

**Allergenic power reduction of
food proteins by nonthermal
technologies**

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*Ph.D. Course in Chemical Engineering
(XIII Cycle-New Series)*

**ALLERGENIC POWER REDUCTION OF
FOOD PROTEINS BY NONTHERMAL
TECHNOLOGIES**

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Abstract

The great interest of the research activity in food allergies could be attributed to the increase of allergic reactions all over the world not only in infants but even in adult age.

As an alternative to the development of an allergen-free diet, many works have been focused on a novel approach for the treatment of allergens: instead of eliminating the allergens from the diet, the immunoresponse can be reduced or even eliminated by inducing some modifications of their molecular structure. In fact, changes in allergen conformation can modulate its identification by the specific antibody produced by immune system in allergic reactions.

Structural modifications in allergens could be induced by conventional thermal treatments as well as by non-thermal technologies, namely High Hydrostatic Pressure (HHP), Pulsed Electric Fields (PEF), Pulsed Light (PL) and γ -radiations.

Non-thermal technologies have been widely used in the last years for food preservation, having the advantage of increasing the shelf-life and freshness of the raw food products. These technologies are able to affect the food nutritional and organoleptic properties only slightly thanks to the use of a non-thermal stress to treat foodstuffs. Among them High Hydrostatic Pressure technology has been successfully used in food pasteurization, but also in processes involving the sol-gel transition such as the production of jams, jellies and dairy products. The ability of High Pressure to determine structural changes in foods was studied in order to assess if proteins unfolding and/or aggregation and gelation can be induced and if the treatment affects the functional properties and digestibility of proteins. These effects were studied on particular proteins, namely the allergens, for which unfolding and structural modification have been proven. However, the effectiveness of the High Pressure processing on the reduction of immunoresponse reduction was not clearly assessed so far.

The objective of this PhD thesis was the study of the modifications induced by High Pressure Process on allergenic proteins and the possibility of obtaining hypoallergenic peptides by means of a combined High Hydrostatic Pressure hydrolysis. In particular, the effect of the HHP on the allergens structural modification was investigated in a wide range of operating conditions, including both gelling and ungelting conditions. Rheological behavior and functional properties of HHP processed allergens was also determined.

After having identified the product characteristics and the operating conditions at which the physical transition from unfolding to aggregation takes place, also the effects of HHP on hydrolysis reaction by proteolytic enzymes were analyzed.

Chemical-physical properties and allergenicity of peptides obtained by HHP were determined. For the sake of comparison, also the characteristics of the peptides produced at atmospherical pressure were measured.

Finally to quantify the effect of the optimized process on the allergenicity of HHP peptides, their immunoresponse was analyzed by *in vivo* tests.

The results of the experimental activity have demonstrated that:

- a) High Hydrostatic Pressure is a technology able to induce protein modifications whose extent depends on processing conditions and product characteristics. In gelling proteins, such as Bovine Serum Albumin (BSA), operating conditions namely pressure level, temperature and holding time as well as product characteristics, namely protein concentration and isoelectric point, can affect the unfolding/gelling transition.
- b) The rheological behavior of HHP induced allergen gels depends on the treatment conditions, which determine gel hardness/consistence. Furthermore, higher deviations of the gels from Newtonian behavior can be observed if higher pressure levels and holding time are applied.
- c) At pressure levels lower than that corresponding to the unfolding/gelling transition is also possible to detect the effect of high pressure on allergens at the microscopic scale. At these conditions, the application of high hydrostatic pressure induces the anticipation of thermal denaturation by reducing the sol/gel transition temperature.
- d) High Hydrostatic Pressure causes conformational changes and the unfolding of allergens, as demonstrated by the increase in foaming properties and the exposure of thiols reactive groups. These effects are likely to occur up to certain values of pressure and holding time and up to certain values of protein concentration while in more drastical treatment conditions and with higher protein concentrations, the aggregation replaces the unfolding.
- e) If the hydrolysis is assisted or anticipated by High Hydrostatic Pressure treatments carried out at the operative conditions maximizing protein unfolding, higher hydrolysis degree are detected and the peptides show a lower size. At very high pressure, due to the formation of protein aggregates and the reduction of enzymatic activity, the hydrolysis reaction rate is drastically reduced.
- f) High Hydrostatic Pressure treatment can be applied also to non-globular proteins, such as caseins. Also with this protein, hydrolysis

reaction is enhanced and lower dimension and more homogenous peptides mixture can be produced.

- g) The kinetics of protein hydrolysis by proteolytic enzymes is faster under high hydrostatic pressure than at atmospheric conditions, as shown by the increase in the reaction constant.
- h) Peptides produced by hydrolysis assisted or induced by High Hydrostatic Pressure can be considered as hypoallergenic compounds being their immunoresponse against antibodies reduced as shown by in vivo tests (*Prick tests*).

Chapter I

Forewords

1.1 Introduction

Allergy is an abnormal response of the immune system against a substance (antigen), which induces the production of a specific antibody, which in turn reacts with it and stimulates the production of histamine responsible for the allergy symptoms. Nowadays, an allergy cure does not exist; for this reason the only approach to solve this disease is the consumption of allergen-free products.

As an alternative to allergen elimination, several technologies have been studied in order to modify allergen structure, and thus reducing its allergenic power, such as thermal or enzymatic treatments. Similarly, non-thermal treatments, namely High Hydrostatic pressure (HPP), pulsed electric fields (PEF) or high intensity pulsed light (LP), are able to induce allergen modifications, avoiding thermal damages and quality loss.

In the scientific literature many different techniques were investigated in order to determine their effects on allergens modifications in terms of unfolding or aggregation. The results suggest that these treatments cause only conformational changes in allergen structure, which do not affect the primary structure.

Even though high hydrostatic pressure is able to induce protein modifications, in many cases its application seems to be not able to affect immunoresponse, and therefore the allergenicity is not reduced.

This PhD thesis aims to setup a novel process based on the High Hydrostatic Pressure assisted hydrolysis by proteolytic enzymes to produce hypoallergenic peptides.

1.2 Structure of the thesis

The thesis is organized in 8 chapters:

Chapter 1 is a brief introduction to the work.

Chapter 2 presents an analysis of both conventional and innovative food preservation technologies having the potential to be used for the treatment of food allergens. In particular, for each treatment the physical principles,

which control allergen modification with particular attention to the effects on allergenicity, are analyzed.

Chapter 3 describes the objectives of the work and the work plan.

Chapter 4 analyses the effect of one of the non-thermal technologies described in state of the art, the High Hydrostatic Pressure, on allergen denaturation and aggregation. The effect of product and process parameters on rheological behavior of BSA was studied in both stationary and dynamic regime and a mathematical model was used for the analysis of the rheological behavior.

Chapter 5 aims to analyze the effect of HHP on allergens structure in ungelting conditions. Functional properties of BSA were analyzed such as the number of SH free groups, the foaming capability as well as the changes in secondary structure by means of FT-IR analysis. The results achieved also allow to identify the operating conditions and the product properties of the allergen at which the transition between the allergen unfolding and the formation of aggregates takes place.

Chapter 6 focuses on the hydrolysis assisted by High Hydrostatic Pressure applied to two allergens with different structure: BSA and casein. The effect of HHP process on the reaction of enzymatic hydrolysis with two proteolytic enzymes (chymotrypsin and trypsin) was evaluated in terms of hydrolysis degree with the aim of identifying the optimal processing conditions. Furthermore the chapter contains an analysis of the effect of process and product parameters on HHP treated peptides and non-treated peptides in terms of structure (by HPLC-RP spectra and FT-IR analysis) and dimension (by Particle Size Distribution and Peptide Profile Patterns at SDS-Page).

Chapter 7 aims at investigating the effect of HHP treatment on the kinetics of the assisted hydrolysis by chymotrypsin and trypsin. The constants of hydrolysis reactions carried out under high pressure and at atmospheric pressure were estimated by fitting the experimental curves of hydrolysis with a generalized model equation.

Chapter 8 aims to characterize the peptide samples in terms of allergenicity. The results of immunoresponse analyses carried out by *in vivo* test (Prick tests) on allergic patients were reported.

Chapter 9 contains the main conclusions of the work.

Chapter II

Background

2. Introduction

Allergies strongly increased in the last years, especially in industrialized countries (Schafer 2008; Cochrane et al., 2009) not only in infants, but even in adults.

The World Allergy Organization estimated that there are approximately 220-250 million people suffering from food allergies and incidence varies from 5% to 8% for infants while is from 1% to 2% for adults (Fiocchi et al., 2011). For this reason, the research of novel solutions to face the problem of allergies in the food industry has strongly increased. Nowadays no perfect treatment for food allergy is available (Mills et al., 2009) and allergic patients must avoid the food causing a specific allergy since even small concentration of the allergen can be responsible of an abnormal response of the immune system.

