everything and I couldn’t have completed this project without you.
<table>
<thead>
<tr>
<th>Table of contents:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>List of Figures ..................................................................................................................................</td>
<td>vii</td>
</tr>
<tr>
<td>List of Tables ....................................................................................................................................</td>
<td>viii</td>
</tr>
<tr>
<td>Abstract ..............................................................................................................................................</td>
<td>1</td>
</tr>
<tr>
<td>1.0 Literature Review ....................................................................................................................</td>
<td>3</td>
</tr>
<tr>
<td>1.1 Introduction ..................................................................................................................................</td>
<td>3</td>
</tr>
<tr>
<td>1.2 Functional foods ..........................................................................................................................</td>
<td>4</td>
</tr>
<tr>
<td>1.2.1 Current trends in the dairy industry and consumer demands ..............................................</td>
<td>4</td>
</tr>
<tr>
<td>1.2.2 Bioactive peptides ....................................................................................................................</td>
<td>5</td>
</tr>
<tr>
<td>1.2.3 Production of bioactive peptides ...............................................................................................</td>
<td>9</td>
</tr>
<tr>
<td>1.2.4 Sodium caseinate and casein hydrolysates ...............................................................................</td>
<td>11</td>
</tr>
<tr>
<td>1.3 Introduction to non-thermal technologies ....................................................................................</td>
<td>13</td>
</tr>
<tr>
<td>1.3.1 Non-thermal processing technology ............................................................................................</td>
<td>14</td>
</tr>
<tr>
<td>1.3.2 Pulsed electric field technology ...............................................................................................</td>
<td>14</td>
</tr>
<tr>
<td>1.3.3 Low frequency high intensity ultrasound ..................................................................................</td>
<td>19</td>
</tr>
<tr>
<td>1.3.4 High pressure processing ........................................................................................................</td>
<td>23</td>
</tr>
<tr>
<td>1.3.5 Effects of novel technologies on bioactivity ............................................................................</td>
<td>26</td>
</tr>
<tr>
<td>1.4 Effects of non-thermal processing on protein structure ..............................................................</td>
<td>28</td>
</tr>
<tr>
<td>1.4.1 Protein structure ......................................................................................................................</td>
<td>28</td>
</tr>
<tr>
<td>1.4.2 Effects of pulsed electric field treatment on protein structure ............................................</td>
<td>29</td>
</tr>
<tr>
<td>1.4.3 Effect of ultrasound on protein structure ................................................................................</td>
<td>31</td>
</tr>
<tr>
<td>1.4.4 Effects of high pressure processing on protein structure ......................................................</td>
<td>33</td>
</tr>
<tr>
<td>1.5 Use of low frequency ultrasound to enhance hydrolysis ................................................................</td>
<td>35</td>
</tr>
<tr>
<td>1.5.1 Enhanced diffusion generated by cavitation ..............................................................................</td>
<td>35</td>
</tr>
<tr>
<td>1.5.2 Use of ultrasound for enhanced hydrolysis rates ......................................................................</td>
<td>36</td>
</tr>
<tr>
<td>1.6 Use of non-thermal technologies to terminate hydrolysis ............................................................</td>
<td>42</td>
</tr>
<tr>
<td>1.6.1 Residual enzyme activity in processed foods ............................................................................</td>
<td>42</td>
</tr>
<tr>
<td>1.6.2 High pressure treatment for enzyme inactivation ....................................................................</td>
<td>43</td>
</tr>
<tr>
<td>1.7 Objectives .....................................................................................................................................</td>
<td>46</td>
</tr>
<tr>
<td>2.0 The application of non-thermal technologies as pre-treatments to enzymatic hydrolysis of sodium caseinate</td>
<td>48</td>
</tr>
<tr>
<td>Abstract ................................................................................................................................................</td>
<td>48</td>
</tr>
<tr>
<td>2.1 Introduction ...................................................................................................................................</td>
<td>49</td>
</tr>
<tr>
<td>2.2 Materials and methods ..................................................................................................................</td>
<td>51</td>
</tr>
<tr>
<td>2.2.1 Materials ...................................................................................................................................</td>
<td>51</td>
</tr>
</tbody>
</table>
List of Figures

Figure 1.1: Arrangement of casein into micellar structure.................................................................12
Figure 1.2: Schematic of continuous PEF system ..............................................................................15
Figure 1.3: PEF waveforms; (a) exponential, (b) oscillatory (c) bipolar and (d) square waveforms ...........................................................................................................................................16
Figure 1.4: Oscillation of cavitation bubbles in response to pressure changes during ultrasound cycles ...............................................................................................................................................17
Figure 1.5: Schematic diagram of a high pressure system ....................................................................24
Figure 2.1: Hydrolysis curve for incubation of 10% (w/v) sodium caseinate with Protamex® at 50°C and stirred at 400 rpm, control (■) and pulsed electric field pre-treatment of 34 kV cm⁻¹ and frequency of 420 Hz (●) ........................................................................................................20
Figure 2.2: Hydrolysis curve for incubation of 10% (w/v) sodium caseinate with Protamex® at 50°C and stirred at 400 rpm, control (■), ultrasound pre-treatment at frequency of 20 kHz and 170 μm amplitude (●) and 136 μm amplitude (▲) ........................................................................................59
Figure 2.3: Hydrolysis curve for incubation of 10% (w/v) sodium caseinate with Protamex® at 50°C and stirred at 400 rpm, control (■) and high pressure pre-treatment of 400 MPa (●) ..................................................................................61
Figure 2.4: TCA solubility index for incubation of 10% (w/v) sodium caseinate with Protamex® at 50°C and stirred at 400 rpm, control (■) and high pressure pre-treatment of 400 MPa (●)........................................................................62
Figure 3.1: (a) Setup 1 application of ultrasound in external flow cell (b) Setup 2 application of ultrasound directly to reaction vessel throughout hydrolysis ......................................................................71
Figure 3.2: Hydrolysis curve for incubation of 10% (w/v) sodium caseinate with Protamex® at 50°C and stirred at 400 rpm, control (■) and ultrasound (●) ......................................................................................74
Figure 3.3: Hydrolysis curve for incubation of 10% (w/v) sodium caseinate with Protamex® at 50°C and stirred at 100 rpm, control (■), 20 μm amplitude (▲) and 40 μm amplitude (●) ...............76
Figure 3.4: TCA solubility index of control (■), 20 μm amplitude (▲) and 40 μm amplitude (●) samples read at 280 nm when stirred at 100 rpm ........................................................................................................76
Figure 3.5: Hydrolysis curve for 10% (w/v) sodium caseinate incubated with Protamex® at 50°C and stirred at 200 rpm, control (■), 20 μm amplitude (▲) and 40 μm amplitude (●) .........77
Figure 3.6: TCA solubility index of control (■), 20 μm amplitude (▲) and 40 μm amplitude (●) samples read at 280 nm when stirred at 200 rpm ........................................................................................78
Figure 3.7: Hydrolysis curve for 10% (w/v) sodium caseinate incubated with Protamex® at 50°C with stirring at 400 rpm, control (■), 20 μm amplitude (▲) and 40 μm amplitude (●) ..........79
Figure 3.8: TCA solubility index of control (■), 20 μm amplitude and (▲) 40 μm amplitude (●) samples read at 280 nm when stirred at 400 rpm ........................................................................................................80
Figure 3.9: Hydrolysis curve for 10% (w/v) sodium caseinate incubated with Protamex® at 50°C, with stirring speed of 400 rpm (■) and 600 rpm (●) ..................................................................................81
Figure 3.10: Hydrolysis curve for 5% (w/v) sodium caseinate incubated with Protamex® at 50°C, control (■) and ultrasound applied in situ at 40 μm amplitude (●) .................................................83
Figure 3.11: Hydrolysis curve for 15% (w/v) sodium caseinate incubated with Protamex® at 50°C, control (■) and ultrasound applied in situ at 40 μm amplitude (●) .................................................83
Figure 4.1: Thermal inactivation of Protamex® in distilled water at 55°C (●), 60°C (■), 65°C (▲), 70°C (▲), 75°C (►), 80°C (◄), 85°C (▼), 90°C (○) and 95°C (X) .........................................................................................91
Figure 4.2: Thermal inactivation of Protamex® in hydrolysed sodium caseinate at 55°C (■), 60°C (■), 65°C (▲), 70°C (▲), 75°C (►), 80°C (◄), 85°C (▼), 90°C (○) and 95°C (X) ........................................................................92
Figure 4.3: High pressure inactivation of Protamex® in distilled water under atmospheric pressure (●), 400 MPa (■), 500 MPa (▲) and 600 MPa (▲) .........................................................................................94
Figure 4.4: High pressure inactivation of Protamex® in hydrolysed sodium caseinate at atmospheric pressure (●), 400 MPa (■), 500 MPa (▲) and 600 MPa (▲) .........................................................95
List of Tables

Table 1.1: Bioactive peptides sourced from milk proteins ............................................................... 6
Table 3.1: Experimental parameters employed to investigate the effect of ultrasound on hydrolysis rate in situ ................................................................. 72
Abstract

In this thesis the potential applications of non-thermal technologies, including pulsed electric fields, low frequency ultrasound and high pressure, at various stages in the production process of protein hydrolysates were investigated. These technologies have the potential to induce structural changes at a molecular level to protein substrates.

The first area investigated was the use of selected non-thermal technologies as pre-treatments for a caseinate substrate prior to enzyme hydrolysis. It has been shown by other authors that pulsed electric field, low frequency ultrasound and high pressure treatments can induce protein denaturation under specific settings and this may increase their susceptibility to subsequent hydrolysis. The pre-treatments of 10% (w/v) sodium caseinate employed were: pulsed electric field treatment of 34 kV cm\(^{-1}\) with a total specific energy of 276.81 kJ kg\(^{-1}\), low frequency ultrasound at a frequency of 20 kHz (amplitudes of 170 µm and 136 µm) and a high pressure treatment of 400 MPa, each applied for 30 minutes. The results from this investigation indicated that low frequency ultrasound applied for 30 minutes at an amplitude of 136 µm is the most promising pre-treatment as a ~33% reduction in the hydrolysis time was achieved, while all other pre-treatments investigated had, at best, a marginal effect on the hydrolysis rate.

Low frequency ultrasound (24 kHz) was also applied during enzymatic hydrolysis of sodium caseinate to investigate if the cavitation effect could lead to increased mixing in the system, thus leading to an enhanced rate of hydrolysis. Amplitudes of 20, 40 and 60 µm were investigated with stirring speeds ranging from 100-600 rpm and protein concentrations of 5-15% (w/v). It was found that
the rate of the hydrolysis could be enhanced by applying ultrasound but only under very specific conditions. For a stirring speed of 400 rpm with a protein concentration of 10% (w/v), application of ultrasound at amplitudes of 20 µm and 40 µm decreased the hydrolysis time by ~25% and ~45% respectively. All other ultrasound conditions investigated lead to either an increased hydrolysis time or had a marginal effect over the control indicating that there is a narrow optimum range of conditions in which this phenomenon occurs.

Finally high pressure was compared to thermal treatments as a means to inactivate enzyme activity (e.g. for the termination of a hydrolysis reaction). The enzyme, Protamex®, was subjected to thermal and high pressure treatments when dispersed in water or in a 10% (w/v) sodium caseinate substrate solution. The presence of the caseinate substantially increased the stability of the enzyme to heat treatment. Similarly the enzyme was more pressure susceptible in water than in the presence of sodium caseinate, again indicating a protective effect of the substrate. Over 90% inactivation was achieved at pressures ≥400 MPa with holding times of 30 minutes in water, whereas this level of inactivation required 600 MPa in the sodium caseinate solution. No significant inactivation occurred at pressures <400 MPa.

During the course of this work it became apparent that these non-thermal technologies only have a positive effect on hydrolysis under a very narrow range of conditions and careful process optimisation is required. All of the investigations conducted were also at laboratory scale and up-scaling of these experiments may result in different outcomes.
1.0 Literature Review

1.1 Introduction

There is currently a lot of interest in manufacturing bioactive peptides from food protein sources (Hartmann & Meisel, 2007). These peptides confer a number of beneficial activities to the consumer to improve health and possibly treat pre-existing ailments (Korhonen & Pihlanto, 2006). However, there have been obstacles to the large scale production of these products as a high degree of hydrolysis needs to be achieved and the product must also be highly purified. The lengthiest step in the manufacture of these peptides is the hydrolysis of the raw protein material (Agyei & Danquah, 2011).

Thermal processing of food products can cause adverse effects on the taste, appearance, texture and nutritional quality of the foodstuff (Korhonen, Pihlantoleppala, Rantamaki, & Tupasela, 1998). It is also well established that excessive heating can cause denaturation of proteins. This is a major consideration when processing foods containing bioactive peptides. If the native configuration of the protein is altered the bioactivity may also be lost. Due to these facts there is interest in developing alternative non-thermal processing methods for bioactive preservation.

Non-thermal processing technologies such as pulsed electric field, low frequency ultrasound and high pressure processing have been shown to have various applications in food processing and preservation (Balasubramaniam, Farkas, & Turek, 2008; Chandrapala, Oliver, Kentish, & Ashokkumar, 2012; Mohamed & Amer Eissa, 2012). Studies have also indicated that these technologies may have little effect on bioactivity of food proteins (Soliva-Fortuny,
As these non-thermal technologies have little effect on bioactivity they may have a range of potential uses in the area of bioactive production. All three of these technologies have been shown to cause protein denaturation under specific conditions (Chandrapala, Zisu, Palmer, Kentish, & Ashokkumar, 2011; Li, 2012; Zhang, Jiang, Miao, Mu, & Li, 2012). This could be employed as a possible pre-treatment to facilitate the enzymatic hydrolysis of food proteins. Studies have been conducted which have shown that the application of ultrasound can enhance the rate of enzymatic hydrolysis under certain conditions (Babicz, Leite, de Souza, & Antunes, (2010); Ma et al., (2011)). It has been suggested that the enhancement is due to an increased rate of diffusion in the system (Yachmenev, Blanchard, & Lambert, 2004). High pressure processing has recently been exploited for use as a non-thermal preservation method. From the various studies which have been conducted in this area it is clear that high pressure is a viable option for inactivating both micro-organisms and enzymes in food ingredients (Bayındırlı, Alpas, Bozoğlu, & Hızal, (2006); Rademacher & Hinrichs, (2006)).

1.2 Functional foods

1.2.1 Current trends in the dairy industry and consumer demands

Ireland is the 10th largest dairy exporter in the world and dairy is the largest exporting food sector of the Irish economy generating an annual income of € 2.3 billion. In 2011, 5021 million litres of milk were produced in Ireland. Of this 494 million litres were consumed as liquid milk, while 145,900 tonnes of butter, 66,500 tonnes of milk powder and 179,900 tonnes of cheese were also produced (CSO,
2013). With the milk quota being lifted in 2015 there is an increased interest in exploring novel uses for milk in Ireland. When the quota system was introduced in 1984, Ireland was producing a similar volume of milk as New Zealand but since that time milk production has increased four-fold in New Zealand while remaining at the same level in Ireland (Ryan, 2012). However, it is believed that once the quota has been lifted the volume of milk produced in Ireland is likely to double.

Consumers are focusing more on health aspects associated with food products, which is reflected in the annual growth of the functional food market which is expanding by 4% more than the conventional food market (Bord bia, 2012). Foods which claim to prevent illness in particular are becoming increasingly popular, which is due to consumers becoming more informed about health issues and the fact that there is increasing strain being put on healthcare resources. Bioactive compounds in particular have been shown to reduce the risk of developing certain diseases such as cardiovascular disease and cancer.

1.2.2 Bioactive peptides

Bioactive peptides are defined as specific protein fragments that have a positive impact on body functions or conditions and may ultimately influence health (Kitts & Weiler, 2003). These peptides are present in many ingredients but the specific peptide sequence must be cleaved from the native protein for this activity to become apparent. The peptides are generally of up to 20 amino acids in length. These peptides can confer immunomodulatory (Agyei & Danquah, 2012), anti-tumour (Lin, Lin, Chen, Hui, & Chen, 2010), anti-hypertensive (Erdmann, Cheung, & Schröder, 2008), anti-oxidative (Aloğlu & Oner, 2011) and anti-microbial (Brown & Hancock, 2006) activities *in vivo*. The amino acid sequence and structure of the peptide are the factors that determine the activity of these peptides. Many of the
peptides have more than one bioactive property for example β-casokinin-10 has both ACE inhibitory and immunomodulatory activities (Meisel, 1998).

Food proteins are an ideal source of these peptides as they are already known to be safe for human ingestion and can be sourced and produced relatively cheaply. Milk is one such food source that is currently under extensive investigation as a potential source of bioactive peptides. Some of the bioactive peptides which have previously been discovered in milk can be seen in Table 1.1 below.

Table 1.1: Bioactive peptides sourced from milk proteins

<table>
<thead>
<tr>
<th>Protein source</th>
<th>Amino acid composition</th>
<th>Bioactivity</th>
<th>Released by</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Para κ-casein</td>
<td>Phe-Phe-Ser-Asp-Lys</td>
<td>Immunomodulatory</td>
<td>Trypsin</td>
<td>Gill, Doull, Rutherfurd, &amp; Cross, 2000</td>
</tr>
<tr>
<td>κ-casein</td>
<td>Met-Ala-Ile-Pro-Pro-Lys-Lys-Asp-Gln-Asp-Lys</td>
<td>Anti-thrombotic</td>
<td>Gastrointestinal digestion</td>
<td>Hartmann &amp; Meisel, 2007</td>
</tr>
<tr>
<td>β-lactoglobulin</td>
<td>Ile-Ile-Ala-Glu-Lys</td>
<td>Hypocholesterolemic</td>
<td>Trypsin</td>
<td>Hartmann &amp; Meisel, 2007</td>
</tr>
</tbody>
</table>

Peptides with bioactive properties can be produced through recombinant DNA techniques resulting in formation of highly specific peptides, for example Kim, Yoon, Yu, Lönnerdal, & Chung, (1999) expressed ACE inhibitory peptides based on sequences derived from human αs1-casein, in Escherichia coli. This method is however extremely expensive and, as such, large scale production of the desired peptide is not economically feasible. Therefore there has been increased interest in deriving bioactive peptides directly from food proteins. Methods such as enzymatic hydrolysis or bacterial fermentation of the food protein can be used to release the bioactive peptide.
The modes of action of bioactive peptides

As previously discussed bioactive peptides have a range of activities including immunomodulatory, anti-tumour, anti-hypertensive, anti-oxidative and anti-microbial activities. The modes of action of these peptides will be discussed briefly in the following paragraphs.

The immunomodulatory effects of bioactive peptides can be either an immune stimulating or suppressing action. Bovine lactoferrin isolated from whey protein has been shown to cause increased production of immunoglobulin A, which is important in mucosal immunity, in Peyer’s patch cells in the lymph node (Miyauchi, Kaino, Shinoda, Fukuwatari, & Hayasawa, 1997). The immune suppression conferred by peptides is generally as a result of increased levels of interleukin-10 which down-regulates the stimulation of T cells (Prioult, Pecquet, & Fliss, 2004). Gauthier, Pouliot, & Saint-Sauveur, (2006) have written a review article concerning the immunomodulatory peptides which have been isolated from whey protein.