On the other side the allergen free diet, which seems to be nowadays the only solution to allergy, presents many limits such as the low nutritional value of the allergen-free products but also the need of specific and expensive production processes.

In this contest, novel approaches to the allergy problem are emerging based on allergens modification during treatment of foodstuffs in order to induce allergenic power reduction. For a better understanding of the possible solutions for allergy reduction it is important to know what is an allergy, how it works and which are the parameters influencing it.

In this chapter the mechanism of the allergic reaction was discussed in details and the main allergens in human diet have been presented. An overview on the *conventional methods* traditionally used to reduce allergenicity or to increase food digestibility, such as thermal and enzymatic treatment of allergenic protein for the production of protein hydrolysates, was also given.

The state of the art on the use of *novel non-thermal technologies* on allergens was presented and discussed. A particular attention was paid to the analysis of the major scientific results achieved in the application of conventional and innovative technologies on the immunoreactivity reduction of the most common allergens. Moreover, for each technology, the principles

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regulating the mechanisms of allergy reduction and/or modification were deeply described.

2.1. Food Allergies

An **allergy** is an abnormal response of the immune system, which is very similar to the defence response of the body in presence of virus, bacteria or toxins. The immune system recognizes the allergens as dangerous compounds and reacts by producing an antibody (IgE), which is specific for each allergen (Figure 2.1).

The antibody reacts with the allergen causing a secondary reaction with mast cells (tissues cells) and basophils (blood cells). The mast cells release a substance, namely histamine, and other substances, such as leukotrienes and prostaglandins, which induce other reactions responsible for the typical symptoms of the allergy.

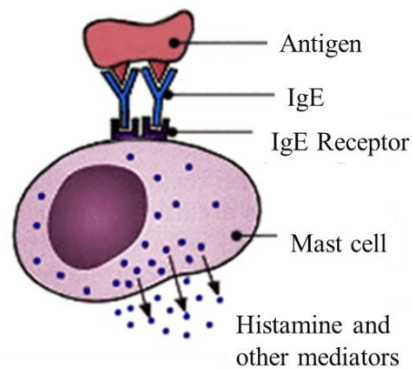


Figure 2.1. Mechanism of allergenic reaction between an antigen and the specific antibody produced by immunosystem.

An **antigen**, which activates the mentioned mechanism of the allergy, is generally indicated as an “allergen,” even if there is a difference between the allergenic source and the allergens. The allergenic source is a food containing one or more allergens (such as eggs, milk, etc.), while the allergen is the specific compound contained of the allergic source responsible for the allergy.

An **allergen** is a protein, glycoprotein or a hapten related to a carrier with a molecular weight between 5 and 150 kDa and isoelectric point between 2 and 10. Every allergen has different epitopes (reacting site) and this characteristic is called “immunologic multivalence”. Different parameters

influence the reaction between the antigen and antibody (Alessandri et al., 2010):

- **Epitopes (antigenic determinants):** the part of an antigen reacting with the antibody (the part of an antibody that recognizes the epitope is called paratope). An epitope can be linear or conformational: a linear epitope is recognized by an antibody through its primary structure (sequence of contiguous amino acids) while the conformational epitope displays its immunoreactivity through its conformational structure.
- **Allergen spatial conformation:** the allergen tridimensional structure.
- **Avidity between antigen and antibody:** the measure of the overall binding strength of an antigen having many antigenic determinants with multivalent anti bodies.
- **Affinity between antigen and antibody:** the strength of the reaction between a single antigenic determinant and a single combining site on the antibody.

Allergen conformation strongly affects its exposure to the antibody in an allergen reaction and, thus, its immunoresponse can be reduced by inducing some structural modification.

For this reason, as an alternative to produce allergen-free products, traditional techniques (e.g., thermal and biochemical treatments like enzymatic hydrolysis) and novel technologies (pulsed electric fields, pulsed light, high hydrostatic pressure) have been applied to modify allergen structure and to reduce its allergenic power (Vallons et al., 2010; Renzetti et al., 2008, 2010).

Table 2.1. Most common allergen and allergenic sources. ImmunoCAP ISACR Allergen (Immuno Solid-phase Allergen Chip, VBC-Genomics, Vienna, Austria) component.

| <i>Food</i> | <i>Molecule</i> | <i>Organism</i> | <i>Code</i> | <i>Function</i> |
|--------------|-----------------|------------------|-------------|------------------------|
| Cow milk | Bos d4 | Bosdomesticus | 163 | α -lactalbumin |
| | Bos d5 | Bosdomesticus | 164 | β -lactoglobulin |
| | Bos d6 | Bosdomesticus | 165 | Serum albumin |
| | Bos d8 | Bosdomesticus | 167 | Casein |
| | Bos d | Bosdomesticus | 1065 | Transferrin |
| Chicken eggs | Gal d1 | Gallusdomesticus | 359 | Ovomucoid |
| | Gal d2 | Gallusdomesticus | 360 | Ovoalbumin |
| | Gal d3 | Gallusdomesticus | 361 | Ovotransferrin |
| | Gal d5 | Gallusdomesticus | 363 | Serum Albumin |
| Fish | Cyp c 1 | Cyprinus carpio | 263 | Parvalbumin |
| | Gad c 1 | Gaduscallarias | 264 | Parvalbumin |
| Celery | Api g1 | Apiumgraveolens | 40 | PR -10 protein |

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|------------------|---------------|----------------------|------|----------------------------|
| Carrot | Dau c1 | Daucus carota | 287 | PR -10 protein |
| Kiwi | Act d1 | Actinidia deliciosa | 1 | Cysteine protease |
| | Act d2 | Actinidia deliciosa | 747 | Thaumatococin like protein |
| | Act d5 | Actinidia deliciosa | 2821 | Kiwiferritin |
| | Act d8 | Actinidia deliciosa | 3546 | PR - 10 protein |
| Apple | Mal d1 | Malus domestica | 1454 | PR - 10 protein |
| Peach | Pru p1 | Prunus persica | 602 | Legumin-like protein |
| | Pru p3 | Prunus persica | 603 | Lipid transfer protein |
| Cashew | Ana o2 | Anacardium | 3077 | Legumin-like protein |
| Peanut | Ara h1 | Arachis hypogaea | 50 | 7S Globulin (vicilins) |
| | Ara h2 | Arachis hypogaea | 51 | Storage protein |
| | Ara h3 | Arachis hypogaea | 52 | Globulin |
| | Ara h8 | Arachis hypogaea | 3100 | PR 10 protein |
| Brasilian walnut | Ber e1 | Bertholletia excelsa | 3134 | Storageprotein(2Salbumin) |
| Hazelnut | Cor a1.0401 | Corylus avellana | 239 | PR-10 protein |
| | Cor a8 | Corylus avellana | 3219 | Lipid transfer protein |
| | Cor a9 | Corylus avellana | 246 | 11SGlobulin(legumins) |
| Soy | Gly m4 | Glycine max | 3297 | PR-10 protein 7S |
| | Gly m5 | Glycine max | 5816 | Globulin (vicilins) 11S |
| | Gly m6 | Glycine max | 5821 | Globulin (legumins) |
| Celery | Sesi 1 | Sesamum indicum | 624 | Storageprotein(2Salbumin) |
| Wheat | Tri a18 | Sesamum indicum | 650 | Agglutinin/Isolectin 1 |
| | Tri a gliadin | Triticum aestivum | 3677 | Crude gliadin |
| | Tri a19.0101 | Triticum aestivum | 3502 | Gliadin |
| | Tri aA_TI | Triticum aestivum | 1051 | Amilase/trypsin inhibitors |

Among the proteins listed in Table 2.1, Food & Drug Administration identified the most common eight allergens in order to help people in recognizing them, thus avoiding food allergies. The major allergenic foods are milk, eggs, peanuts, tree nuts (such as almonds, cashews, and walnuts), fish (such as bass, cod, and flounder), shellfish (such as crab, lobster, and shrimp), soy, and wheat which are responsible for 90% of allergic reactions.

It is very important to clarify that allergy differs from intolerance in many aspects, although these diseases are often confused, especially because many foods can cause both allergenic and intolerance reactions (Table 2). While the *intolerance* is related to the digestive apparatus, allergy involves the immune system. The two diseases differ also in their impact on society: allergies affect 1-2 people out of 10 while food intolerance more commonly affects 5-6 people out of 10.

Table 2.2. Differences between allergy and intolerance (Mandatory M. & Rizzo C., 1999)

| Food Allergies | Food Intolerance |
|--------------------|------------------|
| Immediate reaction | Delayed reaction |

| | |
|--------------------------|-----------------------------------|
| Acute reaction | Chronic reaction |
| Dose dependent | Not dose dependent |
| Sporadically eaten foods | Daily foods eaten 1 or more a day |
| IgE antibody | IG4- AG/ACComplexes |
| No cross-reactivity | Cross-reactivity |
| Defined target organs | Every organ, apparatus, system |

Food intolerances are due to the incapability of the human body to digest certain substances, for example proteins or sugars, because of the deficiency of some digestive enzymes (e.g., β -galactosidase lack for lactose digestion in milk). For this reason, food is not digested with consequent difficulty of being absorbed by the organism, thus inducing inflammation and bodily dysfunctions (www.foodintol.com). An intolerance may cause heartburn, cramps, belly pain or diarrhea and can occur even after the consumption of little amount of foods such as chocolate, eggs, strawberries, citrus fruits and tomatoes, wine (especially red wine), histamines and other amines and food additives (Reid Hospital & Health Care services). The most common food intolerances are related to:

- corn products
- cow's milk and dairy products (lactose intolerance)
- wheat and other grains that contain gluten (celiac disease)

Celiac disease (CD) is triggered by peptide sequences inside the prolamine fractions of wheat (gliadins), barley (hordeins), rye (secalins) and oats (avenin) (Wieser and Koehler 2008). Only a gluten-free diet seems to be the possible approach to avoid CD symptoms, but gluten-free products can be very expensive, with lower palatability than the conventional foods (Arendt et al., 2008), and with many additives used to compensate nutritional deficiencies.

2.2.Allergen free-products

Allergen-free products are foods specifically produced for allergic people are characterized by special formulations without one or more allergens. Food industry produces these products by replacing the allergenic proteins with different ingredients, additives and flours (Marconi & Careca, 2001).

Gluten-free products are a typical example. Bread contains a high percentage of gluten (75-86%) (Bloksma & Bushuk, 1998), which forms a hard matrix together with carbohydrates and lipids, and is responsible for dough extensibility, resistance to stretch and other structural properties. Starches, dairy products, gums and hydrocolloids (Anon., 2002; Laureys, 1996;

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Salama, 2001; Ward & Andon, 2002) were tested as gluten substitutes in order to improve the texture and the flavour of bread (Anon., 2002; Laureys, 1996; Salama, 2001; Ward & Andon, 2002).

Acs et al. (1996a, 1996b) tested several binding agents including xanthan, guar gum, locust bean gum and tragant as substitute of gluten in gluten-free products. The authors found that these binding agents increased loaf volume and affected the crumb structure. Other *additives* used by different authors were fibers (Mariani et al., 1998; Lohniemi et al., 2000; Thomson, 2000; Grehn et al., 2001) and dairy ingredients used to improve nutritional and functional properties including flavour and texture (Cocup & Sanderson, 1987; Kenny et al., 2001; Mannie & Asp, 1999).

Finally, another technique for improving the quality of gluten-free bread is the use of sourdough (SD) technology, which is already being used to improve texture, flavor and shelf life of wheat and rye bread (Hansen and Schieberle, 2005) and for microbial fermentation. The use of additives and the high prices which characterize the allergen-free products, led the researchers to develop novel processes able to induce allergen modification.

2.3. Treatments for allergens modification

Allergen-free diet had many disadvantages related to the high costs to access the proper foods and also from a nutritional point of view. As a consequence, novel approaches, based on the structural modification of the food allergens, were developed. Different thermal and non-thermal treatments have been applied to food allergens with the aim of modifying their secondary, tertiary and quaternary structures, which are responsible of allergen spatial conformation as shown in Figure 2.2.

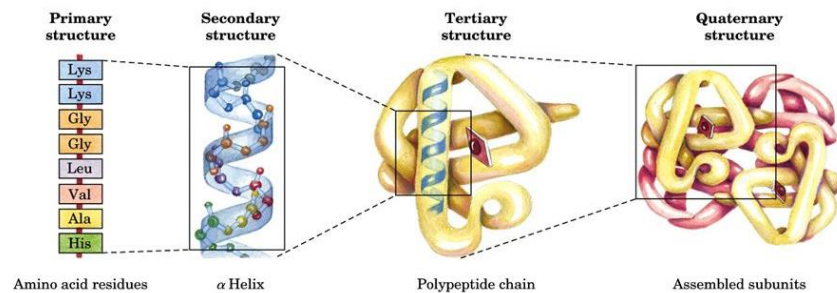


Figure 2.2. Levels of protein organization: primary, secondary and tertiary structure (www.biocode.it).

Traditional treatments of food allergens have been applied since many years, namely thermal treatments able to modify the conformational structure of

proteins and enzymatic treatments for the production of hypoallergenic peptides. Similarly, non thermal technologies have shown a great potential to affect the allergen structure by reducing their immunoresponse and allergenicity. Allergens have revealed a different sensitiveness to thermal or non-thermal processes which allow their classification in processing label and processing stable allergens.

Allergen resistance to structure modifications induced by a certain treatment is a strict function of its structure. Indeed, some proteins can be more easily modified with the application of less drastic processing conditions. For example the less resistant allergens belong to the Bet v1 superfamily, which is responsible for a pollen-fruit/vegetable cross-reactivity syndrome, like Bet v1 homologues from fruits such as Mal d1 in apple and Pru av1 in cherry. The low resistance of these proteins can be related to the presence of only conformational epitopes (Neudecker et al., 2001; Gajhede et al., 1996) but also to the absence of intermolecular disulphide bonds, which are very well known to enable protein structure stabilization.

Processing stable allergens are able to retain their structure during treatments and their stability is mainly due to the higher quantity of disulphide bonds. The proteins belonging to the prolamine superfamily, for example, show a higher stability to denaturing process due to the presence of three or four disulphide bonds, able to preserve the native protein folding. Typical processing stable allergens are Cupins allergens (like Ara h1 from peanut) and Lipocalins (such as β -Lactoglobulin and α -lactoalbumin from milk).

The reduction of the allergenicity in this case is only possible if drastic processing conditions are applied. Sancho et al. (2005) reported that IgE reactivity of Mal d3 processed at 100 °C for 2 h was reduced because of disulphide bonds oxidation. However, sometimes even severe processing conditions do not affect the allergenicity. For instance, Brenna et al. (2000) who processed peach samples at 121°C for 30 min did not observe a significant effect on the allergenicity. In case of stable allergens, a more efficient denaturation can be obtained by using wet heating than by applying a dry heating like roasting, because the denaturation is improved in presence of water (Hansen et al., 2003).

2.3.1. Thermal processes

Thermal treatments have the potential to reduce food allergenicity inducing allergen structure modification. In fact, similarly to mixing and shearing processes occurring during food treatment, heating is able to induce protein denaturation and/or aggregation (Clare Mills et al., 2009).

In thermal treatments proteins gradually loses their native structure undergoing modifications of the conformational structure, namely

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secondary, tertiary and quaternary structure, but no modifications in the primary structure are likely to occur. During denaturation only weak bonds like electrostatic, hydrophobic and disulphide interactions, are modified while covalent interactions involved in primary structure remain unbroken. The extent of denaturation ranging from unfolding to aggregation depends on different factors like temperature and pH variations, flow, and salt or sugar addition (Goetz et al., 2005), which can lead to reversible or irreversible denaturation.

Several authors studied the effects of thermal treatments on food allergens by means of *in vitro* and *in vivo* tests, observing that allergen modifications and thus reduction of immunoreactivity occur at different processing conditions. *In vitro* tests demonstrated that thermal treatment of egg white at 90°C for 10 min lead to a reactivity reduction of 50% with IgE in all the tests with radioallergo absorption (RAST) carried out on 16 patients with egg allergies (add reference).

Previous results have been confirmed by *in vivo* tests: Urisu et al. (1997), for example, found that people with egg white allergy showed no reaction to cooked egg white. Similarly, Eigenmann et al. (2000) observed that patients showing anaphylactic shock after consuming eggs showed no reaction after cooked eggs consumption.

Recently, Tong et al. (2012) studied the effects of thermal treatments on allergenic power of Ovotransferrin (OVT). Treatments were carried out at different temperature levels and the allergenic power analysis was carried out using ELISA. Results showed that good effects in terms of allergy power reduction are observed only at high temperature (80 °C), while at mild temperature allergenic power can be increased (55-60 °C). The same authors showed that thermal denaturation is not always able to induce allergenic power reduction, but that an increase of allergenicity is possible.

In fact, thermal treatments modify protein tridimensional structure, affecting only conformational epitopes, without breaking the covalent bonds of the allergen primary structure. Moreover, the exposure of linear epitopes to the external part of the protein is likely to take place, this causing an increase of the allergenic power of the protein due to the increased exposure of the allergen towards the antibody.