Many of the anti-tumor peptides which have been investigated cause cell death by altering the membrane potential of tumour cells. Tachyplasin, a peptide of 17 amino acids length isolated from horseshoe crab, has been shown to have anti-tumour activity. Tachyplasin binds to hyaluronan (which is known to play a role in cancer metastasis) on the surface of the tumour cell to activate the innate immune system (Chen et al., 2005). Hoskin & Ramamoorthy, (2008) have written a comprehensive review of the literature concerning anti-tumour peptides.

ACE (angiotensin I converting enzyme) inhibitors are commonly administered to individuals that have high blood pressure. ACE cleaves angiotensinogen I resulting in the liberation of angiotensin II which is a known
vasoconstrictor (Fitzgerald, Murray, & Walsh, 2004). ACE inhibitory drugs can cause serious side effects on the individual, such as nausea, hypotension and renal failure, whereas naturally derived inhibitors are not associated with such problems. These naturally derived inhibitors have been isolated from milk proteins. Two peptides isolated from fermented milk (valyl-prolyl-proline and isoleucyl-prolyl-proline) have been shown to lower blood pressure in individuals over a prolonged time period (Seppo, Jauhiainen, Poussa, & Korpela, 2003). ACE inhibitory peptides were also shown to inhibit ACE activity more effectively than most ACE inhibitory drugs. A review of anti-hypertensive peptides has been written by Erdmann et al., (2008).

Oxidative and free radical species in the body are known to cause detrimental modifications to proteins, enzymes and DNA. These modifications can disrupt the normal function of the cell and can contribute to diseases such as rheumatoid arthritis, diabetes and cancer (Pihlanto, 2006). Anti-oxidant peptides which have been identified in casein hydrolysates include a peptide with superoxide anion scavenging activity (Suetsuna, Ukeda, & Ochi, 2000), and a peptide with oxygen scavenging activity (López-Expósito, et al., 2007). Pihlanto, (2006) has written a review of anti-oxidant peptides derived from milk proteins.

The emergence of anti-microbial resistance has become a huge threat to public health and has serious knock on effects in society (WHO, 2012). Anti-microbial peptides may provide part of the solution to this problem as the anti-microbial activity exhibited occurs through an alternative method than that of conventional antibiotic drugs (Kim & Wijesekara, 2010). The peptides alter membrane permeability or interact with internal receptors to cause cell death in microbial cells in vitro however, further investigations must be carried out to
determine the mechanism which occurs in vivo (Brown & Hancock, 2006). Anti-microbial peptides have been isolated from hydrolysates of $\alpha_{s2}$-casein, these anti-microbial peptides have activity against *Escherichia coli* and *Staphylococcus carnosus* (Zucht, Raida, Adermann, Máger, & Forssmann, 1995). Anti-microbial peptide activity has been reviewed by Agyei & Danquah, (2012).

A number of bioactive containing products are currently available in the market place; these include BioZate 1 (Davisco Foods International, USA), Recaldent (Cadbury Adams, USA) and BioPure-GMP (Davisco Foods International, USA). BioZate 1 is a hydrolysed whey protein isolate which contains $\beta$-lactoglobulin derived peptides (www.daviscofoods.com). These peptides exhibit ACE inhibitory activity thus this product claims to reduce the blood pressure of the consumer. Recaldent contains casein phophopeptides which have calcium binding properties and are bound to amorphous calcium phosphate. Recaldent is available as a chewing gum and toothpaste and claims to re-mineralise the teeth (www.recaldent.com). BioPure-GMP is a hydrolysed whey protein isolate which contains a bioactive glycomacropeptide derived from $\kappa$-casein. This peptide has anti-microbial activities and so the product claims to confer protection against viruses and bacteria (www.daviscofoods.com).

1.2.3 Production of bioactive peptides

Although bioactive peptides are currently being isolated from food proteins there are still some problems in the scale up process for commercial manufacture of purified peptides. The initial step is to liberate the peptide from the protein and can be achieved by microbial fermentation or hydrolysis by enzymes from plant or microbial sources (Agyei & Danquah, 2011).
The use of microbial fermentation can achieve a high degree of hydrolysis; however this method can be quite slow and the desired specificity of the hydrolysis may not be achievable. Belem, Gibbs, & Lee (1999) carried out microbial fermentation of whey protein isolate using *Kluyveromyces marxianus* to determine if bioactive peptides could be produced using this strain. However, they realised that the protease secreted by this strain was not able to release the specific bioactive peptide they were attempting to isolate. The authors concluded that the use of commercially available enzymes may be required to target this specific peptide.

Enzymatic hydrolysis of the food protein is the most widely used method of producing bioactive peptides. Food enzymes comprise 25% of the world enzyme market (Cherry & Fidantsef, 2003), which clearly indicates the widespread use of these enzymes in the industry. Commercially available enzymes are highly purified thus ensuring the required specificity of the hydrolysis can be achieved.

A high degree of hydrolysis is often required to generate the desired mix of peptides from the food protein; due to this fact the hydrolysis reactions can be quite lengthy. For example, Toldrá, Molina, Vinuesa, & Aristoy (2005) reported that when using immobilised porcine cathepsins it took up to 48 hours to achieve degradation of α- and β-casein to obtain polypeptides of 15-23 kDa.

The running cost of hydrolysis reactions can be very high due to the necessity of maintaining ambient conditions throughout the duration of the reaction. A reduction in the enzyme hydrolysis reaction time would therefore be extremely desirable as it would reduce the overall manufacturing costs by a considerable amount.
1.2.4 Sodium caseinate and casein hydrolysates

Casein comprises 80% of the protein content of bovine milk. The casein fraction is composed of $\alpha_{s1}$-casein (40% of the casein fraction), $\alpha_{s2}$-casein (12% of the casein fraction), $\beta$-casein (35% of the casein fraction) and $\kappa$-casein (13% of the casein fraction). The $\alpha$-casein fraction is composed of single chain polypeptides with 8-13 phosphate residue sites. Calcium ions within the milk system bind to these sites creating an insoluble protein under normal pH and temperature of milk. The $\beta$-casein fraction is also composed of single chain polypeptides which contain a phosphate residue cluster to which calcium binds, however unlike the $\alpha$-casein fraction the solubility of the $\beta$-casein is related to temperature e.g. at 1°C the protein is soluble. The $\kappa$-casein contains a carbohydrate component which is linked to serine or threonine, this carbohydrate tail plays a role in stabilising the casein micelle. The $\kappa$-casein polypeptide contains only one phosphate residue, therefore this protein is soluble (Rosenthal, 1991). The casein exists as colloidal particulate micelles (Figure 1.1) dispersed within the milk system. These micelles are formed as a result of hydrophobic interactions and calcium phosphate binding which causes aggregation of the individual caseins.

Sodium caseinate is produced by precipitating the casein fraction from milk. This is achieved by lowering the pH to 4.6, the isoelectric pH of casein. The casein is then washed and the pH is altered to 7 by dissolving the casein in NaOH. The resulting mixture is then spray dried (Muller, 1982).
Sodium caseinate is routinely used as a food additive due to its excellent functional properties (Flanagan & Fitzgerald, 2002). It is commonly used as a food additive to enhance the processing capabilities of the food, as it can improve foaming, emulsification and texture of food products (Dickinson, 2006). Casein can also improve the nutritional value of foods as it contains all of the essential amino acids.

Casein is commonly hydrolysed as casein hydrolysates have a wide variety of applications in the food industry. The two methods by which this is achieved are acid protein hydrolysis and enzymatic hydrolysis. Acid protein hydrolysis using 6 M HCl is commonly employed in industry as high degrees of hydrolysis can be achieved in a rapid time using this method. Despite this advantage acid protein hydrolysis causes formation of undesirable salts and other toxic by-products and induces damage to acid sensitive amino acids such as tryptophan, tyrosine, serine and threonine, thus reducing the nutritive value of the product (Fountoulakis & Lahm, 1998). Enzymatic hydrolysis of casein has several advantages over acid hydrolysis some of which include the fact that there are no toxic by-products produced, amino acids are not damaged and the resulting products are safe for
human consumption. The disadvantage of enzymatic hydrolysis is the long processing times required to achieve a high degree of hydrolysis.

As discussed previously casein hydrolysates constitute the raw material for the isolation of many bioactive peptides. In addition, supplementation with casein hydrolysates facilitates the rapid absorption of amino acids in the body. Due to this fact it has found application in infant formulas (Miquel, Alegría, Barberá, & Farré, 2006) and as a supplement (PeptoPro manufactured by DSM nutritional products Europe Ltd., Switzerland) to be taken following vigorous exercise to increase the rate of muscle recovery (Korhonen & Pihlanto, 2006).

1.3 Introduction to non-thermal technologies

Food processing is necessary to ensure that the microbiological safety of the food can be guaranteed and can be maintained upon storage of the food. The most common method of ensuring that the microbial load in the food is at an acceptable level is by applying heat (Awuah, Ramaswamy, & Economides, 2007). However, thermal processing of food ingredients can be quite harsh and may cause damage to the nutrients in the foodstuff; the texture, appearance and flavour of a food product can also be altered when a heat treatment is employed (Zhou, Xu, Sun, & Wang, 2002; Klensporf & Jeleń, 2008). It has also been noted that thermal processing of food proteins can cause denaturation of the protein, the extent of which is dependent on the heat treatment employed. This denaturation can have a negative impact on the bioactivity of the food (Korhonen et al., 1998). Heat treatment of dairy products in particular can cause formation of aggregates of whey proteins (Oldfield, Singh, Taylor, & Pearce, 2000). In terms of bioactive production these aggregates can also hinder the process of
enzymatic hydrolysis as they create mass transfer limitations in the system. For these reasons non-thermal preservation methods could yield major advantages in the nutritional quality and consumer acceptability of food products, particularly bioactive food products, while maintaining safety.

1.3.1 Non-thermal processing technology

The use of novel food processing technologies for many applications is currently being widely investigated by many groups. Many of these novel food processing techniques are non-thermal which has the potential benefit of having little or no effect on the quality of the food product (Barbosa-Canovas, Pothakamury, Palou, & Swanson, 1997).

Of these non-thermal processing technologies pulsed electric fields, low frequency ultrasound and high pressure appear to be the most promising (Rawson et al., 2011). Early investigations employing these technologies focused on their potential application in reducing microbial loads in food products and subsequent investigations found various applications for non-thermal technologies in food processing as discussed in the following sections.

1.3.2 Pulsed electric field technology

Pulsed electric fields (PEF) are applied in the form of short pulses (from micro to milliseconds) of high electric field strengths from 15-35 kV cm\(^{-1}\) and specific energy from 50-700 kJ kg\(^{-1}\) of product (Puértolas, López, Condón, Álvarez, & Raso, 2010). PEF equipment consists of a capacitor bank which is charged by a power source (Figure 1.2), the energy is then discharged from the bank across the food held in the treatment chamber (Qin, Pothakamury, Barbosa-Cánovas, & Swanson, 1996).
PEF can be operated in batch or continuous modes. The batch mode is suitable for lab scale studies while continuous operation is employed in pilot plant studies and for industrial applications (Pataro, Senatore, Donsì, & Ferrari, 2011). The voltage, current and electric field strength can be measured by an oscilloscope. Heat can be generated by this process, for example when a field strength of 27 kV cm\(^{-1}\) was applied for 2.4 µs for 8 pulses a temperature increase of up to 17°C was noted (Lindgren et al., 2002), however this problem can be overcome by means of a cooling system. Four different voltage waveforms can be employed, these are exponential, square wave, oscillatory and bipolar pulses (Figure 1.3) with exponential decay and square wave pulses being the most commonly applied (Toepfl, Heinz, & Knorr, 2007).
Figure 1.3: PEF waveforms; (a) exponential, (b) oscillatory (c) bipolar and (d) square waveforms

For exponential pulses the voltage increases to a peak value and decreases exponentially. The food is subjected to the peak pulse for a short time period. For square wave pulses a high voltage transmission line is connected to a matching load. The resistance of the food must be matched with the impedance of the transmission line to ensure the highest transfer to the food. Bipolar pulses are generated using two capacitor banks, one generates a positive pulse and the
second a negative pulse. Oscillatory pulses are generated by discharging energy from a bank of capacitors (Barbosa-Canovas et al., 1997).

Pulsed electric field technology has been shown to have a wide range of uses in food processing and preservation. Some of these applications include use as a means of inactivating microbes and enzymes in food and as a pre-treatment for extracting oils from plants and a pre treatment for drying of foods (Janositz, Semrau, & Knorr, 2011).

Pulsed electric fields are thought to inactivate micro-organisms by altering the transmembrane potential of the cell. This is achieved when the transmembrane potential is increased up to 1 V at which point poration of the cell occurs, thus increasing cell permeability resulting in cell death (Toepfl et al., 2007). Many studies have been conducted on microbial inactivation by PEF, for example, a 2.5 log reduction in *Listeria innocua* (a pathogen found in milk) was achieved by applying an electric field intensity of 50 kV cm$^{-1}$ with 32 pulses (Calderón-Miranda, Barbosa-Cánovas, & Swanson, 1999).

PEF also has the ability to inactivate enzymes in liquid foods, for example complete inactivation of peroxidase activity was achieved when orange juice was treated at 35 kV cm$^{-1}$ for 1500 µs, the temperature never exceeded 35°C during treatment (Elez-Martínez, Aguiló-Aguayo, & Martín-Bellos, 2006).

PEF has been successfully employed for the extraction of oil from rapeseed (Guderjan, Elez-Martínez, & Knorr, 2007). An electric field strength of 7 kV cm$^{-1}$ with 120 pulses (30 µs duration) lead to an increase of yield from 23% to 32% when hulled seeds were processed. This increased yield was attributed to an increase in cell permeability as a result of PEF.
Osmotic dehydration of food relies on mass transfer effects and the process can be enhanced by employing elevated temperatures and increased concentrations of osmotic solutions (Ispir & Toğrul, 2009). However, an increase in these parameters alters the flavour and texture of the food product. PEF has been employed to induce electroporation of cell membranes thus enhancing the mass transfer rate in this process while minimising adverse changes in the sensorial attributes of the food. For example, carrots were treated with a field strength of 0.6 kV cm\(^{-1}\) with a pulse duration of 0.05 seconds prior to osmotic dehydration with a sugar solution (Amami, Fersi, Vorobiev, & Kechaou, 2007). After 2 hours the PEF treated samples showed a water loss of 42% compared to 38% for untreated samples.

A major advantage of PEF processing is the fact that in most cases this technology has little or no impact on the texture, flavour and appearance of the food product which is to be treated. This was demonstrated in the case of an orange/carrot juice blend which was treated by PEF at a field strength of 25 kV cm\(^{-1}\) for 112 pulses of a duration of 280 µs (Rivas, Rodrigo, Martínez, Barbosa-Cánovas, & Rodrigo, 2006). The PEF treated sample showed a turbidity level similar to that of the untreated sample and had better colour retention than thermally pasteurised samples. Additionally, PEF treated samples were more acceptable to a sensory panel than the thermally treated samples when judged on odour and taste.

However, as this is a relatively novel technology further research is required to design an industrial scale model which will ensure uniform treatment of the sample and can achieve a high throughput in a cost effective manner (Puértolas et al., 2010). The cost of employing pulsed electric fields must also be...
considered. The cost of an industrial scale PEF system is approximately €2 million with a capability of processing 5 tonnes/hour, which when compared to thermal processing would increase production cost by ~€8/ton (Puértolas et al., 2010).

1.3.3 Low frequency high intensity ultrasound

The application of low frequency ultrasound has recently become a topic of intense interest. Low frequency ultrasound occurs at frequencies just above the scope of human hearing which are in the region of 20-100 kHz (Chisti, 2003; Leonelli & Mason, 2010). The mechanism through which ultrasound exhibits its effect is by means of cavitation, which is the formation and vibration of cavities in a liquid. The cavities, or cavitation bubbles, grow and expand until they eventually collapse as the ultrasonic wave passes through the liquid media (Figure 1.4). At the point of collapse of the cavitation bubbles, temperature and pressure extremes are generated (>4000°C and ~1000 atm) while the bulk of the media remains at ambient conditions (Knorr, Zenker, Heinz, & Lee, 2004; Gogate & Kabadi, 2009; Yachmenev, Condon, Klasson, & Lambert, 2009).

This collapsing of cavitation bubbles causes acoustic micro streaming to occur within a liquid system. This has the advantage of enhancing mixing in heterogeneous systems. This micro-streaming can be quite violent also and some studies have claimed that this mechanism can be used to cause break up of bulky substrates prior to enzymatic hydrolysis thus increasing hydrolysis rates (Yachmenev et al., 2009; Nikolić, Mojović, Rakin, Pejin, & Pejin, 2010).

The ultrasonic energy applied to a liquid media is measured in amplitude or intensity. The amplitude indicates the variation occurring in an acoustic variable
and is measured in µm (Figure 1.4). The intensity is the rate at which energy passes through a unit area and is measured in watts per meter squared (Wm²).

Figure 1.4: Oscillation of cavitation bubbles in response to pressure changes during ultrasound cycles (Soria & Villamiel, 2010)

Low frequency ultrasound has been investigated for preservation of foodstuffs by reducing microbial loads and reducing activity of undesirable enzymes. The cavitation phenomenon, which generates high shear forces, is thought to be the cause of the inactivation by causing damage to cell walls of microbes (Piyasena, Mohareb, & McKellar, 2003). One study conducted applied ultrasound to UHT milk inoculated with $1 \times 10^4$ cfu ml$^{-1}$ *Escherichia coli*. The milk was treated with a frequency of 20 kHz, intensity of 9.95 W cm$^2$ for 10 minutes which resulted in a 4.42 log$_{10}$ reduction of *E. coli* (Cameron, McMaster, & Britz, 2008). Low frequency ultrasound has also been investigated for enzyme inactivation; however there have been varying reports on the success of this application as in certain cases an enhancement of the activity has been observed (Ma et al., 2011).
The application of low frequency ultrasound in preservation techniques is extremely beneficial as microbial contaminants are destroyed in the food product while minimising any alteration to the appearance, texture, flavour or nutritional value of the food product. For example ripe strawberries were treated with 40 kHz frequency with an intensity of 0.015 W cm\(^2\) for 30 minutes and subsequently stored for 8 days at 5°C (Cao et al., 2010). The ultrasonically treated samples were shown to remain 17.5% firmer than untreated samples; this was thought to be as a result of inactivation of microbial species. Interestingly it was also noted that ultrasonically treated samples had significantly higher vitamin C levels than that of control samples.

Various applications for ultrasound in biological processes have been documented, for example the use of ultrasound in biofuel production (Stavarache, Vinatoru, & Maeda, 2007), in the food industry for defoaming, meat tenderisation and emulsification (Chemat, Zill-e-Huma, & Khan, 2011) and for the treatment of wastewater (Mahamuni & Adewuyi, 2010).