2.3.2 Enzymatic treatments

While denaturation is often used for allergen conformational modifications, enzymatic treatment can be used to induce the breakage of peptide bonds stabilizing *primary structure* of the allergens. Antibodies specifically produced after allergen consumption are not more able to recognize antigens because of their new assessment in the form of peptides, resulting in the reduction of immunoresponse.

Proteinases represent the specific class of enzymes which are able to catalyse the hydrolysis of peptide bonds by attacking the peptide bonds of proteins in *three stages*. In the first stage, the breakage of the covalent bond between the amino group and the carboxylic group occurs, followed by proton exchange between COOH and NH₂, and finally the titration of amino groups (Márquez et al. 1999).

1. $-\text{CHR}'\text{-CO-NH-CHR}''\text{-} + \text{H}_2\text{O} \rightarrow -\text{CHR}'\text{-COOH} + \text{NH}_2\text{-CHR}''\text{-}$
2. $-\text{CHR}'\text{-COOH-NH}_2\text{-CHR}'\text{-} \rightarrow -\text{CHR}'\text{-COO}^- + \text{N}^+\text{H}_3\text{-CHR}''\text{-}$
3. $\text{N}^+\text{H}_3\text{-CHR}''\text{-} + \text{OH}^- \leftrightarrow \text{NH}_2\text{-CHR}''\text{-} + \text{H}_2\text{O}$

Peptides deriving from this reaction show different properties with respect to native protein, such as smaller size and better nutritional, functional and biological properties, because the structural rearrangements tend to expose some hydrophobic regions (originally buried within the protein molecule) to the aqueous phase (Philips & Beuchat, 1981). For these reasons, the protein peptides are usually used in food, pharmaceutical and parachemistry industries (Kong et al. 2007).

Several enzymes have been used for protein hydrolysis, such as pepsin, trypsin, papain, bromelain and subtilis, and the type of enzyme together with the type of protein and hydrolysis conditions are all parameters influencing the characteristics of peptides obtained with the hydrolysis.

Enzymatic hydrolysis has been widely used to produce highly digestibility peptides from different proteins like casein (Clegg and McMillan, 1974, Clegg, 1973) and gluten (Mimouni, Azanza & Raymond, 1999). Different processing systems have been used as enzymatic reactors to produce hydrolysates, such as continuous membrane reactor (Iacobucci and Myers, 1974) or reverse osmosis cells. Enzymatic hydrolysis of wheat gluten has been studied to modify physicochemical properties such as foaming (Kong et al., 2007) and solubility (Mimouni, Azanza & Raymond, 1999) and to produce peptides with biological activity, especially for celiac disease (Suetsuna & Chen, 2002) and to obtain structural or genetic modification (Masson, Tomè & Popineau, 1986; Shewry et al., 1984b).

Several authors have demonstrated that most proteins are more easily hydrolyzed in their denatured form than in their native form (Evans, 1946). The higher resistance of the native protein to proteolysis is due to the higher compactness of globular structure protecting peptides bonds, while in denatured/unfolding form they are available to enzymatic attack.

2.4. Innovative treatments

The development of new products as well as the increasing attention to the quality and safety of foodstuff have promoted the investigation of novel processing technologies as an alternative to thermal treatments. As an example, to pasteurize foodstuff with traditional treatments long treatment time and high temperature level should be used to inactivate microbial contaminants this leading to the reduction or loss of thermolabile compounds responsible of the nutritional and organoleptic properties. This also occurs even when in HTST processes are set up. Thus, non-thermal technologies have been studied and are nowadays used in some cases in industrial scale to obtain the preservation of foods without detrimental effects on their quality and represent a valid alternative to the traditional thermal treatments.

Beside their effectiveness for microbial inactivation was demonstrated, the application of non-thermal technologies for the treatment of food allergens has been recently proposed in the literature.

Non-thermal treatments, namely Pulsed Electric Fields (PEF), Pulsed Light (PL), High Hydrostatic Pressure (HHP) and irradiation, are currently under investigation.

2.4.1. Pulsed Electric Fields

PEF (Pulsed Electric Fields) is a novel food technology consisting on the application of high voltage pulses of short duration (0.1-40 kV/cm) to an electroconductive food placed between two electrodes. The electric field is able to induce a trans-membrane voltage, which causes the electroporation and permeabilization of the biological membrane. Therefore, this technology is used in several applications, such as microbial inactivation and/or extraction.

PEF technology is able to induce structural modification of allergens (soybean protein isolates) causing denaturation/aggregation (Johnson et al., 2010), but has no effect on allergen immunoreactivity. Zhao and Yang, (2009) showed that PEF - induced aggregation is possible and that the main bonds involved in protein aggregation are disulphide bonds and hydrophobic interaction.

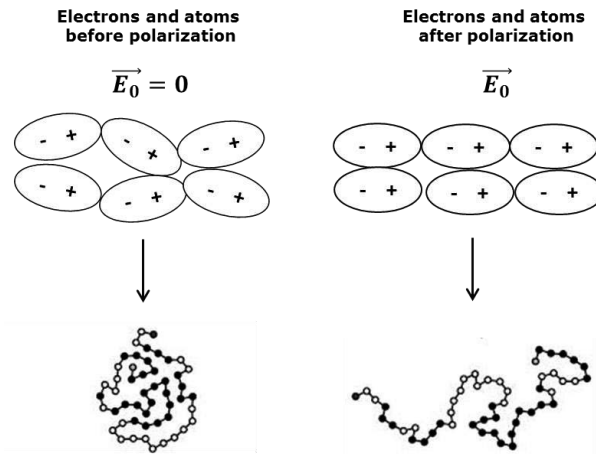


Figure 2.3. Mechanism of proteins unfolding after PEF treatment.

Proteins are sensitive to PEF because polypeptide chains have a strong dipole moment and local electric fields are disrupted by external electric fields. PEF causes movement of free electrons and ions, causing the polarization of electrons in ions and ions in molecules, thus inducing the orientation of molecular dipole moment and the increase of dielectric constant of protein. Dielectric constant increase causes protein unfolding, i.e. the loss of secondary and tertiary native structure. As a consequence, the structure of the protein subunits changes, causing quaternary structure modification and the formation of protein aggregates (Zhao et al., 2013).

2.4.2 Pulsed Light

Pulsed Light (PL) treatment is a non-thermal technology widely used for food preservation consisting of the application of light pulses with high intensity and short duration belonging to a wide spectrum of light (100-1100 nm) including ultraviolet, visible light and infrared spectrum. PL treatment is used as a decontamination technology, and has also the potential to be used for inducing protein modifications. The energy, emitted by the xenon lamp, is able to induce molecule ionization as well as molecule vibration (visible light) and rotation (infrared light). Thus, these effects suggested the possible application of the technology on food allergens. Several authors applied Pulsed Light on nuts which are a well-known source of allergens, such as almonds or peanuts and peanuts extracts.

Li et al. (2013) exposed crude almonds to the effect of PL for different treatment times: 0.5, 1, 2, 3, 4, 6, 7 and 10 min at a vertical distance from the lamp of 10 cm. The authors reported that while shorter treatments (1-4 min) were not effective on allergens, longer exposure to the flashes (10 min)

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allowed the reduction of allergens immunoreactivity (against IgE binding). In fact, PL treatments induced protein cross-linking and/or fragmentation due photothermal, photochemical or photophysical effects.

Similarly, Chung et al. (2008) applied PL treatment on peanut extracts and liquid peanut butter in the following processing conditions: 3 pulses/s, 14.6 cm distance from the lamp and duration of 4 min (extract) and 3 min (liquid peanut butter). The authors showed that allergens immunoresponse of PL treated samples against IgE antibodies was 6/7 times lower than in untreated samples (ELISA) and also that PL treated samples had a lower antigenity than that of thermal treated samples. The effect of PL was explained by the reduction in allergens solubility of some peanut allergens, as shown by the formation of insoluble aggregates observed in SDS-Page analyses in all the tested allergens except for Ara h 2 (18 to 20 kDa) which is the major allergen in peanut.

Yang et al. (2012) treated extracts of raw and roasted peanuts for 2, 4 and 6 min and those contained in peanut butter slurry for 1, 2 and 3 min and they reported that treatment was effective not only on Ara h1 and Ara h3 but also on Ara h2. In fact they observed a reduction of the immunoresponse in all peanut allergens against IgE antibody (ELISA), which was higher by increasing treatment time and decreasing vertical distance from the lamp in the energy doses between 111.6 and 223.2 J/cm². The reduction in immunoresponse was explained by a decrease of the protein band corresponding to Ara h 1, Ara h 2 and Ara h3 as shown by SDS-Page analysis.

The reduction in IgE binding response was also detected on shrimps extract by Shriver et al. (2011), who observed a relevant crosslinking and a reduction in the level of tropomyosin. Similarly, Yang et al. (2010) obtained soy extracts with lower allergenicity with PL treatment with a decrease in soy allergens (i.e. glycinin and β -conglycinin). In particular, IgE binding was reduced of 20%, 44% and 50% after 2, 4 and 6 min of exposure to PL, respectively.