Another advantage that has been associated with ultrasound is the ability to enhance the rate of enzymatic reactions. This phenomenon has been noted by various authors (Sakakibara, Wang, Takahashi, Takahashi, & Mori, 1996; Yachmenev et al., 2009; Wang, Cao, Wang, & Sun, 2011) and is thought to be due to increased mixing in the system as a result of acoustic micro-streaming. The increased efficiency could lead to major cost savings and higher profitability in many manufacturing processes, particularly in the area of bioactive peptide production where enzyme hydrolysis is one of the lengthiest and thus costliest steps associated with production.
Ultrasonic probes can be quite expensive so there would be a substantial initial expense associated with this process; despite this the running costs are comparatively low. There have been some issues in scaling up ultrasonic reactors as it is difficult to achieve a uniform treatment on a large scale, currently most systems are on laboratory or pilot scale (Kumar, Gogate, & Pandit, 2007). This would pose a problem from an industrial point of view as only relatively small volumes can be processed. Therefore further investigations would be necessary to develop a suitable ultrasonic reactor for industrial scale applications. There is also the issue of heat generation when ultrasound is applied, for example, when a frequency of 20 kHz with intensity of 1.42 W cm\(^{-2}\) was applied to rapeseed oil for 20 minutes a temperature increase of 160°C was noted (Su et al., 2013). However, as with PEF processing this can be overcome by employing a suitable cooling system.

Another disadvantage to the use of ultrasound is ensuring a uniform application of the cavitational activity. Use of an ultrasonic probe will confer cavitation in a direct manner to the bulk media, with the cavitational activity dissipating as you move away from this point (Chisti, 2003). To overcome this problem there has been extensive work carried out to design ultrasonic reactors which would ensure uniform application of the ultrasonic energy (Del Campo, Coles, Marken, Compton, & Cordemans, 1999; Kumar, Gogate, & Pandit, 2007; Asakura, Nishida, Matsuoka, & Koda, 2008). Ultrasonic water baths are frequently used as they are readily available, easy to use and economical. Despite these advantages many authors have noted that non-homogeneous application of the ultrasonic frequency occurs in these baths (Barton, Bullock, & Weir, 1996; Sakakibara et al., 1996) and preliminary investigations are required
prior to conducting any study to determine the location in the bath which has the highest ultrasonic amplitude.

1.3.4 High pressure processing

High pressure processing (HPP) has been identified as a possible non-thermal treatment for the preservation of food products. High pressure is generated by direct compression, indirect compression or heating of the pressure medium (Mertens & Deplace, 1993). Direct compression involves pressurising a medium by means of a piston; this allows for rapid compression but is only suitable for use on laboratory and pilot scale systems. Indirect compression employs a pump to pressurise the medium to the desired level; this method is the most widely used on an industrial scale (Figure 1.5). Heating of the pressure medium causes the medium to expand to generate a high pressure; however this method involves high temperature and as such is not suitable as a non-thermal treatment. Cold isostatic systems are the most suitable for use in the food industry and employ pressures in the range of 50-600 MPa. Pressure treatments are generally carried out in batch systems as this reduces the wear to the machine and avoids the need to clean the system between each treatment (Barbosa-Canovas et al., 1997).

This method of preservation has the benefit of causing little alteration to sensory attributes and overall quality of the food (Barbosa-Canovas et al., 1997). HPP was initially investigated as a means of non-thermal preservation to reduce the levels of spoilage micro-organisms in foodstuff and has been applied to fruit juices, wine, pre-cooked meats and seafood (Riahi & Ramaswamy, 2004; Bover-Cid, Belletti, Garriga, & Aymerich, 2011; Buzrul, 2012).
High pressure causes increased cell permeability resulting in cell death and is also thought to disrupt DNA replication in microbial cells (Barbosa-Canovas et al., 1997). It was previously noted that a pressure of 350 MPa applied at 20°C for 5 minutes to kiwi fruit juice lead to a more than 5 log$_{10}$ reduction of both *E. coli* and *Listeria innocua* (Buzrul, Alpas, Largeteau, & Demazeau, 2008).

There are also a range of reports on enzymatic inactivation as a result of high pressure. This is due to the fact that high pressures cause protein denaturation (discussed in further detail in Section 1.4.4), which in the case of enzymes may cause an alteration in the active site. One investigation carried out indicated that amylase in apple juice could be inactivated by high pressure processing (Riahi & Ramaswamy, 2004). The apple juice required the application of a pulse of pressure at 400 MPa at pH 3 under ambient temperature for 50 minutes to achieve complete inactivation of amylase.
HPP has also been applied in the shucking of oysters. Traditional oyster shucking methods involve use of an oyster knife; however this method of shucking prevents a high throughput and requires skilled labourers. HPP has been shown to overcome this problem. He, Adams, Farkas, & Morrisey, (2002) found that applying a pressure of 310 MPa resulted in release of the adductor muscle from the shell. This approach has widely been applied in the processing of oysters.

HPP has also been successfully applied in processing of both cooked ham and guacamole; these products are currently available on the marketplace. Fressure foods™ have a range of guacamole products available, high pressure is employed as a method of cold pasteurisation of these products (Fressure Foods, 2013). In a similar manner high pressure is employed in the pasteurisation of a range of cooked meats by Greta Farms (Greta Farms, 2013).

HP can also be used to aid in the freezing and thawing processes of foods. A food product can be frozen more rapidly under elevated pressures allowing control of the growth of ice crystals, this means that the quality of the food remains more acceptable to the consumer than using conventional freezing methods (LeBail, Chevalier, Mussa, & Ghoul, 2002). One study conducted investigated the application of high pressure during the freezing of potatoes. It was found that when freezing under a pressure of 345 MPa the textural properties of the potatoes after subsequent thawing were improved over those processed by conventional freezing methods. This was attributed to the size of the ice crystals formed being reduced in the pressure frozen product due to a shorter freezing time (Luscher, Schlüter, & Knorr, 2005).

Thawing of food products under high pressure also occurs more rapidly than conventional thawing methods; however this has proven to cause some alteration
of the texture and appearance of the food product due to protein denaturation. For example, when salmon was thawed under a pressure of 200 MPa the drip loss was higher than that of conventional air thawed salmon, the peak force value was also significantly higher than control samples which may have been due to a tougher texture following pressurisation (Zhu, Ramaswamy, & Simpson, 2004).

Despite the range of potential applications of HPP, high pressure rigs require quite a high initial capital cost with prices ranging from $500,000 to $2.5 million (Balasubramaniam et al., 2008). Large scale throughput in many cases is achieved through use of a number of high pressure vessels. However, the running costs appear to be quite economical once the system is in place, when employing holding times from 5-20 minutes the cost of processing is between 10 and 25 cent/kg and throughputs of 200-800 kg/hour are achievable using a high pressure system with a 420 L capacity at a pressure of 600 MPa (Tonello, 2008).

1.3.5 Effects of novel technologies on bioactivity

Heat denaturation of most dietary proteins occurs at temperatures ranging from 60-90°C. In the case of milk this means that significant denaturation of whey proteins occurs during pasteurisation leading to a significant reduction in bioactivity (Korhonen et al., 1998). Non-thermal processing of whey protein may provide a solution to overcome this problem of reduced activity and potentially yield higher levels of bioactive peptides.

It is known that PEF processing can cause protein denaturation (discussed in Section 1.4.2) and the full impact of PEF treatments on the range of potentially bioactive compounds is not yet known. Despite this, the immunoactivity of milk appeared to be minimally effected by PEF treatment when compared to the effects associated with thermal treatments (Qin et al., 1996). Additionally it has
been proven that PEF has little effect on vitamin and fatty acid content in foods (Soliva-Fortuny et al., 2009). Another study indicated that antioxidant activity of polyphenols from orange peel was significantly higher (192%) when PEF (field strength of 7 kV cm\(^{-1}\) with a pulse duration of 15-150 µs) was employed for extraction in comparison to samples obtained by conventional extraction methods (Luengo, Álvarez, & Raso, 2013).

As previously discussed ultrasound improves mass transfer in systems and as such can improve the extraction of bioactives from food sources without causing alterations to the structure or function of the bioactive compound. An example of this is the extraction of polyphenols from arecanut, a seed from the areca palm tree. A frequency of 20 kHz, intensity of 30 W cm\(^{-2}\) for a duration of 50 minutes was used to obtain a high yield of polyphenols with a high anti-oxidant activity. This proved a much more rapid extraction method than conventional methods without having a negative effect on bioactivity (Chavan & Singhal, 2013).

An investigation was also carried out comparing the effects of ultrasonication with those of thermal treatments on vitamin C levels in tomato (Erçan & Soysal, 2011). A 40% loss of vitamin C was noted when a temperature of 67°C was applied for 10 minutes, while only a 12% loss was noted when ultrasound was applied (frequency of 23 kHz, intensity 2.36 W cm\(^{-2}\) for 90 seconds).

HPP is known to cause protein denaturation under certain conditions but employing ambient temperatures in combination with high pressure appears to have little effect on bioactivity in foods (Oey, Van der Plancken, Van Loey, & Hendrickx, 2008). HPP has been employed in the extraction of bioactive compounds from plant sources yielding higher levels of the target compounds.
with increased activity over those of conventionally extracted compounds (Azmir et al., 2013).

1.4 Effects of non-thermal processing on protein structure

While non-thermal processing technologies have received a lot of interest for application in food preservation there has been limited investigation of alternative applications in the food industry. Many studies have indicated that these non-thermal treatments can induce protein denaturation (Zhao & Yang, 2010; Arzeni, Pérez, & Pilosof, 2012; Mazri, Sánchez, Ramos, Calvo, & Pérez, 2012). This protein denaturation or unfolding could be employed as a pre-treatment of substrates prior to enzymatic hydrolysis, the loosening of the three dimensional structure of the protein increases the surface area for the enzyme biocatalysts to interact with thus potentially enhancing the overall rate of the hydrolysis reaction (Carlsson, Lagerkvist, & Morgan-Sagastume, 2012; Sindhu et al., 2012; Zhang, Jiang, Miao, Mu, & Li, 2012).

1.4.1 Protein structure

The structure of a protein is extremely important as this determines the functional properties of the protein. The levels of structure of the protein can be divided into four separate categories which signifies the complexity of these polymers. The primary structure of proteins is composed of a specific sequence of amino acids bonded together to form a polypeptide chain (Malacinski, 2003). The secondary structure involves formation of α-helix and β-sheet domains as a result of hydrogen bonding which occurs between the amine group of one amino acid and the carboxyl group of another. The tertiary structure involves folding of the α-helix and β-sheets to form a more compact structure, this folding occurs
due to hydrophobic interactions but the structure is held in this form by disulfide bonds and hydrogen bonds (Cassimeris, Lingappa, & Plopper, 2011). The quaternary structure of the protein is the complete three dimensional functional protein, which may be composed of multiple subunits held together with disulfide bonds and non-covalent bonds to stabilise the structure (Campbell & Reese, 2002).

1.4.2 Effects of pulsed electric field treatment on protein structure

PEF processing has been noted to cause denaturation of proteins, in particular enzymes (Zhao, Yang, & Zhang, 2012). However, the mechanism of this denaturation is not fully understood (Giner, Gimeno, Martín, & Barbosa-Cánovas, 2001) and may be as a result of increased local temperatures as a result of the high intensities of electric field employed (Huang, Tian, Gai, & Wang, 2012).

Zhao & Yang, (2010) compared the effect of pulsed electric field and thermal treatment (up to 100°C) on the structure of lysozyme. Circular dichroism showed that the application of PEF (35 kV cm⁻¹ for 1 µs) resulted in significant reduction of α-helix, β-sheet and random coil content (by 23, 11 and 10%, respectively), whereas the thermal treatments applied resulted in little structural alteration. This alteration to the structure was thought to be as a result of potential rapid increases and subsequent decreases to ambient temperatures caused by the applied electric field.

Soy protein isolate was treated with a field strength of 30 kV cm⁻¹ for 288 µs and 547 µs (Li, Chen, & Mo, 2007). This resulted in increased solubility of the soya protein isolate which was attributed to unfolding of the protein structure. PEF treatment for 547 µs caused SH groups to be exposed due to disulfide bond
disruption which in turn form disulfide bridges, subsequently leading to protein aggregation after denaturation occurred. Further analysis indicated that PEF treatment caused alteration to the secondary structure of the protein as indicated by a decrease of 3% in α-helical content and an increase of 3% of β-sheet content; however, these alterations were noted as not being statistically significant. The alteration of the soy protein isolate in response to PEF was attributed to molecular unfolding and dissociation of subunits.

Similarly the effects of PEF treatment (12.5 kV cm⁻¹ with 10 pulses of 2 µs) on the structure of β-lactoglobulin and egg white proteins (ovalbumin, conalbumin and lysozyme) were investigated. It was shown that denaturation of β-lactoglobulin occurred as a result of PEF treatment with only 60% of the native configuration of the protein remaining. Further analysis also indicated that secondary structure of the protein was altered leading to formation of aggregates. PEF treatment appeared to have a lesser effect on egg white proteins however with 74% native configuration remaining after treatment; this was thought to be due to the short processing times employed in the study. It was similarly noted that formation of aggregates occurred due to possible alteration of secondary structure (Perez & Pilosof, 2004). The mechanism by which PEF induced alteration to the egg white proteins was thought to be due to molecular unfolding, dissociation of subunits or polarisation of the protein molecule.

The effects of PEF on protein structure are highly variable as indicated by the varying results achieved in each of these studies. This may be due to issues associated with chamber design, it has been noted by many authors that non uniform PEF treatment can occur which can lead to formation of hot spots within the system (Jaeger, Meneses, & Knorr, 2009; Knoerzer, Baumann, & Buckow,
2012). If the treatment chamber is not optimised the results achieved may be the result of thermal effects rather than PEF processing.

1.4.3 Effect of ultrasound on protein structure

It has been noted by many authors that the cavitational phenomenon generated as a result of ultrasound could have the ability to physically disrupt the three dimensional structure of protein substrates prior to enzymatic hydrolysis leading to an enhancement in the rate of the hydrolysis (Czechowska-Biskup, Rokita, Lotfy, Ulanski, & Rosiak, 2005; Peña-Farfal et al., 2005; Yachmenev et al., 2009). This disruption of protein structure is attributed to the physical disruption of molecular bonds in response to shear forces generated by the collapse of cavitation bubbles.

The effects of ultrasound on the structure of whey protein concentrate (WPC) was investigated by Chandrapala, Zisu, Palmer, Kentish, & Ashokkumar, (2011). The samples (5% WPC (w/v)) were treated with a frequency of 20 kHz, intensity of 31 W cm\(^{-2}\) for 60 minutes at a temperature of 6\(^\circ\)C. It was noted that when treated for 5 minutes the viscosity of the samples was reduced significantly, however when the treatment time went beyond this point the viscosity began to increase. It was thought that this was due to initial disruption to secondary/tertiary structure of the whey protein followed by formation of aggregates. The \(\beta\)-lactoglobulin fraction was investigated further by circular dichroism spectroscopy and the results indicated that there were minor alterations to the secondary structure with \(\alpha\)-helix content increasing by 10\% and \(\beta\)-sheet content decreasing by 6-9\% compared with untreated \(\beta\)-lactoglobulin.

Gülseren, Güzey, Bruce, & Weiss, (2007) examined the effect of ultrasonication at an intensity of 20 W cm\(^{-2}\) and frequency of 20 kHz for up to 90
minutes on the structure of bovine serum albumin. Alterations to the secondary structure of the protein were noted, the α-helical content specifically increased from 61% to 74.5% when ultrasound was employed. It was also observed that at prolonged sonication times particle size increased which may indicate the formation of aggregates which was thought to be the result of interaction of exposed hydrophobic regions.

It was similarly noted that ultrasound treatment with frequency of 30 kHz and intensity of 4.27 W cm² applied to egg white caused an increase in particle size of 10% (Carolina Arzeni et al., 2012). It was also noted that the surface hydrophobicity of the protein increased following treatment indicating unfolding of the three dimensional protein structure. Despite this it was noted that there was little or no free surface SH groups after the egg white was treated with ultrasound, suggesting that the tertiary structure remained largely unaffected.

Soy protein isolate was treated with an ultrasound frequency of 20 kHz and intensity of 138 W cm² for 30 minutes (Hu et al., 2013). It was found that the free sulfhydryl content increased following ultrasound treatment, this was thought to be due to partial interruption of the tertiary structure. Surface hydrophobicity also increased which further indicates the disruption of the tertiary structure. Another finding from this work indicated possible alteration of the secondary structure also occurred following ultrasonication as an increase in α-helix and random coil content was noted and a decrease in β-sheet content.

It has been noted that low frequency ultrasound has a greater effect on protein structure than high frequency. For example, a study was conducted in which high frequency ultrasound, at 500 kHz, and low frequency ultrasound, at 20 and 40 kHz, was applied to whey protein concentrate and whey protein isolate
(Jambrak, Mason, Lelas, Herceg, & Herceg, 2008). The results indicated that there was no change to the foaming capacity or stability in the samples when 500 kHz was applied while these parameters were significantly altered when both 20 and 40 kHz was applied.

It should also be noted that the effects of ultrasound on protein structure shows highly varied results as can be seen from the above studies. However, this may be the due to variations in the ultrasonic settings employed in these investigations.

1.4.4 Effects of high pressure processing on protein structure

It is well established that high pressure processing can induce protein denaturation. In this case denaturation is induced as a result of volume changes caused by high pressure which can lead to bond breakage according to Le Chatelier’s principle. Van der Waals forces and hydrophobic interactions in particular are subject to disruption as a result of high pressure while hydrogen and covalent bonds are generally unaffected (Barbosa-Canovas et al., 1997). The effects of high pressure on protein structure has been comprehensively reviewed by Huppertz, Fox, de Kruif & Kelly, (2006).

Lactoferrin and lactoperoxidase are bioactive proteins which have been isolated from skim milk and have exhibited antimicrobial effects in vivo. High pressure treatments of skim milk were carried out to determine if the pressure treatment would have a detrimental effect on bioactivity (Mazri et al., 2012). Lactoperoxidase appeared to be extremely pressure resistant even when treated at 700 MPa for 15 minutes. However, lactoferrin appeared to be more susceptible to pressure induced denaturation, when treated at 400 MPa for 15 minutes there was a 25% decrease in the native configuration of lactoferrin.
The effects of high pressure processing on the structure of the major whey proteins was investigated (Hinrichs & Rademacher, 2005). At 500 MPa with holding times of 10-15 minutes 90% of the native configuration of β-lactoglobulin was denatured as analysed by reversed phase HPLC. At higher pressures again the same level of denaturation could be achieved after only 5 minutes. However, although the tertiary structure of α-lactalbumin appeared to be weakened (more S-S linkages were accessible to disulfide exchange) by pressure treatment of 500 MPa the structure remained unchanged even at prolonged holding times suggesting that it is quite pressure resistant, this is thought to be due to the fact that α-lactalbumin contains 4 intramolecular disulfide bonds while β-lactoglobulin contains only 2 such bonds.

An investigation was also carried out to determine the effects of high pressure on casein micelle structure. It was found that pressures between 400 and 600 MPa with a holding time of 15 minutes caused total breakdown of large micelles with the size reducing from 150-200 nm to ~40 nm (Needs, Stenning, Gill, Ferragut, & Rich, 2000). This was thought to be due to the interruption of hydrophobic interactions in the casein micelle.

As indicated each of these technologies can induce some form of protein denaturation. Therefore each of these technologies could potentially be employed as a pre-treatment to enzymatic hydrolysis. When the substrate is denatured the surface area increases which could allow a greater area for enzyme interaction thus leading to an increased rate of hydrolysis.
1.5 Use of low frequency ultrasound to enhance hydrolysis

While there has been much work carried out in the area of enzyme inactivation by ultrasound there has been quite a limited amount of work carried out which investigated the application of ultrasound to enhance enzyme hydrolysis reactions. As previously discussed reducing the length of enzyme hydrolysis reactions would be highly desirable for a wide range of applications. Ultrasound appears to be an ideal choice as little modification would be required to the system as transducers could be mounted on a pre-existing vessel wall or an ultrasonic probe could be employed which can be either directly inserted into the reaction vessel or it can be externally mounted and liquid can be pumped through a jacketed vessel with the probe inserted (Ma et al., 2011). The running costs of ultrasound are comparatively low (Vouters, 2004).

1.5.1 Enhanced diffusion generated by cavitation

Increased mass transfer in a hydrolysis system should increase the rate of the hydrolysis reaction as substrates would be brought into closer proximity to the enzymes and products would be moved away from the vicinity of active sites by the mixing action (Samaniuk, Scott, Root, & Klingenberg, 2011). The cheapest method of increasing mass transfer in conventional systems is to increase the stirring rate of the impeller. However, it is well established that high stirring rates can cause inactivation of enzyme preparations, for example lysozyme preparations (Colombié, Gaunand, & Lindet, 2001). This inactivation is caused as a result of foam formation created by the increased stirring speeds. The three dimensional configuration of the protein is altered as hydrophobic regions align towards the air bubbles in the foam (Phillips, Hawks, & German, 1995), resulting in enzyme inactivation. Babicz et al. (2010) also observed that high stirring speeds can cause
inactivation of commercial enzyme preparations due to the formation of foam which leads to aggregation of enzyme biomolecules on vessel walls.

It has been proposed that the cavitation process leads to efficient mixing in heterogeneous systems (Yachmenev et al., 2009; Babicz, Leite, de Souza, & Antunes, 2010). Phase boundaries between substrate and enzyme which occur in liquid heterogeneous systems create a physical barrier between the enzyme and substrate, the rate of diffusion across these boundaries is the rate limiting step in the enzymatic hydrolysis reaction (Yachmenev, Blanchard, & Lambert, 2004). Micro-streaming events which occur in the medium in response to cavitation can assist mass transfer across these boundaries thereby accelerating the rate of hydrolysis (Yachmenev et al., 2009).

1.5.2 Use of ultrasound for enhanced hydrolysis rates

The application of ultrasound to accelerate the enzymatic bleaching of textiles has proven to be successful (Basto, Tzanov, & Cavaco-Paulo, 2007). Cotton was treated at a frequency of 20 kHz and intensity of 1.94 W cm$^{-2}$ for 30 minutes at 50°C. The whiteness of the cotton was increased by 0.56 Berger units over the cotton treated with laccase (an enzyme commonly used for bleaching of materials) alone. The authors attributed this increased whiteness purely to increased mass transfer in the system, as it was noted that the specific activity of the enzyme was reduced when ultrasound was employed. The authors suggested that the effects of cavitation cause micro-jets to form in the system which allows for better diffusion of the enzyme through the substrate and thus increases the effective surface area of cotton for the enzyme to interact with. The result of this improved diffusion was that processing costs were reduced as there was a reduced biocatalyst requirement and a reduced processing time.
Yachmenev, Condon, Klasson & Lambert (2009) investigated the use of ultrasound for accelerating the enzymatic conversion of sugar cane bagasse (the fibrous material that remains after sugar canes are crushed to extract their juice) and corn stover to simple sugars. It was found that applying ultrasound (frequency 50 kHz, intensity 41.5 W cm$^{-2}$ for up to 8 hours) lead to an increase of roughly 15% in the conversion of corn stover (the leaves and stalks of maize left in the field after harvest) to glucose; however the rate of conversion was increased by approximately 32% during the first hour of the hydrolysis. When ultrasound was applied to the sugar cane bagasse the overall rate of conversion to glucose increased by 20.6%. This increase in the rate of conversion was attributed to increased mass transfer within the system. The application of ultrasound to the raw materials was also investigated to determine if the ultrasound would cause initial degradation of the substrate and thus decrease the hydrolysis time. The pre-treatment of raw materials with ultrasound appeared to have a minimal effect on the rate of the hydrolysis reaction for the corn stover while it did appear to increase the rate of hydrolysis when sugar cane bagasse was the raw material. This was thought to be due to the fact that the sugar cane bagasse is a more complex structure and diffusion would be limited through this substrate.

At the very high substrate concentrations used in industrial settings the rate of hydrolysis can be reduced significantly due to high viscosity. Barton, Bullock, & Weir (1996) investigated the use of ultrasound to overcome this problem. The study was carried out using sucrose as the substrate. It was determined that under control conditions a concentration of 0.7 M sucrose was optimum whereas when ultrasound (frequency 38 kHz with intensity of 0.37 W cm$^{-2}$) was applied the optimum sucrose concentration was increased to 1 M. The overall rate of the reaction was also increased by 50% when invertase was used in combination with
ultrasound; this was thought to be due to one of three mechanisms. The ultrasound may have caused substrate degradation through non-enzymatic methods, the enzyme activity may have been increased or the ultrasound may have caused disruption of hydrogen bonds preventing the formation of aggregates of substrate molecules.

A study was conducted employing high frequency (815 kHz) ultrasound to investigate the effect on invertase activity (Sakakibara et al., 1996). It was observed that the activity of the enzyme was reduced by 15% under ultrasonic processing at the highest intensity employed in the study (85 W cm\(^{-2}\)). Despite this, an increased rate of enzyme activity, corresponding to 30% over control, was noted when ultrasound was applied during the hydrolysis of 0.05 M sucrose. This was thought to be the result of ultrasound irradiation overcoming the phenomenon of substrate aggregation which occurs due to hydrogen bonding between sucrose molecules.

Jian, Wenyi, & Wuyong (2008) examined the use of combined ultrasound and enzyme processing of solid leather waste. An ultrasonic frequency of 40 kHz with intensity of 0.64 W cm\(^{-2}\) was employed for a duration of 28 hours. An alkaline protease isolated from Bacillus licheniformis was utilised to degrade pig skin. An initial study was conducted to elucidate the effect of ultrasound processing on the activity of the protease and it was found that there was little effect on the activity when treated for 120 minutes. Although the application of ultrasound did not increase the enzyme activity per se there was a higher rate of conversion of the substrate in the presence of ultrasound. A range of substrate concentrations were investigated and it was clear that the positive effect conferred by the ultrasound became more apparent at higher substrate concentrations, with a 46% increase in soluble proteins achieved at the highest substrate concentration employed.
Following the hydrolysis reaction the amino acid composition of the product was analysed. From this analysis it was indicated that ultrasound processing caused some initial breakdown of the collagen in the pig skin, which increased the diffusion of the enzyme leading to a higher rate of conversion.

Ultrasound was applied during the catalysis of glucose fatty acid esters by lipase. The ultrasound was applied to overcome mass transfer effects and increase the amount of dissolved glucose, thus increasing the rate of activity of the lipase. A frequency of 47 kHz with intensity of 0.11 W cm\(^{-2}\) was applied for a duration of six hours. The dissolution rate of the glucose was increased by 45% which subsequently resulted in a 34% increase of catalysis in the system (Lee, Nguyen, Koo, & Ha, 2008).

The effect of ultrasonic processing on the activity of the proteinase alcalase was investigated (Ma et al., 2011). The substrate employed in this investigation was casein. The alcalase samples were treated with a frequency of 20 kHz and intensity of 0.07 W cm\(^{-2}\) with pulses of 2 seconds duration and 2 seconds off. When the samples were treated for 4 minutes the activity of the alcalase increased by 5.8% over the untreated samples. This increased activity was thought to be as a result of the cavitation activity created by the ultrasonic processing. As the cavitation phenomenon generates efficient mixing, breakdown of substrate aggregates may have occurred resulting in an increased hydrolysis rate. Another possibility proposed was that the extreme temperatures and pressures generated by the ultrasound resulted in conformational changes in the enzyme which lead to increased activity.

Ultrasound has been employed to assist the enzymatic extraction of metals from seaweed (Peña-Farfal et al., 2005). Frequencies of 17 and 35 kHz were
employed with intensity of 0.13 W cm\(^{-2}\). There appeared to be a positive effect when ultrasound was employed with the yield of each metal increasing over the control. Another interesting finding was that there was no significant difference in yields obtained between the two frequencies studied.

Fiametti et al., (2011) employed low frequency ultrasound to enhance the hydrolysis of olive oil by lipase to yield mono- and diacylglycerols. An ultrasonic frequency of 37 kHz and an intensity of 0.74 W cm\(^{-2}\) applied for 2 hours. This intensity was determined to give the highest yields of both mono- and diacylglycerols; higher intensities caused a reduced yield and a reduced enzyme activity. This study did not determine the effect of ultrasonic processing alone, however it was noted that the activity of lipase was reduced by 30% by ultrasound under the conditions employed in this study.

Ultrasonic processing has also been investigated for the extraction of bioactive compounds from food ingredients. One such investigation employed a range of ultrasonic frequencies and intensities to improve the extraction of allicin from crushed garlic (Wang et al., 2011). Allicin has been shown to have a range of bioactivities including antimicrobial, antioxidant, antitumor and anti-inflammatory activities. Extraction of this compound from garlic is difficult as both the allicin and the enzyme alliinase are unstable. However the application of ultrasound at a frequency of 50 kHz, intensity of 0.4 W cm\(^{-2}\), for 30 minutes increased the yield of allicin by 25.2% over extraction using the enzyme alone. According to the authors this increased yield was the result of an increase of 42.8% in the activity of the alliinase. It was thought that the increased activity was caused by the action of cavitation bubbles altering the three dimensional configuration of the enzyme resulting in an exposure of the active site of the enzyme. Another study was
conducted to improve the extraction of lycopene from tomato peel (Konwarh, Pramanik, Kalita, Mahanta, & Karak, 2012). Lycopene has antitumor, anti-inflammatory and antithrombotic activities. When cellulase alone was used for the extraction a yield of 260 µg g⁻¹ was achieved, however when ultrasound was applied (frequency of 24 kHz, amplitude of 460 W cm⁻² for 12 minutes) in addition to cellulase a yield of greater than 600 µg g⁻¹ was achieved. This increased yield was attributed to enhanced mixing in the system allowing increased interaction between substrate and enzyme. The effect of ultrasonic processing on the structure of the lycopene was also investigated and it was determined that there was little or no effect of ultrasound on the structure of the compound.

Greige cotton is currently treated with sodium hydroxide in the presence of other agents to achieve a fibre which is highly absorbent. However, this generates a high level of alkaline wastes. An alternative treatment involves the use of enzymes but these are considered expensive and the enzymatic reactions are slow which have prevented widespread use. Therefore an investigation was carried out employing ultrasound during the enzymatic processing of greige cotton (Yachmenev, Blanchard, & Lambert, 2004). A reactor was designed which contained two transducer with differing frequencies (16 and 20 kHz) to create a continuously oscillating zone with intensity of 3 W cm⁻². The application of ultrasound in combination with pectinase lead to a >50% reduction in wicking time required over that of pectinase alone.

Another study was conducted to determine the effects of low frequency ultrasound on the conversion of cellulose to reducing sugars using the enzyme cellulase (Nakao, 1990). A frequency of 20 kHz with intensity of 0.25 W cm⁻² was employed. However, the results indicated that the application of ultrasound had
little or no effect on the rate of conversion to reducing sugar. Similarly cellulase was employed to investigate the conversion of waste papers to sugars (Li et al., 2005). A frequency of 20 kHz with intensity of 1.04 W cm$^{-2}$ was employed. It was found that ultrasound enhanced the rate of saccharification when office paper and carton paper were the substrates employed but had little effect on the rate of saccharification when newspaper was the substrate.

The majority of authors have indicated that low frequency ultrasound has a positive effect on the rate of enzymatic hydrolysis. As hydrolysis reactions can be quite time consuming any acceleration of the rate of hydrolysis would be significant. As previously discussed ultrasound appears to be an ideal technology to facilitate this acceleration as it is quite economical.

1.6 Use of non-thermal technologies to terminate hydrolysis

1.6.1 Residual enzyme activity in processed foods

Failure to inactivate specific native food enzymes can significantly reduce the shelf life of the food product and also affect the overall quality of the food (Laratta et al., 1995; Schweiggert, Schieber, & Carle, 2005; Limbo & Piergiovanni, 2006). Residual enzyme activity in food ingredients can cause rapid spoiling of the product; therefore it is necessary to ensure that the enzymes have been fully inactivated. Inactivation of most enzymes however requires extreme temperatures which in turn can lead to undesirable characteristics in the food ingredient and may lower the nutritional value of the food considerably (Kuda, Tsuda, & Yano, 2004; Guiavarc’h, Segovia, Hendrickx, & Van Loey, 2005; Anese & Sovrano, 2006). Thus investigations are ongoing to determine non-thermal methods of enzyme inactivation.
1.6.2 High pressure treatment for enzyme inactivation

It has been observed that HPP can be employed to cause enzyme inactivation. The mechanism of this inactivation is thought to be as a result of protein denaturation (Katsaros, Katapodis, & Taoukis, 2009; Rauh, Baars, & Delgado, 2009). This method of inactivation has the benefit of causing little or no alteration to sensory and nutritional quality of the food (Krebbers et al., 2003; Landl, Abadias, Sárraga, Viñas, & Picouet, 2010; McArdle, Marcos, Kerry, & Mullen, 2010).

Alkaline phosphatase is a naturally occurring enzyme in milk, the activity of this enzyme is used to indicate whether an applied treatment has been sufficient for preservation of milk. A study was conducted to investigate the inactivation of alkaline phosphatase (ALP) in milk under high pressure at ambient temperature. At a pressure of 650 MPa and a holding time of 128 minutes the activity of ALP was reduced by 99% (Rademacher & Hinrichs, 2006). However, it was noted that some reactivation of the enzyme can occur at room temperatures following high pressure processing.

The residual activity of plasmin in bovine milk following high pressure treatment was investigated by Scollard, Beresford, Needs, Murphy, & Kelly (2000). Plasmin has a notable effect on the quality of milk as it is responsible for the degradation of casein which can lead to bitterness. The effect of pressures ranging from 50-800 MPa with holding times of 1, 10 and 30 minutes were investigated. It was determined that a pressure of 800 MPa with a holding time of 10 minutes was required to reduce plasmin activity to ~20%, it was also noted that holding times beyond this caused no additional reduction in plasmin activity.
The effects of high pressure processing on the activity of plant proteases ficin and papain was investigated by Katsaros, Katapodis, & Taoukis, (2009). Pressure ranging from 200-900 MPa were employed with temperatures from 40-80°C. It was determined that both enzymes investigated were highly pressure resistant with little or no inactivation occurring below pressures of 450 MPa. It was noted that the higher the pressure employed the lower the temperature dependence of papain inactivation, while the opposite was true for ficin inactivation. At a pressure of 750 MPa at a holding time of 60 minutes and temperature of 50°C there was <30% residual activity of papain. While treatment of ficin for 30 minutes at a pressure of 900 MPa and temperature of 50°C there was ~80% residual activity.

Another study was conducted to assess the effects of high pressure processing on the activity of polyphenol oxidase (PPO) and peroxidase (POD) in strawberry puree (Terefe, Yang, Knoerzer, Buckow, & Versteeg, 2010). Both enzymes were determined to be highly pressure stable at ambient temperatures. Moreover it appears that PPO activity was enhanced by high pressure processing as treatment at 100 MPa for 15 minutes indicated a residual activity of 83%, while this residual activity increased by a further 29% when the pressure was increased to 690 MPa for the same holding time. Peroxidase activity however reduced with higher pressure processing, at 100 MPa with a holding time of 15 minutes the residual activity was determined to be ~77% while at a pressure of 690 MPa with the same holding time lead to a residual activity of ~29%. These results illustrate that the effects of pressure treatment on enzyme activity are highly dependent on the enzyme under investigation.
High pressure processing was employed to investigate the level of inactivation of amylase that could be achieved in apple juice (Riahi & Ramaswamy, 2004). At a pH of 3, temperature of 20°C and pressure of 400 MPa, amylase activity was reduced by 98.1%. The authors also noted that the level of inactivation achieved was highly dependent on the temperature and pH employed in addition to the pressures selected.

The residual activity of polyphenol oxidase (PPO) in apple juice after pressure treatments was investigated by Bayındırlı, Alpas, Bozoğlu, & Hızal, (2006). Polyphenol oxidase is known to cause enzymatic browning in fruit juices. The results of this investigation indicated that a pressure of 450 MPa with a holding time up to 30 minutes at ambient temperature actually increased PPO activity. However when the temperature was increased to 50°C with the same pressure conditions the PPO activity could be reduced by ~93%. This indicates that some enzymes which are highly pressure stable can be inactivated using a combination of mild thermal treatment and high pressure processing.

An investigation was conducted to determine whether high pressure processing could inactivate protease (isolated from *bacillus sp.*) activity in raw, homogenised and pasteurised bovine milk (Bilbao-Sáinz, Younce, Rasco, & Clark, 2009). The most severe treatment employed (600 MPa with holding time of 15 minutes) in this investigation did not cause a significant level of inactivation with 70% protease activity remaining, indicating that the enzyme was quite pressure resistant. It was also suggested that the use of high temperature may have negated the effects of the high pressure on the enzyme activity. Another interesting observation from this investigation was that the protease activity was actually increased in the homogenised milk sample following pressure treatment;
this was thought to be due to increased enzyme activity following the homogenisation process or alteration of the three dimensional structure of the enzyme.

Although it has been reported that high pressure processing can have little effect or even an enhancing effect on enzyme activity the majority of studies conducted indicated that employing pressures above 400 MPa lead to significant levels of inactivation. Therefore this method of processing may be of use for terminating the hydrolysis reaction for food proteins to prevent any adverse effects to the nutritional and sensory attributes of the food which can be induced by thermal inactivation.

1.7 Objectives

The aims of this work are to:

1. Determine if specific novel technologies (PEF, ultrasound and HPP) could be used as a pre-treatment of protein substrates to enhance the rate of enzymatic hydrolysis.

2. Investigate the application of low frequency ultrasound during enzymatic hydrolysis as a means of enhancing the hydrolysis rate.