The immunoresponse of PL treated gluten was studied by Noojii et al. (2010) who combined a PL treatment of 45 sec with thermal treatments at 100 °C. Anugu et al. (2010) detected a reduction of 9.5 times of allergens immunoresponse in eggs treated by PL treatment, except for ovalbumin which still showed reactivity against IgE antibody after pulsed light treatment.

PL showed also the potential to be used for milk allergens treatment as reported by Anugu et al. (2009) on caseins and whey proteins which were not detectable with SDS Page analysis after a treatment of 150 s and their reduction of immunoreactivity of 7.4 times for caseins and of 7.7 times for whey proteins. Worst results were reached by Helmnasser et al. (2008) who applied a treatment of 5, 7 and 10 pulses on milk proteins and did not observe any reduction of their immunoresponse. The combination of the PL

treatment with enzymatic hydrolysis with trypsin and proteinase K could improve the effect of the treatment on milk allergenicity. Manzocco et al. (2013) found no differences in immunoreactivity of eggs treated by PL treatment; on the contrary reactivity higher than that observed in the native proteins was observed on treated samples. This effect is due to the exposure of the protein epitopes induced by fragmentation which causes the marked increase in immunoreactivity.

Despite PL treatment had the potential to be used as a treatment for allergen modification (results are reported in Table 2.3) the variation of allergenicity strictly depends on the type of proteins and of the amino groups responsible for the light absorption by the specific allergen. Furthermore, the major limit of the technology is the weak penetrability of the light in solids which causes the disomogeneity of the treatment and a reduction in the efficiency of the process in case of solids, for which only superficial treatments are possible. Moreover, high energy doses are necessary to affect the immunoresponse of allergens processed in PL treatments, this causing a relevant temperature increase of the sample. Accordingly, the photochemical and the thermal effect of PL on allergens can't be discriminated.

Table 2.3. Main findings in the application of PL on food allergens

| Allergen | Process Conditions | Major findings | Reference |
|--|--|---|---------------------|
| Almond Protein Extracts | 3 pulses/s, 10 cm from lamp, for 0.5, 1, 2, 3, 4, 6, 7, and 10 min | reduction in the levels of allergens and IgE binding, as demonstrated by SDS-PAGE, Western blots, and ELISA | Li et al. (2013) |
| Ara h1, Ara h2, Ara h3- Liquid Peanut Butter | 3min, 14.6cm, 0.92J/cm ² , 3pulses/s | IgE binding 7-fold lower in PUV-treated samples than in nontreated ones. No effect on Ara h2. | Chung et al. (2008) |
| Ara h1, Ara h2, Ara h3- Peanut Extracts | 4min, 14.6cm, 0.92J/cm ² , 3pulses/s | IgE binding 7-fold lower in PUV-treated samples than in nontreated ones. No effect on Ara h2. | Chung et al. (2008) |
| Ara h1, Ara h2 and ara h3 in extracts from peanuts | 2, 4, and 6 min, 10.8, 14.6, and 18.2 cm | reduction in IgE binding of up to 12.9 folds, respectively, compared to control. | Yang et al. (2012) |

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| Ara h1, Ara h2 and ara h3 in peanut butter slurry | 1, 2, and 3 min, 10.8, 14.6, and 18.2 cm | reduction in IgE binding of up to 6.7-folds, respectively, compared to control. | Yang et al. (2012) |
| Atlantic White Shrimp (<i>Litopenaeus setiferus</i>) Extract | 3 pulses/s, 10 cm from light source, for 4 min | Results showed that levels of tropomyosin and IgE binding were reduced following PUV treatment | Shriver et al. (2011) |
| Gluten | 45 sec | Reduction in gluten content | Noojii et al. (2010) |
| Eggs | 2 min | reduction of 9.5 times of allergens immunoresponse in eggs treated by PL treatment except that for ovalbumin | Anugu et al. (2010) |
| caseins and whey proteins | 150 sec | reduction of immunoreactivity of 7.4 times for caseins and of 7.7 times for whey proteins | Anugu et al. (2009) |
| β -lactoglobulin | 4 cm, 2.2 J/cm ² with 1, 3, 5, 7, and 10 pulses. | No significant changes in the amino acid composition of proteins observed | Helmnasser et al. (2008) |
| egg white | 1.75 to 31.5 J/cm ² | Equal or even higher Immunoresponse | Manzocco et al. (2013) |

2.4.3.High Hydrostatic Pressure

High Hydrostatic Pressure (HHP) is a non-thermal technology based on the application of pressure levels ranging from 100 to 1000 MPa on food samples, introduced in flexible packages. HHP treatments allow the microbial decontamination at mild temperatures, a prolonged shelf-life and the retention of color, flavour and nutrients of foods.

Similarly to thermal treatment, HHP process is able to induce protein unfolding and/or denaturation and aggregation, because the complex protein-solvent has a smaller volume in the denatured state than in the native form (Figure 2.4). In fact, after application of high pressure, the presence of cavities in the folded protein or at the oligomer interface facilitates unfolding or dissociation (Weber et al., 1983), the destruction of electrostatic interactions (Kauzmann et al., 1959) and the solvation of polar and hydrophobic groups, which are all phenomena resulting in volume decrease.

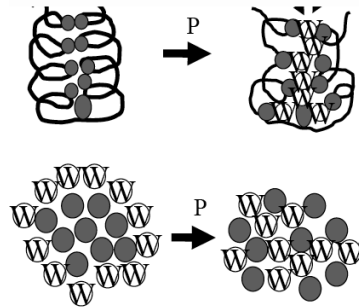


Figure 2.4. Water Penetration Model for Pressure Induced Unfolding of Proteins (Hummer et al., 1998).

HPP promotes the rupture of non-covalent bonds and hydrophobic groups (Jeanjean & Feillet, 1980 and Apichartsangkoon et al. 1997), thus affecting the secondary, tertiary and quaternary structure of protein and causing denaturation, aggregation or gelation phenomena (Messens et al., 1997). The extent of these phenomena depends on the pressure level, type of protein, pH, temperature and holding time. In particular, high pressure modifies the quaternary structure by destroying the hydrophobic interactions, the tertiary structure by reversible unfolding and the secondary structure via irreversible unfolding (Tauscher, 1995). The physical response of a generic protein or of an allergen protein to HHP treatment is described by its pressure-temperature stability diagram in which, for each treatment temperature, the corresponding pressure that it necessary to overcome in order to achieve an irreversible protein denaturation (Figure 2.5).

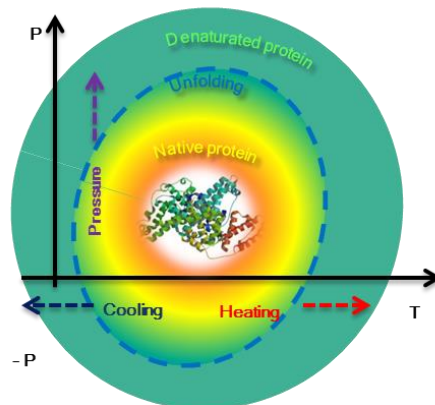


Figure 2.5. Pressure- temperature stability range for proteins.

Background

Many authors studied the effect of high pressure induced denaturation and aggregation of proteins through analysis of their structure modifications. Apichartsrangkoon et al. (1997) studied the effect of high pressure on gluten denaturation by applying different pressure levels (200, 400, 600 and 800 MPa at 20, 40 and 60° C for 20 or 50 minutes). They noticed that HPP induced gels were more elastic with a higher storage modulus with respect to thermally induced. The higher springiness of HPP proteins is due to the fact that HPP leads protein unfolding, which implies SH/SS exchange interactions and the formation of disulphide-bonded aggregates (Funterberger, Dumay & Cheftel, 1995) together with the unmasking of buried hydrophobic groups.

Also Jeanjean and Feillet (1980) and Hosene et al. (1987) noticed that disulphide bonds are the main bonds involved in gluten structure after treatment, and that disulphide bonded aggregates are modified by pressure treatment. Additionally, electrophoresis showed that samples treated at pressure levels of 600 and 800 MPa lost some protein bands, confirming a reduction of gluten solubility because of the newly formed covalent bonds (disulphide bonds).

Somkuti et al. (2010) studied the effect of pressure on the allergen Mal d1 which is the most potent allergen in apple (Vanez -Krebitz et al., 1995) causing also anaphylactic shock (Saraswat and Kumar 2005) and cross reaction with Bet v from birch pollen (Rouge et al., 2009). They analyzed the effect of different environmental factors (pH conditions, sugars and ionic strength) on the level of protein unfolding, as measured by High-pressure Fourier-Transform Infrared technique, which is a suitable tool to determine the secondary structure content of proteins (Barth 2007; Smeller et al., 1995a). The authors observed that Mal d1 native secondary structure changed after pressure treatment in the range 120-260 MPa because protein unfolding and aggregation occurred.