3. Identify if the application of HPP is a suitable means of terminating the hydrolysis of dairy proteins by inactivating protease activity.
Experimental Section
2.0 The application of non-thermal technologies as pre-treatments to enzymatic hydrolysis of sodium caseinate

Abstract

Non-thermal technologies such as PEF, ultrasound and HP have been reported to induce protein denaturation under specific settings. As these technologies have little effect on bioactivity (as previously discussed in section 1.3.5) they could provide a useful substrate pre-treatment prior to bioactive production. Such pre-treatments may reduce the length of protein hydrolysis reactions by causing an initial disruption of the native protein structure which may allow the enzyme to access more labile bonds. Therefore this study was conducted to determine if PEF, ultrasound or HPP could be employed as an effective pre-treatment of sodium caseinate substrate to enhance subsequent hydrolysis rates. PEF pre-treatment of 34 kV cm\(^{-1}\) and a frequency of 420 Hz with a total specific energy of 276.81 kJ kg\(^{-1}\) appeared to have little effect on hydrolysis rate. Similarly a high pressure pre-treatment of 400 MPa for 30 minutes appeared to have little effect on the subsequent hydrolysis rate of the sodium caseinate. Low frequency ultrasound (frequency of 20 kHz and amplitude of 136 µm applied for 30 minutes) gave the only promising results with a decrease of ~33% in the hydrolysis time being achieved, however at a higher amplitude (170 µm) the hydrolysis rate was actually slower than that of the control reaction. Further optimisation of these conditions could maximise this effect.
2.1 Introduction

As discussed in Section 1.2.3, the enzymatic hydrolysis of dairy proteins is routinely performed in the food industry. This is a necessary step in the production of many high value products such as hydrolysed protein powders, which are added to a wide range of food products (for example, infant formulas, food supplements and flavour enhancers) and can improve functional properties (Kim et al., 2007; Chobert, 2012). More recently bioactive peptides have been discovered which confer health benefits (Hartmann & Meisel, 2007; Agyei & Danquah, 2011). However, as previously discussed in Section 1.2.4, hydrolysis reactions are lengthy processes (Toldrá et al., 2005). Due to this fact there is interest in pre-treatments which may accelerate this process which in turn would increase productivity and profit for manufacturers (Panagiotopoulos, Bakker, de Vrije, & Koukios, 2011; Carlsson, Lagerkvist, & Morgan-Sagastume, 2012).

As previously mentioned in Section 1.3.1, non-thermal processing of food ingredients, e.g. milk, cooked meats and guacamole, has received much attention recently due to the minimal effects these processes have on sensory and nutritional attributes of the food (Barbosa-Cánovas, Pothakamury, Palou, & Swanson, 1997). Of particular relevance in this context is the fact that PEF, ultrasound and HPP can also cause denaturation of proteins when applied at extreme settings (Needs, Stenning, Gill, Ferragut, & Rich, 2000; Gülseren, Güzey, Bruce, & Weiss, 2007; Li, Chen, & Mo, 2007). Protein denaturation may be an effective pre-treatment prior to enzymatic hydrolysis as the protein unfolds perhaps allowing the enzyme better access to more labile bonds.

PEF treatments have been conducted on milk proteins, liquid whole egg, soybean proteins and various enzymes (Section 1.3.2), the results of these
studies have indicated that PEF treatment can have a significant effect on the protein structure (Zhao et al., 2012). PEF causes alterations to secondary and tertiary structure of proteins, the alterations are caused as a result of disruption of hydrogen and disulfide bonds (Li et al., 2007; Li, 2012). When PEF was applied to bovine β-lactoglobulin (12.5 kV cm\(^{-1}\) applied for 235 s) it was observed that aggregates formed as a result of inter-molecular disulfide bond exchange. It was also determined that 40% of the ordered structure of the protein was denatured due to the PEF treatment (Perez & Pilosof, 2004).

Ultrasound can disrupt protein structure by physically breaking covalent bonds (Section 1.4.3). This bond breaking occurs due to the extreme shear forces generated when cavitation bubbles collapse (Arzeni et al., 2012 (b)). The alteration of egg white proteins, dairy proteins and soy protein isolate by ultrasound has been investigated (Hu et al., 2013; Chandrapala, Zisu, Palmer, Kentish, & Ashokkumar, 2011; Arzeni et al., 2012 (a), respectively). In the case of other substrates, such as the conversion of sugarcane and corn meal to biofuels, ultrasound pre-treatment also shows promising results. For example, when ultrasound was applied to corn meal (40 kHz, intensity of 7.96 W cm\(^{-2}\) for 5 minutes at 85°C) as a pre-treatment to the α-amylase catalysed conversion to glucose the yield was increased by 3.3% over that of untreated samples (Nikolić et al., 2010).

HPP causes the system to react to counter the applied pressure, according to the Le Chatelier-Braun principle, which in turn can lead to the disruption of hydrophobic and Van der Waals forces (Huppertz et al., 2002) as previously discussed in Section 1.4.4. Needs et al., (2000) investigated the effects of high pressure treatment (600 MPa for 15 minutes at ambient temperature) on skim
milk; they found that there was a reduction in casein micelle size of ~75% as measured by transmission electron microscopy and turbidity. The solubility of whey protein concentrate was decreased by 30% following pressure treatment. In both cases these results were thought to be due to disruption of hydrophobic interactions and disulfide bonds.

Overall, while there have been some studies suggesting that these technologies can be used to induce changes in protein structure, limited work has been performed in which these technologies have been employed as pre-treatments prior to enzyme hydrolysis of dairy proteins. Therefore this study was conducted to determine if PEF, ultrasound and HPP could be employed as pre-treatments to subsequently enhance the rate of enzyme hydrolysis of a sodium caseinate substrate.

2.2 Materials and methods

2.2.1 Materials

Sodium caseinate (88.6% protein) was obtained from Kerry ingredients ltd. (Listowel, Co. Kerry). Protamex®, a commercially available bacillus protease, was obtained from Novozymes A/S, Bagsvaerd, Denmark and had a declared activity of 1.5 AU/ g.

2.2.2 Preparation of substrate

The substrate employed in all cases was a 10% (w/v) sodium caseinate solution and was prepared as follows: 169 g (equivalent to 150 g of protein) of the sodium caseinate was dispersed in 1.4 L of distilled water. The powdered sodium caseinate was incorporated using a laboratory mixer (Model L4RT,
Silverson Machines Ltd., Chesham Bucks, England). The solution was then stored at 4°C overnight to allow collapse of any foam.

2.2.3 Pulsed electric field pre-treatment of substrate

For the PEF treatments, a lab scale customized system (ELCRACK HVP 5, DIL, German Institute of Food Technologies, Quackenbruck, Germany) was used. The maximum achievable voltage and frequency were 25 kV and 1 kHz, respectively, and the pulse width could be adjusted between 4 and 32 μs. The treatment module consisted of three co-linear treatment chambers with a refrigerated cooling module integrated. Each chamber held two parallel stainless steel electrodes separated by a 5.0 mm gap, with the electrode diameter being 3.0 mm, the total area of the chamber was 0.0707 cm²; which resulted in a total treatment volume of 0.106 cm³. The substrate was subjected to 10 μs pulses at a field intensity of 34 kV cm⁻¹ and a frequency of 420 Hz which gave a total number of pulses of 14. The flow rate through the system was 11.38 L hr⁻¹. The conductivity of the 10% sodium caseinate solution was determined to be 3.4 mS cm⁻¹. The temperature was monitored throughout the treatment (Appendix I), and water was circulated through the system at 2°C to minimise the risk of thermal denaturation of the substrate solution. The resistance of the solution in the system was calculated by:

\[
\text{Resistance} = \frac{\text{Gap (cm)}}{\text{Area} \times 0.001 \times \text{Conductivity}} \quad \text{(equation 2.1)}
\]

This was used to calculate the energy per pulse by the following equation:

\[
\text{Energy per pulse} = \frac{((\text{Input voltage} \times 1000)^2 \times (0.000001 \times \text{Pulse width}/\text{Resistance}))}{0.001 \times \text{Diameter} \times 1000 \times 2} \quad \text{(equation 2.2)}
\]
Using these values the total specific energy for the treatment was calculated as 275.13 kJ kg\(^{-1}\) by the following:

\[
\text{Total specific energy} = \text{Total pulses} \times \text{Energy per pulse} \quad \text{(equation 2.3)}
\]

### 2.2.4 Ultrasonic pre-treatment of substrate

A Hielscher UIP1000hd ultrasonic probe (Hielscher Ultrasonics GmbH, Teltow, Germany) was used to treat the substrate prior to hydrolysis. The probe had a frequency of 20 kHz and maximum power output of 1000 W. A 1.4 L sample of the substrate was subjected to 30 minutes ultrasonic treatment at 100\% (170 \(\mu\)m) or 80\% amplitude (136 \(\mu\)m). The temperature of the substrate was kept below thermal denaturation values (Appendix I) by employing a 2 L jacketed vessel with water circulating through the vessel at 15\°C.

### 2.2.5 High pressure pre-treatment of substrate

High pressure processing of substrate was carried out in a Stansted Fluid Power high pressure vessel (Stansted Fluid Power Ltd., Harlow, Essex, UK). The vessel can be operated at a maximum pressure of 900 MPa. The dimensions of the vessel were 37 mm x 300 mm with a capacity of 1 L and the pressure transmitting fluid employed was 15\% castor oil in ethanol. 200 ml aliquots of sodium caseinate were transferred to plastic centrifuge tubes and triple vacuum packed. The samples were then treated for 30 minutes at a pressure of 400 MPa. For the high pressure treated samples the degree of hydrolysis was monitored using the OPA method and TCA solubility index, the OPA method has been previously shown to correlate well with the pH stat method (Appendix I).

### 2.2.6 Enzymatic hydrolysis

The enzymatic hydrolysis of the substrate after PEF or ultrasound processing was carried out in a FerMac 200, fitted with FerMac 230 (agitation...
and temperature) and FerMac 260 (pH control) modules (Electrolab Ltd., Gloucestershire, UK). Prior to hydrolysis the substrate was heated to 50°C and the pH was adjusted to 7, which are optimal conditions for Protamex® activity (Liaset, Nortvedt, Lied, & Espe, 2002). The pH was maintained at 7 for the duration of the reaction by the addition of 1 M NaOH. The amount of NaOH added was recorded using SartoCollect software (Sartorius AG, Goettingen, Germany) and this formed the basis of the pH stat technique which was used to monitor the hydrolysis reaction for the pulsed electric field and ultrasound pre-treated samples. The reaction was terminated once a degree of hydrolysis (DH) of 7 was achieved.

The DH was calculated by the following equation (Adler-Nissen, 1986):

\[
\text{DH} = \frac{B \times MB \times \frac{1}{\alpha} \times \frac{1}{MP} \times \frac{1}{htot} \times 100\%}{\text{(Equation 2.4)}}
\]

Where \( B \) is the volume of NaOH consumed (ml), \( MB \) is the molarity of the NaOH, \( \alpha \) is the average degree of dissociation of the \( \alpha \)-amino groups with a given value of 0.44 at pH 7, \( MP \) (g) is the amount of the protein in the reaction mixture, and \( htot \) (meqv g\(^{-1}\)) is the sum of the millimoles of individual amino acids per gram of protein. The \( htot \) value used for the casein substrate was 8.2 meqv g\(^{-1}\) (Adler-Nissen, 1986).

2.2.7 OPA method

The increase in free NH\(_2\) groups during incubation was monitored by the OPA assay using the method of Nielsen, Petersen, & Dambmann, (2001) as follows: Aliquots (1 ml) were removed from the reaction vessel at appropriate
intervals. The samples were quickly transferred to boiling tubes and immediately submerged in a water bath heated to 95°C for 15 minutes to thermally inactivate the Protamex®. 400 µl of the inactivated reaction mixture were added to 3 ml of OPA reagent. The samples were mixed using a vortex mixer and allowed to stand for 2 minutes before the absorbance was recorded at 340 nm (UVmini 1240, Shimadzu Corporation, Kyoto, Japan). A blank sample was prepared using 400 µl of distilled water with 3 ml OPA reagent which has a typical value 0.07. A standard serine sample was prepared by diluting 50 mg serine in 500 ml distilled water. A standard sample was then prepared using 400 µl of the serine standard with 3 ml OPA reagent which has a typical value of 0.8. The percentage degree of hydrolysis was calculated by:

\[ DH = \frac{h}{htot} \times 100 \]  
(Equation 2.5)

Where \( h_{tot} \) is given as 8.2 (Adler-Nissen, 1986), and \( h \) is obtained by the following:

\[ \text{Serine NH}_2 = \frac{\text{OD sample} - \text{OD blank}}{\text{OD standard} - \text{OD blank}} \times 0.9516 \times 0.1 \times 100 \times \frac{P}{Z} \]  
(Equation 2.6)

Where serine NH\(_2\) = meqv serine NH\(_2\) g\(^{-1}\) protein; \( Z = g \) sample; \( P = \) protein % in sample; 0.1 is the sample volume in litre (L).

2.2.8 TCA solubility index

A modified version of the assay described by Gradisar et al., (2005) was also used to monitor the increase in TCA soluble peptides during hydrolysis. Aliquots (3 ml) were removed at 30 minute intervals from the reaction vessel. Ice cold 24% (w/v) TCA (3 ml) was added immediately to precipitate unhydrolysed protein. The samples were then filtered through Whatman no. 1 filter paper. The absorbance of the filtrate was recorded at 280 nm using a UV-Vis
spectrophotometer (UVmini 1240, Shimadzu Corporation, Kyoto, Japan). A blank sample was also prepared using 3 ml distilled water and 3 ml 24% (w/v) TCA.

2.2.9 Statistical analysis

All experiments were repeated at least three times. Statistical analysis was carried out using SPSS software (IBM Corporation, New York, USA). One-way ANOVA was used to determine difference between control and non-thermal pre-treated hydrolysis reactions. The Tukey post hoc test was used for pair wise comparison.

2.3 Results and Discussion

2.3.1 Pulsed electric field pre-treatment

The pulsed electric field conditions employed as a pre-treatment to enzyme hydrolysis appears to have had little or no effect on the rate of the hydrolysis (Figure 2.1). This may indicate that there was little or no modification of the protein structure occurring under the conditions investigated. The electric field strength applied may not have been sufficient to induce protein modification. Another possibility is that the pulse duration was inadequate to allow protein denaturation to occur, at lower flow rates thermal denaturation could be an issue. Casein is also known to have a naturally highly unordered structure (Fox & McSweeney, 1998(b)) which may mean that the PEF treatments employed would only have had a marginal effect on the structure.
A previous study (Li, 2012) indicated that soybean protein isolate could be denatured by a pulsed electric field treatment of 30 kV cm\(^{-1}\) with frequency of 400 Hz, the flow rate through that system however was 60 ml min\(^{-1}\) which was ~3 times slower than the flow rate employed in this present study which meant the PEF power input was ~3 times greater.

Perez & Pilosof, (2004) also suggested that a longer treatment time leads to more significant denaturation of proteins and confirmed that applying a field strength of 12.5 kV cm\(^{-1}\) for up to 235 seconds caused significant denaturation of β-lactoglobulin. Following the PEF treatment the β-lactoglobulin was found to be denatured by 40%. However, a batch mode was employed which generates a more intense treatment than the continuous mode used in the present study. It should also be noted that this was a very extreme treatment with the treatment time employed being ~10 times longer than that in most other studies conducted and could lead to a significant temperature increase in the system. Therefore
these results could possibly be due to thermal denaturation rather than due to the PEF treatment.

2.3.2 Low frequency ultrasound pre-treatment

Many investigations employing ultrasound to induce protein denaturation have indicated that the disruption of the structure is due largely to interruption of disulfide bonds (Gülseren et al., 2007; Hu et al., 2013). However, sodium caseinate is known to have very few thiol groups (Rasmussen et al., 1999) and so any disruption which may occur is more likely to be caused by interruption of non-covalent interactions such as hydrogen bond or hydrophobic interactions.

Low frequency ultrasound was investigated under two power settings as a pre-treatment to hydrolysis. Under both settings it was noted that an odour similar to burnt rubber was generated when the ultrasound was applied to sodium caseinate. At an amplitude of 170 µm it became apparent that there was actually an increased hydrolysis time over that of the control as indicated in Figure 2.2.

This increase in hydrolysis time was significantly different (p≤ 0.05) to that of the control time at degrees of hydrolysis of 4, 5, 6 and 7. A possible explanation for this result may have been due to the formation of aggregates. Hydrophobic regions of the protein may have become exposed following the treatment; this may consequently have led to the formation of aggregates. Such aggregates could reduce access of the enzyme to labile bonds, thus reducing the rate of the hydrolysis reaction.
Figure 2.2: Hydrolysis curve for incubation of 10% (w/v) sodium caseinate with Protamex® at 50°C and stirred at 400 rpm, control (■), ultrasound pre-treatment at frequency of 20 kHz and 170 µm amplitude (●) and 136 µm amplitude (●). Within each degree of hydrolysis, points with different superscripts are significantly different (p≤ 0.05)

This phenomenon was noted by (Lin et al., 2014) when casein was subjected to ultrasound treatment (frequency of 20 kHz and intensity of 42 W cm⁻² applied for 10 minutes). Following the ultrasound treatment these workers observed that particle size increased by ~43% due to the formation of aggregates. The authors concluded that this aggregation was the result of interaction between the casein and free radicals (which were produced as a result of cavitation).

The ultrasonic pre-treatment carried out at 136 µm on the other hand resulted in a decrease of ~33% in the time required to reach a DH of 7% over that of the control (Figure 2.2). The time required to achieve a degree of hydrolysis of 6 and 7% was significantly (p ≤0.05) quicker in the hydrolysis reaction which was pre-treated with 136 µm ultrasound. This milder treatment may have had a lesser effect on the structure of the casein causing unfolding of the structure to expose hydrophobic regions while having little or no effect on disulfide bonds. This trend has been noted previously by Arzeni et al., (2012 (a)) when whey protein
concentrate was subjected to ultrasound treatment (frequency of 20 kHz and amplitude of 114 µm applied for 20 minutes). The results of that investigation indicated that there were no free surface sulfhydryl groups generated following ultrasound treatment, which may suggest that there was little disruption of disulfide bonds, while surface hydrophobicity increased by ~34%. This was attributed, by the authors, to unfolding which exposed hydrophobic regions of the protein. These results are similar to the findings of Chandrapala et al., (2011) who also subjected whey protein concentrate to an ultrasound treatment of 20 kHz and intensity of 3.41 W cm\(^{-2}\) for up to 60 minutes, which resulted in increased surface hydrophobicity due possibly to protein unfolding. There was also no observed change in the true thiol content following the ultrasound treatment indicating that there was little disruption of disulfide bonds.