Husband et al. (2011) studied the effects of HPP treatment (700 MPa at 20 °C and 115 °C) on the immunoreactivity of Mal d1, Mal d3 in apple and Api g1in celeriac by means of SDS-PAGE and Western Blot techniques observing a great resistance of Mal d 1 to high pressure treatment. Similar results were obtained by Johnson et al. (2010) who did not observe any changes in the same allergen Mal d1 conformation by applying high pressure (150-800 MPa).

Heroldova et al. (2009) studied the application of HPP treatment to reduce allergenicity of rDau c1 and carrot juice using in vivo and in vitro tests. After treatment, samples were analyzed with SDS-PAGE (Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis), WB (Western Blot), SPT (Skin Prick Test) and basophil activation test BAT (Basophil Activation Test). The first two techniques were used to investigate protein structure, while with the latter techniques were used to evaluate the effects of HPP on immunoreactivity. The authors could not confirm the influence of

high pressure on allergenicity of rDau c1 and carrot juice with neither with HPP carried out with pressure from 400 to 550MPa applied for 3 and 10 min, nor working at 500 MPa for 10 min and temperature of 30 °C, 40 °C and 50 °C. Higher structural changes were observed in rDau c1 samples treated for 10 min at 500 MPa and temperature 50 °C. Also Kleber et al. (2007) observed that the antigenicity of β -lactoglobulin treated by high pressure (P = 200, 400, 600 MPa, T = 30-68°C and t = 0, 10, 30 min) was not reduced by the treatment but it was even increased at higher levels of pressure and longer holding time.

According to the scientific literature, even though HPP is suitable to induce protein modifications, secondary structure modifications, gelation and aggregation depending on processes parameters and on the stability of the allergen, high pressure has only a weak effect in terms of immunoresponse and, in some cases, can even induce an increase in allergenicity. This can be explained taking into account that HHP treatment has an effect only on weak bonds of the conformational structure of the allergen, while any modification of the allergen primary structure hold by covalent interactions is detected. For this reason HHP might not affect the linear epitopes of the allergen primary structure or can even cause the exposure of the conformational epitopes, thus, increasing the final antigenity of the allergens.

Moreover, HPP processes enhance the exposure of polypeptide chains, facilitating the enzymatic attack of some allergenic proteins, such as albumin (López-Expósito et al., 2008) and whey proteins (Foegeding et al., 2002) in appropriate operative conditions (pressure, temperature and holding time). This finding suggests that the combination of hydrolysis and by high pressure could lead to an increase of the enzymatic reaction rate.

Table 2.4. *Effect of HHP treatment on food allergens.*

| Technology | Allergen | Process Conditions | Major findings | Authors |
|------------|--------------------------------|---|--|-------------------------|
| HHP | rDAu c1 and carrot juice | P=400-500MPa; t=3-10min P=500MPa t=10min T=30,40,50°C | Main changes were seen in rDAu c1 samples treated 10 min at 500 MPa and temperature 50°C (CD). | Hereldova et al. (2009) |
| HPP | Mal d1 Mal d3 Api g1 | P=700MPa T=20 or 115°C T=10 min | Significant reduction of immunoreactivity (SDS-PAGE and Western blot) | Husband et al. (2011) |
| HPP | Ara h2,6 Mal d3 Mal d2 b | P=150-800 MPa T=20°C t=10 min | No structural changes in Ara h 2,6 or Mal d 3; minor changes in Mal d 1b (at DC) | Johnson et al., (2010) |

Background

| | | | | |
|-----|---------------|--|--|-----------------------|
| HPP | β -Lg | P=200-400-600MPa T=30 to 68 °C t=0,10,30 min | Antigenity increased increasing pressure and holding time (ELISA) | Kleber et al. (2007) |
| HHP | Maldl (apple) | P=100-1000 MPa T=30-85°C t=5min | Protein unfolding at pressure of 150-250 °C depending on the environmental conditions (at Fourier Transform Infrared, FTIR). | Somkuti et al. (2010) |

2.4.4. Enzymatic treatment assisted by High Hydrostatic Pressure

Despite High Hydrostatic Pressure is able to modify the conformational structure of proteins, changes in primary structure of allergy proteins do not occur (Tanford, 1968). Allergenicity may be due to linear epitopes and, therefore, in order to reduce allergenic power is also necessary to modify the primary structure.

For this reason as an alternative to HHP treatment, the HHP assisted hydrolysis may be tested in order to induce also the cleavage of peptide/covalent bonds. In addition, modifications of the allergen structure may enhance the proteolytic activity of the enzymes. Several authors demonstrated that several proteins are more easily hydrolyzed in their denatured form than in their native one (Fukushima, 1969, 1959; Evans, 1946). The resistance of the native protein to proteolysis is due to its compact tertiary structure, namely its globular form, which protects peptide bonds, while in denatured form, the peptide bonds are available to enzymatic attack.

Hydrolysis assisted by high pressure was applied on whey proteins (Foegeding et al., 2002), such as β -lactoglobulin. In fact, for a long time these proteins have been considered to be the major allergens of milk (Wal. 2004); they are very resistant to gastric acid and proteolytic attack (Reddy et al. 1988; Sorv et al., 1994).

Also, Vilela et al. (2006) observed that the HPP process can improve in vitro enzymatic digestion (pepsin and pancreatic) of whey proteins. This treatment was used to generate low molecular weight (< 1 kDa) peptides which provided anti-inflammatory benefits to cystic fibrosis (CF); proteolytic digestion of WPI (whey proteins isolates) increases intracellular glutathione (GSH) concentration. Vilela et al. (2006) concluded that native whey proteins were more resistant to pepsin hydrolysis; after 30 min of pepsin digestion, only 30.9% decrease in protein content occurred, whereas WPI subjected to single (550 MPa) or triple cycle pressure treatment (400

MPa) showed a decrease in protein content of 68% after 30 min of pepsin digestion.

The antigenic activity of protein hydrolysates has been reported to be reduced when high hydrostatic pressure is applied prior to or during enzymatic hydrolysis (Bonomi et al., 2003). López-Expósito et al. (2008) studied the changes in OVA (ovalbumin) proteolysis profile by high pressure treatment and its effect on IgG and IgE binding. The authors applied an enzymatic treatment with pepsin after high pressure application (400 MPa). The results of *in vivo* tests (ELISA test) showed that peptides produced after hydrolysis on samples treated for few minutes at pressure level below 400 MPa have an allergenic power lower than that of peptides produced by hydrolysis carried out at ambient pressure after long treatment time (of the order of hours) possessing only one binding site for the Ig-E antibody.

Immunoreactivity and digestibility of high-pressure treated whey proteins isolates (WPI) were also studied by Chicón et al. (2008). They treated β -lactoglobulin and WPI with porcine pepsin under high pressure (200 and 400 MPa) at pH 6.8 and pH 2.5 and observed the formation of protein aggregates at pH 6.8 and a higher digestibility of high pressure treated proteins than non-treated ones. The increased susceptibility of β -lactoglobulin to proteolysis was progressively lost during the refrigerated storage of the HPP-treated WPI. The authors did not observe any correlation between the increase of digestibility and IgE-binding of the proteolytic products. In fact, in both cases, specific IgE peptides were released after hydrolysis with Corolase PP, and some immunoreactive fragments persisted.

Penãs et al. (2006) analyzed the effect of HPP processes (100-300 MPa for 15 min at 37°C) before hydrolysis of dairy whey proteins by three different enzymes (trypsin, chymotrypsin and pepsin). HPP process increased the degree of hydrolysis and chymotrypsin, and trypsin showed the highest proteolytic activity at 100 and 200 MPa, followed by pepsin at 300 MPa. The major results in terms of immunoreactivity reduction were observed after HPP treatment with pepsin or trypsin, obtaining dairy whey hydrolysates to be used as a source in hypo-allergenic infant formula.