It is possible that there is an optimum range in which low frequency ultrasound induces modification of the protein structure. As previously discussed sodium caseinate is a naturally aggregated substrate and it is possible that the milder ultrasound treatment (136 µm amplitude) caused some disruption of these aggregates leading to exposure of hydrophobic regions. This effect would have particular benefit with the enzyme employed in this study, Protamex\(^\text{®}\), which has broad specificity for hydrophobic amino acids (Sidayikengera & Xia, 2006). The disruption of intermolecular aggregates mediated by hydrophobic interactions, as a result of ultrasound treatment, could increase the availability of labile bonds, which may explain the reduced hydrolysis time achieved when 136 µm amplitude was employed. However, it may be that increasing the amplitude beyond this point may induce re-aggregation of the sodium caseinate. This would reduce the access of the Protamex\(^\text{®}\) to labile bonds, thus reducing the rate of the subsequent hydrolysis reaction (as was observed when 170 µm amplitude was employed).
2.3.3 High pressure pre-treatment

The results of the high pressure pre-treatment are indicated in Figure 2.3 where it can be seen that the pre-treatment had little effect on the hydrolysis rate up to the end point of the hydrolysis at which time the hydrolysis rate, if anything, appears to be slower than that of the control.

![Figure 2.3](image-url)

**Figure 2.3: Hydrolysis curve for incubation of 10% (w/v) sodium caseinate with Protamex® at 50°C and stirred at 400 rpm, control (•) and high pressure pre-treatment of 400 MPa (●)**

A possible explanation for this result is the formation of aggregates due to hydrophobic interactions due to modification of the protein structure as a result of the high pressure applied. Such aggregates could reduce access of the enzyme to hydrophobic residues which would otherwise be susceptible to hydrolysis thus lowering the rate of the hydrolysis reaction. This observation is consistent with the results of the TCA solubility index (Figure 2.4) where it can be seen that the hydrolysis reaction appears to slow significantly after the first 30 minutes.

A similar effect was noted by Zhang, Jiang, Miao, Mu, & Li, (2012) when chickpea protein isolate was treated with high pressure followed by alcalase
catalysed hydrolysis. When pressures above 300 MPa were employed it was noted that, while the protein was denatured, the formation of aggregates led to a significantly reduced hydrolysis rate. However, when pressures in the range of 100-300 MPa were applied this aggregation was not noted and the hydrolysis rate was enhanced.

![Graph showing TCA solubility index](image)

**Figure 2.4: TCA solubility index for incubation of 10% (w/v) sodium caseinate with Protamex® at 50°C and stirred at 400 rpm, control (■) and high pressure pre-treatment of 400 MPa (●)**

In the context of other dairy proteins, another investigation indicated that pressures of 100-200 MPa caused unfolding of β-lactoglobulin, while pressures >200 MPa caused the formation of aggregates due to disulfide exchange (Hinrichs & Rademacher, 2005). It has also been noted that the application of high pressure, >200 MPa, can lead to disruption of casein micelles which can subsequently re-aggregate due to interaction of exposed hydrophobic regions (Needs et al., 2000).
2.4 Conclusions

The results of this study indicate that milder ultrasound treatments can provide an effective pre-treatment for substrate prior to enzyme hydrolysis. This effect may be due to a limited exposure of hydrophobic regions in such manner as increases the surface area of the protein thus increasing the available binding sites for the enzyme. However, there appears to be an optimum set of conditions which will have a positive effect on the subsequent hydrolysis reaction. Employing treatment conditions beyond this optimum can actually lead to an increased hydrolysis time. This may be due to a net positive/negative effect conferred by the application of ultrasound as will be discussed further in chapter 3. Therefore, further investigations would be necessary to further optimise the pre-treatment to establish if an even greater reduction in hydrolysis time might be achieved.

The application of pulsed electric fields and the field strength and treatment times investigated in this study appears to have had little effect on the rate of subsequent enzymatic hydrolysis which appeared to be unchanged in the PEF treated samples. This may be the result of an insufficient field strength or pulse duration which meant that the protein structure was unaltered by this treatment. As previously discussed many authors have observed that PEF treatment can modify the protein structure thus further investigations employing a broader range of PEF settings and treatment times may yield more positive results.

The high pressure treatment applied in this investigation may have caused the undesirable formation of aggregates which hindered the enzymatic hydrolysis. Thus further investigations could be conducted at lower pressures to investigate whether unfolding of the protein without disruption to hydrophobic
interactions could be achieved to enhance the rate of subsequent hydrolysis. It may also be beneficial to establish the degree of aggregation which occurs under the high pressure conditions employed in this study.
3.0 Investigation of the impact of ultrasound on the hydrolysis rate of sodium caseinate catalysed by Protamex®

Abstract

The isolation of bioactive peptides from food proteins has received much attention in recent years. One of the major drawbacks of large scale production of these peptides is the lengthy hydrolysis reactions required. There have been reports of enhancement of enzymatic hydrolysis reactions when low frequency ultrasound is applied. Therefore, this study was conducted to determine if application of low frequency ultrasound at 24 kHz, with amplitudes of 20 and 40 µm and stirring speeds ranging from 100-600 rpm, during the enzymatic hydrolysis of sodium caseinate would reduce hydrolysis times. At a stirring speed of 400 rpm and ultrasonic amplitudes of 20 µm and 40 µm the rate of hydrolysis was increased by ~25% and ~45% respectively. However, this rate enhancement was only observed under very specific conditions and the ultrasound appears to enhance the rate of hydrolysis up to a degree of hydrolysis of 7% beyond which there was no additional benefit.

3.1 Introduction

Bioactive peptides are defined as specific protein fragments that have a positive impact on body functions or conditions and may ultimately influence health (Kitts & Weiler, 2003). These peptides can confer immunomodulatory (Agyei & Danquah, 2012), anti-tumour (Lin et al., 2010), anti-hypertensive (Erdmann et al., 2008), anti-oxidative (Aloğlu & Oner, 2011) and anti-microbial
activities in vivo as previously discussed in Section 1.2.2. Many of these peptide sequences have been identified in milk proteins. The casein fraction of milk in particular has been shown to provide an excellent source of bioactive peptides with varying functionalities, for example both an immunomodulatory peptide and an antithrombotic peptide have been isolated from κ-casein (Agyei & Danquah, 2011).

The bioactivity of these peptides is not evident until they are cleaved from the native protein. Therefore, enzymatic hydrolysis of the protein is the initial step in production of these peptides. As previously discussed in Section 1.2.3, the fact that these peptides are so small, i.e. usually no more than 20 amino acids in length, a high degree of hydrolysis must be achieved to isolate the desired peptide fragment. This has proven to be an obstacle to large scale production of these peptides as the hydrolysis reactions are quite lengthy, generally 24-48 hours duration (Toldrá et al., 2005).

Low frequency ultrasound has a wide range of applications in the food processing industry (Section 1.3.3). Many of these applications are well established and routinely used, for example emulsification (Behrend, Ax, & Schubert, 2000), degassing of liquids (Gondrexon et al., 1997), accelerated ageing of alcoholic drinks (Chang, 2005), pasteurisation (Cameron, McMaster, & Britz, 2009) and tenderisation (Chang & Wong, 2012). The possibility of using low frequency ultrasound to enhance the rate of enzyme reactions however has received limited investigation.

Enhancement of various bioprocesses, such as wastewater treatment, biofuel production and extraction of bioactive compounds, has been achieved by applying ultrasound as previously discussed in Section 1.5.2. Low frequency,
high intensity ultrasound (20-100 kHz) creates a cavitational phenomenon caused by oscillating bubbles within the liquid. These oscillating bubbles expand to the point of collapse when they cause violent effects, namely localised extreme pressure (up to 5000 atm) and temperature (up to 5000°C) within the system, (Mason, 1990; Gogate & Kabadi, 2009, O’Donnell, Tiwari, Bourke, & Cullen, 2010; Soria & Villamiel, 2010). Violent shock waves and liquid jets also occur in the system when these bubbles collapse and this is thought to enhance mixing (Soria & Villamiel, 2010). This enhanced mixing may overcome any reaction rate limitation caused by mass transfer effects.

Sonication has proven to be an effective tool to enhance the extraction of various bioactive compounds when used in combination with enzyme preparations. For example, the extraction of lycopene from tomato peel (Konwarh et al., 2012) and the extraction of allicin from garlic (Wang et al., 2011) by enzymatic means. The yield of lycopene was increased from 260 µg g⁻¹, when cellulase alone was employed, to more than 600 µg g⁻¹, when cellulase and ultrasound were utilised in combination. Additionally, a 40% reduction in time required to achieve this yield was observed when ultrasound was employed. This increased yield was attributed to enhanced mass transfer in boundary layers due to collapsing cavitation bubbles, however it was noted that there was a slight decrease of cellulase activity in the presence of ultrasound. The yield of allicin from garlic was similarly improved when ultrasound was used in combination with enzymatic hydrolysis. Under the optimised conditions the activity of allinase was increased by ~43% per se when ultrasound was applied, this increased activity gave rise to a 25.2% increase in yield.
Various other authors also noted an increase in the rate of enzymatic hydrolysis when ultrasound was employed. One study indicated that the hydrolysis of corn stover and sugar cane bagasse to glucose by cellulase could be accelerated with ultrasound. This resulted in an increase of \(~15\%\) in the conversion of the corn stover and an increase of \(~20.5\%\) for the sugar cane bagasse (Yachmenev, Condon, Klasson, & Lambert, 2009). Another study involved the use of lipases in the hydrolysis of soy bean oil to yield diacylglycerols (Babicz et al., 2010). When ultrasound was applied there was a \(50\%\) increase in the yield. Similarly it was reported that the application of ultrasound could increase the rate of hydrolysis of a solution of \(1\ M\) sucrose by invertase by \(50\%\) (Barton et al., 1996).

There appears to be some dispute between authors as to the actual mechanism by which the enhanced hydrolysis rate is achieved when ultrasound is applied. Some authors claim that the conformation of the enzyme is altered thus exposing the active site in a way that enhances substrate binding and release of subsequent products (Fiametti et al., 2011; Ma et al., 2011; Wang et al., 2011). However, the majority of authors attribute the enhancement to improved mass transfer occurring within the system as a result of micro-streaming effects (Barton et al., 1996; Lee, Nguyen, Koo, & Ha, 2008; Yachmenev et al., 2009; Babicz et al., 2010).

While there have been some studies focused on enhancing enzymatic hydrolysis using ultrasound, there are few studies which investigate whether ultrasound can reduce the time required for the hydrolysis of soluble food proteins derived from dairy sources by proteases. There has also been limited work carried out investigating the effect of combined stirring and ultrasonication on the
enzyme hydrolysis reaction. If the hydrolysis of proteins could be enhanced by ultrasound this could reduce the production times associated with the high degrees of hydrolysis required to produce bioactive peptides. Thus the aims of the present work were to investigate the effects of varying ultrasonic amplitudes, stirring speeds and substrate concentrations on the rate of hydrolysis of sodium caseinate using Protamex®.

3.2 Materials and methods

3.2.1 Materials

Sodium caseinate and Protamex® were as described in Section 2.2.1. All other chemicals were obtained from Sigma chemicals (Wicklow, Ireland) or Fischer Scientific (Dublin 15, Ireland) and were of reagent grade.

3.2.2 Temperature change studies

A preliminary investigation was carried out to determine the temperature increases which occur when ultrasound is applied to the protein solution. For this preliminary investigation 1.5 L of distilled water was heated to and maintained at 50°C. The water was then pumped through a flow cell (1.03 ml capacity) containing the ultrasonic probe (Figure 3.1 (a)). An orthogonal factorial design with three variables and three intervals each was used to observe the temperature fluctuations when using combinations of the variables (Appendix II). The residence time ($\tau$) of the sample in the ultrasonic flow cell was calculated by:

$$\tau = \frac{V}{q}$$

(equation 3.1)
Where \( V \) is the volume of the cell and \( q \) is flow rate in the system. A second system setup was also investigated in which the ultrasonic probe was applied directly to the bulk media (Figure 3.1 (b)). In both cases a stirring speed of 400 rpm was employed.

3.2.3. Enzyme Hydrolysis reaction

3.2.3.1 Preparation of substrate solution

The standard conditions for the hydrolysis reaction were a 10% (w/v) protein solution and stirring speed of 400 rpm. Sodium caseinate was prepared as discussed in Section 2.2.2. Prior to the hydrolysis reaction the sodium caseinate substrate solution was heated to 50°C and the pH was adjusted to 7, which are the optimum conditions for Protamex® activity (Liaset et al., 2002). Protamex® (0.669 g) was diluted to 100 ml in distilled water and added to the sodium caseinate solution to give a final volume of 1.5 L in the reaction vessel and an enzyme to substrate concentration of 0.446 g Protamex®/100 g protein.

3.2.3.2 Hydrolysis reaction

The hydrolysis experiments employing the ultrasonic flow cell (Figure 3.1 (a)) were carried out in as discussed in Section 2.2.6. Stirring speeds ranging from 100-600 rpm were employed to ensure the mixture was homogenous.

3.2.4 Ultrasonic processing

A Hielscher UP400S ultrasonic probe (Hielscher Ultrasonics GmbH, Teltow, Germany) was used to induce cavitation effects in the system. This probe has maximum amplitude of 100 µm with frequency of 24 kHz and output power of 400 W. The probe tip has a diameter of 2 cm. Two methods of introducing the ultrasound to the reaction mixture were employed. The first involved pumping the reaction mixture to an external flow cell (1.03 ml capacity) where the ultrasound...
was applied (Figure 3.1 (a)). Samples were pumped from the reaction vessel to the flow cell using a MasterFlex Economy L/S drive (Fischer Scientific, Ballycoolin, Dublin 15, Ireland).

Figure 3.1: (a) Setup 1 application of ultrasound in external flow cell (b) Setup 2 application of ultrasound directly to reaction vessel throughout hydrolysis
In the second method (Figure 3.1 (b)) the ultrasonic probe was introduced directly into the reaction vessel and operated continuously throughout the hydrolysis. The probe was placed at depth of 4.5 cm into the vessel and the stirrer (with a 6 cm diameter) was placed at a depth of 8.5 cm in the vessel. The diameter of the beaker used was 14 cm and the depth of the hydrolysis mixture was 11.5 cm. A Hielscher UP400S ultrasonic probe, Hielscher Ultrasonics GmbH, Teltow, Germany, was used to induce cavitation effects in the system. The probe has maximum amplitude of 100 µm and a diameter of 2 cm. Following on from the results obtained in the temperature increase study (Appendix II) a flow rate of 25 ml per minute, amplitude of 40 µm and a jacketed heating water temperature of 45°C were selected as optimum conditions.

The effect of various ultrasonic amplitudes, stirring rates and protein concentrations on the rate of the hydrolysis reaction were investigated as indicated in Table 3.1.

Table 3.1: Experimental parameters employed to investigate the effect of ultrasound on hydrolysis rate in situ

<table>
<thead>
<tr>
<th>Amplitude (µm)</th>
<th>Stirring speed (rpm)</th>
<th>Substrate concentration (g/100g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>100</td>
<td>10</td>
</tr>
<tr>
<td>20</td>
<td>100</td>
<td>10</td>
</tr>
<tr>
<td>40</td>
<td>100</td>
<td>10</td>
</tr>
<tr>
<td>-</td>
<td>200</td>
<td>10</td>
</tr>
<tr>
<td>20</td>
<td>200</td>
<td>10</td>
</tr>
<tr>
<td>40</td>
<td>200</td>
<td>10</td>
</tr>
<tr>
<td>-</td>
<td>400</td>
<td>10</td>
</tr>
<tr>
<td>20</td>
<td>400</td>
<td>10</td>
</tr>
<tr>
<td>40</td>
<td>400</td>
<td>10</td>
</tr>
<tr>
<td>-</td>
<td>400</td>
<td>5</td>
</tr>
<tr>
<td>40</td>
<td>400</td>
<td>5</td>
</tr>
<tr>
<td>-</td>
<td>400</td>
<td>15</td>
</tr>
<tr>
<td>40</td>
<td>400</td>
<td>15</td>
</tr>
<tr>
<td>40</td>
<td>600</td>
<td>10</td>
</tr>
</tbody>
</table>
3.2.5 TCA solubility index

The TCA solubility index was obtained as discussed in Section 2.2.8

3.2.6 Viscosity of substrate solution

The viscosity of the sodium caseinate solution at different protein concentrations was determined using a physica MCR 301 rheometer (Anton Paar GmbH, Österreich, Austria), fitted with a cup (diameter 26.66 mm) and bob (diameter 28.92 mm), at a shear rate of 525 s\(^{-1}\) and temperature of 50\(^\circ\)C.

3.2.7 Statistical analysis

All experiments and assays were repeated at least three times. Statistical analysis was carried out using SPSS software (IBM Corporation, New York, USA). One-way ANOVA was used to determine significant differences between control and ultrasound assisted hydrolysis reactions. The Tukey post-hoc test was used for pair wise comparison.

3.3 Results and Discussion

3.3.1 Application of ultrasound through external flow cell

The results obtained when ultrasound was introduced in an external flow cell are presented in Figure 3.2 below. From this Figure it can be seen that there was no enhancement of the hydrolysis rate when ultrasound was introduced, in fact the hydrolysis reaction carried out in the presence of ultrasound took \(\sim1.5\) times longer to reach a degree of hydrolysis of 7\%. There was a statistically significant difference (\(p \leq 0.05\)) in the time taken to reach degrees of hydrolysis of 5, 6 and 7\% between the control and ultrasound hydrolysis reactions, perhaps suggesting that it was the latter stage of the ultrasound assisted hydrolysis that...
was slower than the control. This may be due to the fact that only 1.03 ml of the hydrolysis mixture was passing through the ultrasonic flow cell at a given time and an amplitude of 40 µm may have been too extreme to apply to such a small volume possibly resulting in some degree of enzyme inactivation, the effect of which may have accumulated with increasing incubation time.

Figure 3.2: Hydrolysis curve for incubation of 10% (w/v) sodium caseinate with Protamex® at 50°C and stirred at 400 rpm, control (■) and ultrasound (●). Within each degree of hydrolysis, points with different superscripts are significantly different (p ≤ 0.05), ultrasound applied in external flow cell.

The temperature of the sodium caseinate was also subject to fluctuation due to the ultrasonication. There was an average increase in temperature from 50°C to 56.5°C within the flow cell during sonication; these temperature fluctuations may also have negatively affected the enzyme activity. Previous studies carried out have noted that enzyme inactivation can occur from the combination of low frequency ultrasound and increased temperatures, for example in a study on pectin methylesterase in tomato juice it was observed that at a temperature of 50°C no inactivation of the enzyme occurred, however when an amplitude of 64 µm was applied in combination with heating to 50°C substantial inactivation occurred (Terefe et al., 2009). Due to the possible enzyme
inactivation and poor temperature control during passage through the flow cell an alternative experimental setup was subsequently used.

### 3.3.2 Application of ultrasound directly to hydrolysis vessel

This design involved introducing the ultrasonic probe directly into the reaction vessel under various stirring speeds as indicated in Figure 3.1 (b). This allowed for continuous application of the ultrasonic power to the bulk of the reaction medium throughout the duration of the hydrolysis reaction. Varying amplitudes and stirring speeds were investigated as indicated in Table 3.1.