Table 2.5. Effect of hydrolysis assisted by HHP treatment on food allergens.

| Technology | Allergen | Process Conditions | Major findings | Authors |
|------------|-------------|--|---|----------------------|
| HHP+enzyme | β -Lg | Chymotrypsin Trypsin P=600MPa T=30,37,44°C t=10min | Higher hydrolysis under HP than at atmospherical pressure; hydrolysates with no immunoreactivity (Western Blotting) | Bonomi et al. (2003) |

Background

| | | | | |
|------------|------------------------------|--|---|---------------------------------|
| HPP+enzyme | WPI β-Lg | P=200-400 MPa T=30-50°C t=10-30 min | HPP at 400 MPa promoted hydrolysis of β-Lg producing hydrolysates with higher digestibility (RP-HPLC, SDS-PAGE) | Chicón et al. (2008) |
| HHP+enzyme | OVA (Ovalbumin) | Pepsin P=400MPa T=37°C t=10,60,120min | Production of hydrolysates (RP-HPLC-MS/MS) with lower antigenicity (binding to IgG) than those produced in hours at atmospheric pressure. | López-Expósito I. et al. (2008) |
| HHP+enzyme | Dairy whey proteins | Chymotrypsin, trypsin, pepsin P=100-300MPa T=37°C t=15min | Important decrease of immunochemical reactivity was found for pepsin and trypsin hydrolysates (ELISA). | Peñas E. et al. (2006) |
| HHP+enzyme | WPI (whey proteins isolates) | <u>1 cycle</u> (P=550MPa; t=3-4 min); <u>3 cycles</u> (P=400 MPa; t=1 min) T<26-27°C | HP enhanced the in vitro digestibility of WPI to proteolytic enzymes (CZE and GC-MS) | Vilela et al. (2006) |

2.5. Final remarks

Conventional and innovative technologies for the treatment of food allergens may be a valid alternative to allergen-free products, which are very expensive and have high nutritional deficiencies. Both enzymatic and thermal treatments are able to induce allergen modifications, but with low effect on immunoreactivity due to the fact that their effect is limited only to specific epitopes (linear or conformational).

Despite thermal technologies, non-thermal treatments can be used for allergens treatment with the advantage of preserving the nutritional quality of the food product. Among the non-thermal technologies, high hydrostatic pressure has shown a significant potential in inducing protein modifications, thus, representing the most promising technology for allergens treatment.

In order to increase the extent of allergen modifications by High Hydrostatic Pressure, the combination of this non-thermal treatment together with an enzymatic treatment could be a more effective approach for the production of hypoallergenic products.

Once the main allergen target has been fixed, process conditions for the combined treatment have to be optimized. This novel approach could lead to the development of hypoallergenic products with high nutritional value.

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Chapter III

Objectives and working plan

3. General Objective

The scientific background on the topic of food allergens demonstrated that non-thermal technologies may represent a valid alternative to an allergen - free diet. In particular, High Hydrostatic Pressure (HHP) showed to be the most promising treatment being able to modify the conformational structure of the allergens as well as to affect the efficiency of the enzymatic hydrolysis reactions. For all these reasons the general objective of the PhD thesis is the *setup of a novel process for the production of hypoallergenic foods with high nutritional value.*

The work was aimed to study the effect of non-thermal technologies as well as of non-thermal technologies combined with proteolytic enzymes on allergenic proteins.

3.1. Specific Objectives

The general objective was achieved through the accomplishment of the following specific objectives:

- **Study of the effect of High Hydrostatic Pressure on protein structure:** the effect of the HHP treatment on protein structure modifications ranging from unfolding to aggregation and/or gelation were evaluated. Furthermore functional properties of target proteins treated by HHP processes were also determined.
- **Study of the effect of High Hydrostatic Pressure on proteolytic hydrolysis of allergens:** the effects of High Hydrostatic Pressure on hydrolysis of BSA and casein were evaluated and the hydrolysates produced were characterized in terms of dimensions and structure by molecular weight distribution, IR-spectra and particle size distribution.

- **Immunoreactivity analysis of allergens treated by High Hydrostatic Pressure or by the combination of High Hydrostatic Pressure with enzymatic treatment:** the allergenicity of the peptides produced by high pressure and hydrolysis assisted by high pressure were detected by in vivo tests on allergic patients (prick tests).

3.2. Working plan

The experimental activity was divided into three different parts corresponding to the three specific objectives of this PhD thesis. The first part aimed to analyze the effect of High Hydrostatic Pressure on the conformational structure of allergens.

The effects of HHP process on allergens were studied in a wide range of process conditions and different product characteristics in order to induce the unfolding/denaturation as well as aggregation and or gelation.

Utilizing the operating conditions optimizing the unfolding of the allergens, the effects of the treatment on the hydrolysis reaction of the allergens by proteolytic enzymes were studied. In particular, the effect of both product and process parameters on the extent of hydrolysis was determined and the hydrolysates produced were characterized in terms of dimensions and structure and compared with the peptides produced in the same conditions at atmospheric pressure.

Once the combined process of enzymatic hydrolysis assisted by high hydrostatic pressure was optimized, the final part was addressed to analyze the produced peptides in terms of immunoresponse in order to determine the effect of the process on the allergenicity.

The working plan is summarized in the following:

1. Study of the effects of high hydrostatic pressure (HHP) on allergens structural modifications:

- Determination of the *transition* between unfolding phenomena and macroscopical aggregation causing gelation in target allergens as a function of operating conditions;
- Study of the effect of product and process parameters on microscopic and macroscopic rheological properties in *dynamic and oscillating regime* both in and modelling of rheological behaviour;
- Study of the effect of product and process parameters on rheological properties in *stationary regime*;
- At the operating conditions causing unfolding, that is at pressure levels lower than those causing gelling, the effects of

HHP on *functional properties* of allergens were studied. In particular, the target allergen was characterized in terms of number of free thiols groups, foaming properties and secondary structure.

2. Study of the effect of high hydrostatic pressure on the enzymatic hydrolysis reaction:

- Investigation of the influence of high pressure induced unfolding on the *hydrolysis* of BSA and casein by chymotrypsin and trypsin.
- Analysis of the effects of HHP processing on the hydrolysis of casein.
- Study of the effect of the reaction conditions and operating conditions (pressure, holding time and temperature) on the extent of *hydrolysis* applied after or during HHP treatment.
- *Characterization of the peptides* produced by high pressure in terms of dimensions (particle size distribution and molecular weight distribution by means of SDS Page) and structure (HPLC profiles and FT-IR spectra).

3. Investigation of the effect of the combined process of enzymatic hydrolysis assisted by HHP on allergens immunoreactivity:

- Investigation of the optimized conditions to enhance the hydrolysis degrees and to produce peptides of lower dimensions;
- *In vivo test* in order to analyze the immunoresponse of the produced peptides to detect the effect of High Hydrostatic Pressure on allergenicity.

Chapter IV

Effects of High Hydrostatic Pressure on allergen structure

4.Introduction

Among the non-thermal technologies, High Hydrostatic Pressure (HHP) showed to have a high potential in inducing protein modifications, thus representing a suitable tool to reduce allergens immunoresponse. Many authors demonstrated that high pressure modifies the protein conformational structure, thus inducing several phenomena, namely protein unfolding, aggregation and/or gelation.

The effects on proteins as well as on allergens depend on several factors related to the compound characteristics, namely the nature of the protein, as well as to processing conditions, such as the applied pressure, treatment temperature and holding time (Messens, Van Camp, & Huyghebaert, 1997).

In general, the protein structures (primary, secondary, tertiary and quaternary) are stabilized by covalent bonds, electrostatic interactions, hydrogen bonds and hydrophilic interactions. The HHP process causes the alteration of the structures which are stabilized by hydrophobic interactions, namely the quaternary, the tertiary (reversible unfolding), and secondary structures (irreversible unfolding). In the case of globular proteins, such as albumin, their structural properties are related to the optimum packing of the hydrophobic core, the minimum hydrophobic surface area and ion pairs within and between subunits (Richards, 1977; Dill, 1990; Jaenicke, 1991). Therefore, since HHP process affects both the tertiary and quaternary structures of globular proteins (Gross and Jaenicke, 1994), it is able to promote the dissociation and assembly of systems with higher complexity on the one hand and the unfolding and disassembly on the other hand (Jaenicke, 1987).

The mechanism of pressure-induced denaturation of several proteins has not been elucidated yet but it has been hypothesized that it takes place through reversible unfolding steps as described in the following. When

pressure is applied, the intermediates formed play an important role in the aggregation upon the depressurization phase. Pressure-induced conformations are more susceptible to aggregation since the hydrogen bonds can be formed at lower temperatures under pressure (Heremans, 2001).

Pressure-induced denaturation can be reversible (Mozhaev et al., 1996) depending on the kind of protein and concentration and treatment conditions (temperature, time and pressure) (Rastogi et al., 2007). Low protein concentrations and pressure levels up to 200 – 300 MPa usually result in a reversible denaturation. Higher pressure levels, above 300 MPa, irreversibly and extensively affect proteins, even causing denaturation, due to the unfolding of monomers, the aggregation or gel formation (Cheftèl, 1992).