The reaction was terminated after a degree of hydrolysis of 7% was obtained as it was observed that the reaction slowed significantly beyond this point in both the control and ultrasonically assisted reaction. This may be due to the fact that the substrate is initially consumed rapidly and once a 7% degree of hydrolysis has been obtained there are less labile bonds for the enzyme to interact with.

#### 3.3.2.1 Stirring speed of 100 rpm

Initially a stirring speed of 100 rpm was investigated; the effect of ultrasound under these conditions can be seen in Figure 3.3. It is clear that there is little or no difference between the control and ultrasound hydrolysis reactions at this stirring speed. The results from the TCA solubility assay (Figure 3.4) corroborate the observation that there was no significant enhancement effect (p >0.05) observed under these conditions.
Figure 3.3: Hydrolysis curve for incubation of 10% (w/v) sodium caseinate with Protamex® at 50°C and stirred at 100 rpm, control (■), 20 µm amplitude (▲) and 40 µm amplitude (●). Ultrasound applied in situ for duration of reaction.

While the application of the ultrasound may have been enhancing diffusion in the system, increasing the rate of the reaction, this effect may have been negated by the low stirring speeds which may not have been sufficient to dissipate local temperature increases caused by the ultrasonic cavitation which in turn may have lead to some level of enzyme inactivation.

Figure 3.4: TCA solubility index of control (■), 20 µm amplitude (▲) and 40 µm amplitude (●) samples read at 280 nm when stirred at 100 rpm.
3.3.2.2. Stirring speed of 200 rpm

Figure 3.5 indicates the reaction times for the control and ultrasound hydrolysis reactions at a stirring speed of 200 rpm.

![Figure 3.5: Hydrolysis curve for 10% (w/v) sodium caseinate incubated with Protamex® at 50°C and stirred at 200 rpm, control (■), 20 µm amplitude (▲) and 40 µm amplitude (●). Within each degree of hydrolysis, points with different superscripts are significantly different (p ≤ 0.05), ultrasound applied in situ for duration of reaction.](image)

When ultrasound was applied to the reaction mixture being stirred at 200 rpm the rate of hydrolysis was actually marginally decreased for both amplitudes investigated (Figure 3.5). This difference was only statistically significant (p ≤ 0.05) between the control hydrolysis reaction and that carried out in the presence of 20 µm amplitude, and then only when a DH of 7% was achieved. This may indicate that the higher amplitude employed was causing no net effect in the system while the lower amplitude appears to cause a slight inactivation of the enzyme, however as it was only significant at the final point of hydrolysis there is most likely little practical difference between the control and ultrasonically assisted hydrolysis at either amplitude. The results from the TCA solubility assay
(Figure 3.6) also indicate that there was little or no difference between the rates of the control and ultrasonically assisted hydrolysis reactions.

![Figure 3.6: TCA solubility index of control (■), 20 µm amplitude (▲) and 40 µm amplitude (●) samples read at 280 nm when stirred at 200 rpm](image)

As argued above this phenomenon may be a result of both a positive and negative effect of the ultrasound on the system. On the positive side the mass transfer may be improved on application of ultrasound but on the other hand localised heating can also occur within the system due to the cavitation action (Gogate & Kabadi, 2009). Therefore the stirring speed of 200 rpm may not have been sufficient to allow for adequate heat dissipation in the system resulting in no net benefit for the application of ultrasound.

### 3.3.2.3 Stirring speed of 400 rpm

From Figure 3.7 it can clearly be seen that at an agitation rate of 400 rpm the application of both ultrasonic amplitudes resulted in a significant increase (p ≤0.05) in the rate of hydrolysis over the control experiment.
Figure 3.7: Hydrolysis curve for 10% (w/v) sodium caseinate incubated with Protamex® at 50°C with stirring at 400 rpm, control (●), 20 µm amplitude (▲) and 40 µm amplitude (●). Within each degree of hydrolysis, points with different superscripts are significantly different (p ≤ 0.05), ultrasound applied in situ for duration of reaction

When ultrasonic amplitude of 20 µm was applied a 25% decrease in the time required to achieve a degree of hydrolysis of 7% was observed relative to the control, when the amplitude was increased to 40 µm the hydrolysis time was ~45% lower than the control. The results of the TCA assay also confirm a positive effect of ultrasound on hydrolysis rate (Figure 3.8). Ultrasonic amplitude of 60 µm was also investigated at this stirring rate however, this increased the temperature by 19.2°C (from 50°C to 69.2°C) in the reaction vessel, which is well beyond the optimum temperature range of the enzyme (Kennedy, Cronin, O'Sullivan, & Lyng, 2012), quite likely leading to substantially reduced enzyme activity.

Thus it seems that under these specific conditions, i.e. 400 rpm and ultrasonic amplitude of 20 or 40 µm, that ultrasound appears to increase the hydrolysis rate. The fact that this effect is evident only at this stirring speed may be due to better mixing within the vessel, which ensured that any local high temperatures induced by the ultrasound are rapidly dissipated thus leading to
minimal loss in enzyme activity allowing the effects of improved mass transfer to become apparent.

![Graph showing solubility index](image)

**Figure 3.8:** TCA solubility index of control (■), 20 μm amplitude and (▲) 40 μm amplitude (●) samples read at 280 nm when stirred at 400 rpm

The finding that the enhancement observed increased with increasing amplitude up to 40 μm is consistent with previous reports that showed the beneficial effect of ultrasound on the lipase catalysed production of diacylglycerols from olive oil (Fiametti et al., 2011). When the ultrasonic probe was applied at a power output of 150 W a 16.5% yield of diacylglycerol was obtained, however when the power output was increased to 175 W a yield of 21.33% was obtained.

### 3.3.2.4 Stirring at 600 rpm

An attempt was made to investigate the effect of ultrasound on the hydrolysis reaction at a stirring speed of 600 rpm, however as is evident from Figure 3.9 the control hydrolysis reaction was extremely slow, taking 3 times as long to achieve 7% degree of hydrolysis as the 400 rpm control included for comparison.
This prolonged hydrolysis was mainly due to extensive foaming which occurred in the reaction vessel at this stirring rate. It is well established that denaturation of proteins can occur at liquid-gas boundaries (Phillips, Hawks, & German, 1995; Bramanti et al., 2006). This is the result of conformational changes in the protein, which align their hydrophobic regions towards the gaseous phase of the foam. This process of enzyme inactivation by over agitation and foaming was noted in another study where inactivation of lysozyme occurred at a stirring rate of 740 rpm (Colombie, Gaunand, & Lindet, 2001). Since any effect of ultrasound was likely to be obscured by the enzyme inactivation effect of foaming no further investigations were carried out at this agitation rate.

### 3.3.2.5 Effect of substrate concentration

From previous work it has been reported that for highly viscous substrates, such as mono- and diacylglycerols, the application of ultrasound overcomes rate limitations thus enhancing the rate of hydrolysis (Stavarache, Vinatoru, & Maeda, 2007; Fiametti et al., 2011). Since the sodium caseinate substrate forms a

![Figure 3.9: Hydrolysis curve for 10% (w/v) sodium caseinate incubated with Protamex® at 50°C, with stirring speed of 400 rpm (■) and 600 rpm (●)](image-url)
relatively viscous solution the effects of ultrasound were investigated at two additional protein concentrations of 5\% (w/v) and 15\% (w/v). The 5\% (w/v), 10\% (w/v) and 15\% (w/v) protein solutions at a shear rate of 525 s\(^{-1}\) had apparent viscosities of 3.78 \times 10^{-3} \text{ Pa s}, 7.54 \times 10^{-3} \text{ Pa s}, and 5.106 \times 10^{-2} \text{ Pa s}, respectively.

The effect of ultrasound on hydrolysis rates was strongly dependent on substrate concentration. At 5\% (w/v) there was no significant effect (p >0.05) as indicated in Figure 3.10, whereas at a substrate concentration of 10\% (w/v) there was a marked increase in the hydrolysis rate (p ≤0.05). At the highest concentration (15\% (w/v)) the application of ultrasound appeared to result in a significant (p ≤0.05) increase in the rate of hydrolysis (Figure 3.11).

Despite this, in the 15\% (w/v) substrate concentration, there was a prolonged hydrolysis time for both the control and ultrasound treated samples, particularly in the control hydrolysis reaction which despite an incubation time of >5.5 hours only achieved a 4\% degree of hydrolysis. One explanation for the observed effects could be that the effect of ultrasound is multi-factorial. Firstly, there is a positive effect due to enhanced mass transfer which would be expected to be most manifest at higher substrate viscosities. On the other hand the localised extremes of temperature and pressure caused by ultrasonic cavitation could give rise to enzyme inactivation. Thus the net effect of ultrasound on hydrolysis may depend on the balance of these opposing effects.
Figure 3.10: Hydrolysis curve for 5% (w/v) sodium caseinate incubated with Protamex® at 50°C, control (■) and ultrasound applied in situ at 40 µm amplitude (●)

In the case of the 5% (w/v) sodium caseinate solution it may be that the viscosity is so low that the benefits due to improved mass transfer are not evident. Additionally the localised temperature increases are more easily dissipated in the low viscosity medium so there is neither a positive nor negative effect at this concentration. At 10% (w/v) the solution is sufficiently viscous to benefit from the
increased mass transfer yet still able to dissipate localised heating resulting in a net benefit to hydrolysis. In the case of the 15% (w/v) substrate solution the high viscosity may have reduced the capacity of the system to dissipate localised high temperatures associated with ultrasound, resulting in an increased level of enzyme inactivation which may have offset any benefits arising from improved mass transfer resulting in a net decrease in hydrolysis rate. It was expected that a greater enhancement in hydrolysis rate would occur at this concentration. However, other authors have also observed that application of ultrasound to highly viscous substrates containing high starch concentrations has limited benefits (Barton et al., 1996).

The results obtained were not as expected from previous investigations carried out in this area as little effect of ultrasound on reaction rate was observed in most cases for this work. This may be explained by the nature of the substrate employed. Previous work reporting positive effects of ultrasound on hydrolysis generally employed insoluble substrates and the observed reduction in the hydrolysis time was attributed to a breaking down of the substrate due to the cavitational action of the ultrasound which in turn allowed for a greater diffusion rate in the reaction system (Barton et al., 1996; Yachmenev, Condon, Klasson, & Lambert, 2009; Babicz, Leite, de Souza, & Antunes, 2010; Fiametti et al., 2011). The sodium caseinate employed in this study was a soluble substrate and it was thought that any benefit from the application of the ultrasound would arise from increased mixing in the boundary layer between the substrate and enzyme. While this effect can be seen, it is only of significant benefit under very specific conditions an observation which is in agreement with findings of previous workers (Guo, Kim, Sung, & Lee, 2010; Ma et al., 2011; Wang et al., 2011).
3.4 Conclusion

It is clear that applying ultrasound to the hydrolysis of sodium caseinate can lead to a reduction in the hydrolysis time at a stirring speed of 400 rpm, amplitudes of 20 and 40 µm and a protein concentration of 10% (w/v). From this work it became clear that the effect was only observed within a specific and narrow range. For industrial application an investigation of this kind would be necessary for process optimisation for each enzyme and substrate of interest. Furthermore, as industrial processes commonly use a combination of enzymes to improve the rate of the hydrolysis this process would need to be further optimised to ensure that there was no reduction in the activity of any component of such a mixed enzyme preparation.
4.0 Investigation of Protamex® inactivation in sodium caseinate and distilled water by thermal and high pressure treatments

Abstract

The use of HPP for enzyme inactivation is currently under investigation by many researchers. It has been noted that HPP has little effect on the bioactivity of food proteins. Therefore this study was conducted to determine whether HPP could be employed as a method of terminating the hydrolysis reaction for the production of bioactive peptides. Pressures ranging from 100-600 MPa were applied to samples of 10% (w/v) sodium caseinate or distilled water containing Protamex®. It was found that the activity of Protamex® in distilled water could be reduced by >90% when treated at pressures ranging from 400-600 MPa for 30 minutes while in hydrolysed sodium caseinate ~90% reduction in activity could be achieved when treated at 600 MPa for 30 minutes.

4.1 Introduction

HPP of foods has many advantages over thermal processing as there is little alteration to sensory and nutritional attributes of food products (Barbosa-Canovas et al., 1997). As previously discussed in Section 1.3.4, high pressure can be employed as an effective preservation technique to decrease microbial loads in food systems (Balasubramaniam, Farkas, & Turek, 2008; Bover-Cid, Belletti, Garriga, & Aymerich, 2011). It also has applications in the food processing industry as a means of oyster shucking (He et al., 2002) and to allow better control of the freezing and thawing process in foods (LeBail et al., 2002).
HPP can also be used to inactivate native enzymes in food products (Section 1.6.2). Residual enzyme activity in food products post processing can have adverse effects on the quality as the enzyme can reduce the shelf life of the food (Laratta et al., 1995; Schweiggert, Schieber, & Carle, 2005). High pressure inactivation could also be employed to terminate the hydrolysis reaction of food proteins for bioactive peptide production. This method of termination would have more benefit than thermal treatments as it is well established that HPP has little or no effect on the bioactivities of food (Oey et al., 2008) whereas thermal treatments can cause protein denaturation leading to a loss of bioactivity (Korhonen et al., 1998).

There have been some studies carried out investigating the inactivation of food enzymes by high pressure and the results of most studies indicate that a sufficiently high level of inactivation can be achieved. This high pressure inactivation occurs due to the denaturation of the enzyme structure. As previously discussed in Section 1.4.4, high pressure can induce protein denaturation by interrupting electrostatic and hydrophobic interactions (Needs, Stenning, Gill, Ferragut, & Rich, 2000; Zhang, Jiang, Miao, Mu, & Li, 2012).

One such study was conducted by Scollard, Beresford, Needs, Murphy, & Kelly, (2000) where the effects of HPP on plasmin activity in milk was investigated. Pressures ranging from 50-800 MPa were applied for 1, 10 and 30 minutes at ambient temperatures. The results indicated that plasmin was quite pressure resistant, with <50% inactivation achieved at pressures below 600 MPa. However, when 800 MPa was applied for 10 minutes the activity was reduced by ~80% but it was noted that no additional inactivation occurred when the holding time was longer than 10 minutes at this pressure.
Another study conducted also examined the effect of high pressure on activity of enzymes in bovine milk (Rademacher & Hinrichs, 2006). The enzymes investigated were γ-glutamyltransferase, phosphohexoseisomerase and alkaline phosphatase. Pressures ranging from 400-800 MPa with holding times of 0-128 minutes were employed at ambient temperatures. The results indicated that pressures of 600 MPa and higher lead to significant inactivation of alkaline phosphatase with >90% inactivation being achieved at a holding time of 128 minutes. Complete inactivation of γ-glutamyltransferase and phosphohexoseisomerase was achieved at a lower pressure of 500 MPa and holding time of 30 minutes, indicating that these enzymes were more susceptible to pressure induced inactivation.

The effects of high pressure on amylase activity in apple juice was also examined (Riahi & Ramaswamy, 2004). Pressures of 100-400 MPa with holding times of 12-60 minutes, pH of 3.5 and ambient temperatures were employed. The results showed that pressures below 340 MPa did not induce significant levels of inactivation (<50%), while at a pressure of 400 MPa with a holding time of 30 minutes the amylase activity was reduced by 82%.

While there has been some work carried out in this area there is little or no information on the inactivation of enzymes by high pressure to terminate hydrolysis reactions. The aim of this study was to investigate the effect of high pressure on the activity of Protamex® in the presence of a 10% (w/v) sodium caseinate substrate or water under ambient temperatures.
4.2 Materials and methods

4.2.1 Materials

As discussed in Section 2.2.1

4.2.2 Sodium caseinate hydrolysis

A 10% (w/v) protein solution of sodium caseinate was prepared as described in Section 2.2.2. This solution was subsequently hydrolysed to a degree of hydrolysis (DH) of 7% as described in Section 2.2.6 and the reaction was terminated by thermally inactivating the enzyme by heating to 95°C for 15 minutes.

4.2.3 High pressure processing

High pressure processing was carried out in a Stansted Fluid Power high pressure vessel (Stansted Fluid Power Ltd., Harlow, Essex, UK). The vessel has maximum pressure of 900 MPa. The dimensions of the vessel were 37 mm x 300 mm and the pressure transmitting fluid employed was 15% castor oil in ethanol. Aliquots (9 ml) of sodium caseinate hydrolysate and distilled water had 1 ml of a Protamex® solution added (0.446 g Protamex®/100 g protein). Each of the samples was triple vacuum packed prior to pressure treatment. The samples were treated at pressures ranging from 200-600 MPa and holding times of 0-30 minutes at a temperature of 20°C. Control samples were prepared by heating 10 ml of sodium caseinate hydrolysate and 10 ml of distilled water both containing Protamex® at 20°C under atmospheric pressure.

4.2.4 Thermal inactivation

3 ml of 7% DH sodium caseinate or distilled water containing Protamex® (0.446 g/100 g protein) were transferred to boiling tubes. Samples were subjected
to thermal treatments from 55-95°C for 2-12 minutes. The activity was then compared to control samples which were left at ambient temperature for 12 minutes.

4.2.5 Azo-casein assay

The azo-casein assay was used to determine the activity of Protamex®. The method was adapted from that of Charney & Tomarelli, (1947) as follows:

3 ml of a 0.4% (w/v) Azo-casein (dissolved in 0.05 M tris-HCl and pH adjusted to 7) solution was added to all test tubes. 100 µl of the pressure treated sodium caseinate hydrolysate/distilled water samples were added to the azo-casein solution. The test tubes were then incubated at 50°C for 90 minutes. After incubation the samples were plunged into a container of ice and 3 ml of a 4% (w/v) TCA solution was added to the tubes to terminate the reaction. The resulting solutions were filtered through Whatman no. 42 filter paper and the absorbance of the filtrate was obtained at 400 nm using a UV-Vis spectrophotometer (UVmini 1240, Shimadzu Corporation, Kyoto, Japan). Controls were prepared by using 100 µl aliquots of the control samples prepared for the high pressure treatment.

4.2.6 Statistical analysis

All experiments and assays were carried out in triplicate. Minitab 16 (Minitab Inc., Pennsylvania, USA) was used to determine decimal reduction time values of samples. Statistical analysis was carried using SPSS software (IBM Corporation, New York, USA). One-way ANOVA was used to determine significant differences between treatments.
4.3 Results and Discussion

4.3.1 Thermal inactivation of Protamex® in water samples

Thermal inactivation of Protamex® in sodium caseinate and distilled water was investigated. The thermal inactivation of Protamex® in distilled water is indicated in Figure 4.1 below.

![Figure 4.1: Thermal inactivation of Protamex® in distilled water at 55°C (●), 60°C (■), 65°C (▲), 70°C (▲), 75°C (▲), 80°C (▲), 85°C (▲), 90°C (▲) and 95°C (X)](image)

As can be seen significant inactivation (>90%) could be achieved at a temperature as low as 60°C for a treatment time of 10 minutes. Increasing the temperature above this lead to higher levels of inactivation, which is in agreement with many studies conducted on thermal inactivation of proteases (Ludikhuyze, Claeys, & Hendrickx, 2000; Bilbao-Sáinz, Younce, Rasco, & Clark, 2009).
4.3.2 Thermal inactivation of Protamex® in Sodium caseinate samples

The thermal inactivation of Protamex® in hydrolysed sodium caseinate is indicated in Figure 4.2.

As indicated in this figure significant levels (~80%) of inactivation were achieved at temperatures of 80°C and above with treatment times as short as 2 minutes. The most significant level of inactivation was achieved at 95°C with a treatment time of 12 minutes where ~90% inactivation was achieved. As the temperature increased more inactivation occurred, as already discussed, which is in agreement with other studies.

It became apparent from this investigation that the sodium caseinate conferred some protective effect on the enzyme creating a higher resistance to thermal treatment. At 55°C there was no inactivation of the Protamex® in the sodium caseinate samples while >60% reduction of activity was achieved in the
distilled water samples. At 95°C there was ~10% activity remaining in the sodium caseinate samples while there was <5% activity remaining in the distilled water samples. Prado, Sombers, Ismail, & Hayes, (2006) noted a similar phenomenon when plasmin inhibitors were subjected to thermal treatments. The activity of the plasmin inhibitors was reduced by ~70% in buffer when heated to 74.5°C for 15 seconds; however when the same treatment was applied to the plasmin inhibitors in bovine milk the activity was only reduced by ~36%. This was thought to be the result of interaction between some component of the milk system and the inhibitor.

4.3.3 High pressure inactivation of Protamex®

Initial investigations indicated that there was little alteration of Protamex® activity at the lower pressure ranges investigated. At 200 MPa and 300 MPa <10% inactivation was achieved in the samples with sodium caseinate as substrate. It has been noted in many investigations that pressures below 400 MPa cause little or no inactivation of enzymes but may cause some alteration of the protein configuration (Barbosa-Canovas et al., 1997; Huppertz, Kelly, & Fox, 2002). Due to this fact it was decided that pressures from 400-600 MPa would subsequently be investigated.

4.3.3.1 High pressure inactivation of Protamex® in water samples

The results of the high pressure inactivation of Protamex® in distilled water are indicated in Figure 4.3 below.
It can be seen in this Figure that significant levels (~90%) of inactivation can be achieved under all pressures investigated with a holding time of 30 minutes. This is in agreement with the study conducted by Scollard, Beresford, Needs, et al. (2000) who noted that the production of proteose peptone was reduced when pressure >300 MPa were applied to raw milk, the authors concluded that this was due to reduced plasmin activity as a result of the pressure treatment.

Surprisingly at holding times less than 30 minutes 500 MPa appeared to cause higher levels of inactivation. This may indicate that pressures in the region of 500 MPa are optimum for reducing Protamex® activity for shorter holding times. A similar trend of high pressure inactivation was reported by Katsaros, Katapodis, & Taoukis, (2009). When ficin (a plant protease) was treated with a pressure of 500 MPa at 70°C for 20 minutes the residual activity was <40%, however when the pressure was increased to 750 MPa under the same conditions the residual
activity recorded was >70%. The authors concluded that the ficin inactivation rate became less pressure dependent under the high temperature employed. However, the temperatures employed in the study were quite extreme whereas in our investigation ambient temperatures were employed to eliminate any thermal effects which could contribute to the inactivation of the Protamex®.

4.3.3.2: High pressure inactivation of Protamex® in hydrolysed sodium caseinate samples

The high pressure inactivation of Protamex® in sodium caseinate is indicated in Figure 4.4.

![Graph showing high pressure inactivation of Protamex® in hydrolysed sodium caseinate samples](image)

**Figure 4.4: High pressure inactivation of Protamex® in hydrolysed sodium caseinate at atmospheric pressure (●), 400 MPa (■), 500 MPa (♦) and 600 MPa (▲).**

From this graph it can be seen that the activity of the Protamex® can be reduced to ~10% when 600 MPa is applied for 30 minutes. Pressures of 400 and 500 MPa also lead to significant inactivation when samples were treated for 30 minutes with ~25% and ~20% residual activity, respectively, remaining in both
cases. This is in agreement with other investigations where the higher the pressure the greater the level of inactivation achieved. One such study examined the inactivation of alkaline phosphatase in bovine milk (Rademacher & Hinrichs, 2006). When the samples were subjected to a pressure treatment of 500 MPa for 128 minutes the level of inactivation achieved was <50%, however when the pressure was increased to 650 MPa the alkaline phosphatase activity was reduced by 99%. This was similar to results obtained when plasmin was treated with high pressure (Scollard, Beresford, Needs, et al., 2000). The activity of the plasmin remained at >40% when a pressure of 600 MPa was applied for 30 minutes, however when the pressure was increased to 800 MPa the activity was reduced to ~20%.

The inactivation of Protamex® in both sodium caseinate and distilled water appears to occur in two stages. In the initial 10 minutes the activity of the enzyme is reduced by ~40-70%, which is followed by a further rapid decrease in activity during the last 10 minutes of treatment (Figure 4.3 and Figure 4.4). This trend has been observed by Bayındırılı, Alpas, Bozoğlu, & Hızal, (2006) when polyphenoloxidase in apple juice was treated with high pressure. The authors observed that when 450 MPa was applied the enzyme activity reduced only slightly in the first 30 minutes followed by more rapid inactivation beyond this time. This trend was thought to be due to interactions occurring between the enzyme and other constituents in the apple juice.

It can be seen from both figures (Figure 4.3 and Figure 4.4) that the inactivation of Protamex® in distilled water occurred more rapidly than in sodium caseinate. This may be due to a protective effect conferred by the sodium caseinate. Bilbao-Sáinz, Younce, Rasco, & Clark, (2009) noted that protease
(isolated from *Bacillus subtilis*) was highly pressure resistant in bovine milk and even at the most severe treatment employed (600 MPa for 15 minutes) the protease retained >70% of its activity. It was similarly noted by Scollard, Beresford, Murphy, & Kelly, (2000) that plasmin was quite pressure resistant in the presence of casein while it became pressure susceptible in the presence of β-lactoglobulin as compared to buffer alone.

### 4.4 Conclusions

High pressure processing appears to be an effective method of reducing enzyme activity in aqueous systems. An interesting observation to note is the effect that the substrate has on the effectiveness of the pressure treatment as it was observed that the sodium caseinate in this study conferred a protective effect on the enzyme activity of both thermal and pressure treatments.

Although high pressure processing lead to significant levels of enzyme inactivation, the treatment times required to achieve similar levels of inactivation under thermal conditions were up to three times shorter. For industrial scale inactivation of enzyme activity in food products high pressure may prove to be quite expensive. Therefore thermal processing may be a more viable option. However, high pressure processing may be beneficial as a means of terminating enzyme activity in the area of bioactive production as it could have a lesser effect on the bioactivity than thermal treatments.
5.0 General Discussion

Although numerous uses for non-thermal technologies in the food processing industry have been identified there are still many potential applications of these technologies to be investigated. The present study focuses on the use of these technologies as interventions in the production process of protein hydrolysates.

Previous research has indicated that PEF, low frequency ultrasound and HPP can induce protein unfolding under extreme conditions (Arzeni et al., 2012; Li, 2012; Zhang, Jiang, Miao, Mu, & Li, 2012), though the results obtained when these technologies were employed as substrate pre-treatments prior to enzymatic hydrolysis were not always promising. Low frequency ultrasound (20 kHz) appeared to confer some modification of the 10% (w/v) sodium caseinate at least in so far as the subsequent hydrolysis time could be decreased by ~33% over that of the control hydrolysis time when an amplitude of 136 µm was applied for a duration of 30 minutes. This could potentially lead to significant savings for manufacturers. It was thought that this particular set of conditions may have led to interruption of hydrophobic interactions in the protein structure (Chandrapala et al., 2011). Protamex® is known to have broad specificity for hydrophobic amino acids (Sindayikengera & Xia, 2006), such as leucine and valine, and thus interruption of hydrophobic interactions could allow this enzyme better access to labile bonds, in turn possibly enhancing the rate of the hydrolysis reaction.

PEF pre-treatment of substrate (34 kV cm⁻¹ with intensity of 420 Hz) appeared to have no effect on the overall rate of the subsequent hydrolysis reaction. The use of a high pressure pre-treatment (400 MPa for 30 minutes) also
indicated that some level of substrate modification may have been achieved, however the effect was negative in that it resulted in an increased hydrolysis time over that of the control reaction. This increase in hydrolysis time was attributed to the formation of aggregates in the system which would reduce the rate of mass transfer thus slowing the hydrolysis reaction (Needs et al., 2000).

Sodium caseinate has a naturally disordered structure (Fox & McSweeney, 1998) and as such the application of the various pre-treatments may not have had a significant effect on the substrate structure. The effects of these pre-treatments may have been more evident if a more ordered protein substrate, such as whey protein (Thompson, Boland, & Singh, 2009), had been employed, this may have been of particular benefit in the case of PEF and high pressure pre-treatments. Li, (2012) noted that under similar PEF conditions (30 kV cm\(^{-1}\) and frequency of 400 Hz applied in a continuous fashion) to those employed in this current study, significant alteration of the secondary structure of soy protein isolate was achieved. In the case of HPP Kromkamp, Moreira, Langeveld, & van Mil, (1996) noted that treatments ranging from 100-300 MPa were optimum for inducing disruption of casein micelles, but that re-aggregation of the casein occurred following these pressure treatments. A similar type of re-aggregation might explain the increased hydrolysis times observed in the present study when high pressure (400 MPa) was employed as a pre-treatment to enzymatic hydrolysis of sodium caseinate.

The application of low frequency ultrasound (24 kHz) during the enzymatic hydrolysis of sodium caseinate yielded some promising results. This investigation highlighted the fact that very specific conditions are required to achieve an enhancement of the hydrolysis rate. Under the conditions of 40 µm amplitude with
a stirring speed of 400 rpm a reduction in hydrolysis time of ~45% could be achieved. This positive result was attributed to increased mass transfer in the system which is most likely generated by collapsing cavitation bubbles. However, outside these precise operating parameters the benefit of the ultrasonic cavitation was not apparent.

While thermal inactivation led to more rapid enzyme inactivation, the use of a non-thermal technology like HPP could find application as a means of terminating the hydrolysis reaction for the production of bioactive peptides, as HP has been shown to have less effect on bioactivity than thermal treatments (Rawson et al., 2011). The use of high pressure as a method of inactivating enzyme activity to terminate hydrolysis appears to be a technically viable option. This effect was most apparent when the enzyme was dispersed in distilled water, where ~90% inactivation could be achieved at a pressure of 400 MPa with a holding time of 30 minutes while in the sodium caseinate solution a higher pressure of 600 MPa was required to reduce the enzyme activity by greater than 80%. These results indicated that the sodium caseinate substrate employed has a protective effect on the enzyme. A similar effect was noted by Scollard, Beresford, Murphy, et al. (2000) where plasmin activity was more difficult to inactivate in the presence of β-casein. This illustrates the importance of the effects of substrate on the system and would need to be considered when developing a suitable method of terminating enzyme activity.

During the course of this work it became apparent that these non-thermal technologies can give rise to highly variable effects and a considerable amount of careful process optimisation is required to achieve the desired effect, this has been previously noted by various authors (Kruus, 1996; Stewart, Tompkin, &
Cole, 2002; Balasubramaniam et al., 2008; Pataro et al., 2011). These systems are extremely complex and many factors such as temperature, substrate concentration, agitation, pH, and viscosity can influence the effects these non-thermal technologies have on the system. Each of these parameters must be considered, especially in the case of PEF, ultrasound and HPP where there is an optimum range of conditions at which the desired effect (i.e. enhancement of hydrolysis or inactivation of enzyme activity) can be achieved, varying the conditions even slightly beyond this range can lead to a loss of the beneficial effect in the system. This can be seen quite clearly in chapter 3 when ultrasound was applied during the hydrolysis reaction. At a stirring speed of 400 rpm and an amplitude of 40 µm the hydrolysis reaction time could be reduced by ~45% over that of the control, however when the stirring speed was reduced to 200 rpm with all other parameters remaining the same there was no difference between the control and ultrasonically assisted hydrolysis times. Comparison of the results obtained in this study with those of other authors also proved difficult as there is a lot of variability in the equipment used and many authors use bespoke equipment; this can make it extremely difficult to determine equivalence of power inputs between systems. All of the experiments conducted in this thesis were carried out at laboratory scale and due to the complexity of these systems it is difficult to determine whether scale-up of these systems to industrial volumes would be viable either technically or economically.
5.1 References


Guiavarch, Y., Segovia, O., Hendrickx, M., & Van Loey, A. (2005). Purification, characterization, thermal and high-pressure inactivation of a pectin methylesterase from white grapefruit (Citrus paradisi). *Innovative Food...


Jaeger, H., Meneses, N., & Knorr, D. (2009). Impact of PEF treatment inhomogeneity such as electric field distribution, flow characteristics and temperature effects on the inactivation of E. coli and milk alkaline phosphatase. *Innovative Food Science & Emerging Technologies, 10*(4), 470–480. doi:10.1016/j.ifset.2009.03.001


Landl, a., Abadias, M., Sárraga, C., Viñas, I., & Picouet, P. a. (2010). Effect of high pressure processing on the quality of acidified Granny Smith apple


Qin, B. L., Pothakamury, U. R., Barbosa-Cánovas, G. V., & Swanson, B. G. (1996). Nonthermal pasteurization of liquid foods using high intensity pulsed electric fields. *Critical Reviews in Food Science and Nutrition, 36*(6), 603–627.


Soliva-Fortuny, R., Balasa, A., Knorr, D., & Martín-Bellos, O. (2009). Effects of pulsed electric fields on bioactive compounds in foods: a review. *Trends in Food Science & Technology*, 20(11-12), 544–556. doi:10.1016/j.tifs.2009.07.003


Terefe, N. S., Gamage, M., Vilkhu, K., Simons, L., Mawson, R., & Versteeg, C. (2009). The kinetics of inactivation of pectin methylesterase and
polygalacturonase in tomato juice by thermosonication. *Food Chemistry*, 117(1), 20–27. doi:10.1016/j.foodchem.2009.03.067


Tonello, C. (2008). *Commercial applications of high pressure processing, processing capacities and costs* (pp. 1–38).


Zhao, W., Yang, R., & Zhang, H. Q. (2012). Recent advances in the action of pulsed electric fields on enzymes and food component proteins. *Trends in Food Science & Technology, 27*(2), 83–96. doi:10.1016/j.tifs.2012.05.007


Figure A1.1: Temperature increase of sodium caseinate substrate when subjected to PEF treatment of 34 kV cm\(^{-1}\) and frequency of 420 Hz for 30 minutes.

Figure A1.2: Temperature increase in sodium caseinate substrate (■) and in the waterbath (■) when ultrasound of 20 kHz and 170 \(\mu\)m amplitude was applied for 30 minutes.
Figure A1.3: Correlation of pH stat and OPA as a means of determining degree of hydrolysis for incubation of 10% (w/v) sodium caseinate with Protamex® at 50°C and stirred at 400 rpm

% Degree of hydrolysis monitored by OPA method

% Degree of hydrolysis monitored by pH stat

\[ y = 0.9465x \]

\[ R^2 = 0.9936 \]
Appendix II

Figure A2.1: Points in the vessel at which temperature increase was monitored when ultrasound of 24 kHz was applied for 30 minutes: T1- Temperature (°C) of sodium caseinate prior to ultrasound treatment, T2- Temperature (°C) of sodium caseinate post-ultrasound treatment, T3- Temperature (°C) of jacketed heating water pre-ultrasound treatment, T4- Temperature (°C) of jacketed heating water post-ultrasound treatment

Table A2.1: Orthogonal factorial design used to monitor temperature increase when ultrasound at 24 kHz was applied to water for 30 minutes

<table>
<thead>
<tr>
<th>Description</th>
<th>Temperature of jacketed heating water (°C) (A)</th>
<th>Amplitude of ultrasonic processor (µm) (B)</th>
<th>Flow rate of product (ml/min) (C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1B1C1</td>
<td>40</td>
<td>20</td>
<td>5</td>
</tr>
<tr>
<td>A1B2C2</td>
<td>40</td>
<td>60</td>
<td>50</td>
</tr>
<tr>
<td>A1B3C3</td>
<td>40</td>
<td>90</td>
<td>95</td>
</tr>
<tr>
<td>A2B1C2</td>
<td>50</td>
<td>20</td>
<td>50</td>
</tr>
<tr>
<td>A2B2C3</td>
<td>50</td>
<td>60</td>
<td>95</td>
</tr>
<tr>
<td>A2B3C1</td>
<td>50</td>
<td>90</td>
<td>5</td>
</tr>
<tr>
<td>A3B1C3</td>
<td>60</td>
<td>20</td>
<td>95</td>
</tr>
<tr>
<td>A3B2C1</td>
<td>60</td>
<td>60</td>
<td>5</td>
</tr>
<tr>
<td>A3B3C2</td>
<td>60</td>
<td>90</td>
<td>50</td>
</tr>
</tbody>
</table>
Table A2.2: temperature increase for each variable after 30 minute ultrasound (24kHz) treatment

<table>
<thead>
<tr>
<th>Treatment condition</th>
<th>Temp. change from T1 to T2</th>
<th>Temp. change from T3 to T4</th>
<th>Residence time</th>
</tr>
</thead>
<tbody>
<tr>
<td>40 °C</td>
<td>4.572321849</td>
<td>0.677945784</td>
<td>4.748</td>
</tr>
<tr>
<td>50 °C</td>
<td>12.55651969</td>
<td>0.649737802</td>
<td>4.748</td>
</tr>
<tr>
<td>60 °C</td>
<td>19.20215242</td>
<td>0.394940324</td>
<td>4.748</td>
</tr>
<tr>
<td>20 µm</td>
<td>7.480850864</td>
<td>0.003442646</td>
<td>4.748</td>
</tr>
<tr>
<td>60 µm</td>
<td>11.67685353</td>
<td>0.622468202</td>
<td>4.748</td>
</tr>
<tr>
<td>90 µm</td>
<td>17.17328957</td>
<td>1.096713062</td>
<td>4.748</td>
</tr>
<tr>
<td>5 ml/min</td>
<td>19.9799437</td>
<td>0.681064315</td>
<td>12.36</td>
</tr>
<tr>
<td>50 ml/min</td>
<td>9.53011742</td>
<td>0.572766257</td>
<td>1.236</td>
</tr>
<tr>
<td>95 ml/min</td>
<td>6.820932839</td>
<td>0.468793339</td>
<td>0.648</td>
</tr>
</tbody>
</table>

Figure A2.2: Average temperature of T1 under each condition investigated

Figure A2.3: Average temperature of T2 under each condition investigated
Figure A2.4: Average temperature of T3 under each condition investigated

Figure A2.5: Average temperature of T4 under each condition investigated
Figure A2.6: Average temperature change from T2 to T1 under each of the conditions investigated

Figure A2.7: Average temperature change from T4 to T3 under each of the conditions investigated