The mechanism of the protein gelation differs according to the protein species, their state and gel-forming ability as well as on pressure-time-temperature combinations (Pérez-Mateos and Montero, 1997, Montero and Pérez-Mateos, 1997 and Fernández-Martín et al., 1998). Gels induced by high hydrostatic pressure exhibit different characteristics than temperature induced ones showing minor strength and higher content of incorporated liquid (Van Camp & Huyghebaert, 1995).

In the literature several studies reported the gelling of different proteins such as ovalbumin (Okamoto et al., 1990 and Galazka et al., 1997), fish protein (Pérez-Mateos and Montero, 1997), and meat protein (Okamoto et al., 1990 and Jiménez Colmenero et al., 1998) under the effect of pressure and the improvement of some rheological properties in pressure-induced gels.

As a first objective of this study, the effect of operative conditions and solution properties on the *critical transition* from allergen unfolding to aggregation was evaluated with the aim of discriminating the two phenomena induced by high hydrostatic pressure.

The critical conditions determining the transition from unfolding to aggregation were determined and the effect of both product (protein concentration and pH of the solution) and process (pressure level, temperature and holding time) parameters on the extent of aggregation and on the conformational structure of the allergens in gelling conditions.

The *extent of protein gelation* of globular proteins, such as BSA (Bovine Serum Albumin), was characterized through a rheological characterization in both dynamic (oscillating tests) and stationary regime.

In particular, strain sweep tests, frequency sweep tests and temperature ramp tests in both stationary and dynamic regime were carried out on BSA samples (concentration of 50 and 100 mg/mL) processed at pressure levels between 600 and 900 MPa. Furthermore the data obtained were worked out and fitted by a mathematical model representing the rheological behavior of HHP induced gels.

4.1. Materials and methods

4.1.1. Preparation of the samples

Bovine serum albumin (BSA) was chosen as target protein to be used in the experimental campaign. BSA is a water soluble globular protein, made of two chains of 583 amino acids, organized in alpha-helices (67% of the whole structure) and joined by 17 S-S groups and one free thiol group. In this configuration the BSA protein is a dimer made of two symmetric chains, thus showing a very resistant structure.

BSA (Sigma-Aldrich, Italy) samples were prepared according to Penãs et al. (2006) by dissolving the protein in a 50 mM Sodium Phosphate Buffer, kept at 25 °C, at different concentrations (12, 25, 50 and 100 mg/mL) under gentle mixing until a homogenous solution was obtained. The pH of the protein solutions was reduced with HCl and measured with a laboratory pH-meter in order to achieve the desired value. The protein solutions were stored under refrigerated conditions (4 °C) before being processed by HHP.

4.1.2. Experimental apparatus

The HHP system U22 (Institute of High Pressure Physics, Polish Academy of Science, Unipress Equipment Division, Poland) was used in the experimental campaign. The laboratory scale U22 unit holds a high pressure vessel with a maximum processing volume of 50 mL) and can be operated in a wide pressure range between 0 and 1400 MPa, and under thermal controlled conditions (0 – 120 °C). A control panel allows to set-up the operating pressure, the ramp rate and the processing time, as well as to control the opening and the closure of the HHP vessel. The temperature in the HHP vessel is set-up and controlled by a portable Temperature Power and control Unit (TCU) connected to the main unit with K-type thermocouples cables. The vessel is provided with electrical heaters to increase the temperature during treatment while the cooling of the vessel is obtained with compressed air. The pressure medium is Plexol (Bis (2-ethylhexyl) sebacate from Sigma-Aldrich, Italy) and the estimated temperature increase due to pressure build-up is 2-3 °C/ 100 MPa.

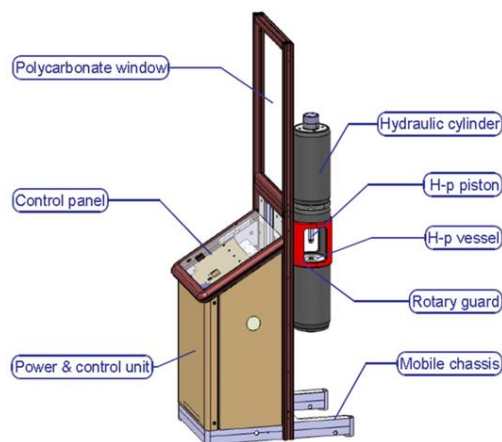


Figure 4.1. Schematic representation of the main components of the experimental apparatus, HHP system U22 (Institute of High Pressure Physics, Polish Academy of Science, Unipress Equipment Division, Poland).

4.1.3. Experimental protocol

For each experiment, BSA samples (5 mL) were sealed in flexible pouches of a multilayer polymer/aluminium/polymer film (Polyethylene-Aluminium-Polypropylene). The pouches were introduced into the U22 vessel and the pressure cycle was set at the desired conditions. All the experiments were conducted at ambient temperature while the pressure was varied in the range between 500 and 900 MPa and the operating time between 15 and 25 min.

At the end of the treatment, the pouches were stored at 4 °C before undergoing the rheological characterization.

4.1.4. Rheological characterizations

A rheometer AR 2000 (TA Instruments, New Castle, DE) was used to detect the structural changes of BSA protein after HHP treatments. Several rheological tests were carried out in *dynamic-oscillating regime* or in *stationary regime*. A plate-cone measuring geometry was used (40 mm diameter, 2°), with a gap width of 1 mm. Samples were loaded onto the rheometer plate and allowed to equilibrate to the measuring temperature (25 °C) for 10 min. Different protocols were used to determine the rheological behaviour of BSA samples.



Figure 4.2. Rheometer used for tests with plate and cone geometry TA Instruments AR 2000 (TA Instruments, New Castle, DE).

4.1.4.1. Strain sweep tests

Preliminary strain sweep tests were carried out in order to individuate the linear viscoelastic region of BSA protein dissolved at two different concentration of 50 and 100 mg/mL in phosphate solution after HHP treatments at 700 MPa and 900 MPa for 15 and 25 min. Storage (G') and loss (G'') moduli were recorded as a function of the strain, in a range between 0.01 and 10 %, at a constant angular frequency of 6.3 rad/s and at a fixed temperature of 25°C.

4.1.4.2. Frequency sweep tests

The protein structure has been further characterized by means of frequency sweep tests at a strain below the critical value. The tests were carried out on BSA samples at a concentration of 50 mg/mL after HHP treatments at 700 MPa and 900 MPa for 25 min. The trends of G' and G'' moduli were measured in the angular frequency range between 1 and 100 rad/s at a fixed strain of 0.1%, lower than the critical value, which, in turn, is obtained from the corresponding strain sweep tests conducted on samples treated in the same HHP processing conditions.

4.1.4.3. Flow measurement tests

Flow measurement tests were carried out on BSA samples with two protein concentrations, namely 50 and 100 mg/mL, processed in HHP

treatments, at pressure level of 600 MPa and 800 MPa, and processing time of 15 and 25 min. The apparent viscosity and shear stress were measured at a fixed angular velocity of 6.3 rad/s in the range of shear rates between 0.01 and 100 Hz.

4.1.5. Mathematical modelling

The *Ostwald de Waele* model (Eq. 1), which assumes a power-law dependence between shear stress, τ , and shear rate, $\dot{\gamma}$, was used to model the rheological behaviour of BSA samples:

$$(1) \tau = K(\dot{\gamma})^{n-1}\dot{\gamma}$$

Where K represents the consistency coefficient and n a dimensionless number, which indicates the deviation from the Newtonian behavior ($n=1$). The model parameters, K and n , were estimated by applying the least squares method. The values of the statistical parameters, R^2 and the standard deviation of the curves from the experimental data, were also estimated in order to evaluate the correctness of the fitting equations.

4.2. Temperature ramp tests

To assess the effect of temperature increase on the structural properties of proteins, dynamic temperature ramp tests were carried out on BSA samples of different concentrations, namely 12, 25, 50 and 100 mg/mL, treated by HHP at 500 MPa and 700 MPa. The rheological tests were conducted in the temperature range between 25 °C to 95 °C utilizing at a ramp rate of 1.5 °C/min, at a constant strain of 0.1% and angular frequency of 6.3 rad/s.

The trends of G' and G'' moduli (in dynamic regime) or of viscosity (in stationary regime) were recorded as a function of temperature and the transition phase temperatures were individuated in the graphs.

4.3. Results and discussion

4.3.1. Preliminary observations

In Figure 4.3 the pictures of BSA samples at pH=8 treated at several processing conditions namely pressure level in the range between 600 and 900 MPa, treatment times of 15 and 25 min, and protein concentration of 50 and 100 mg/mL, are reported. It can be observed that more compact gels are obtained at the highest pressure level of 900 MPa, processing time of 25

min, and protein concentration of 100 mg/mL. At lower pressures (600 MPa) protein denaturation and aggregation occur with increasing the protein concentration, from 50 to 100 mg/mL, or the processing time, from 15 to 25 min.

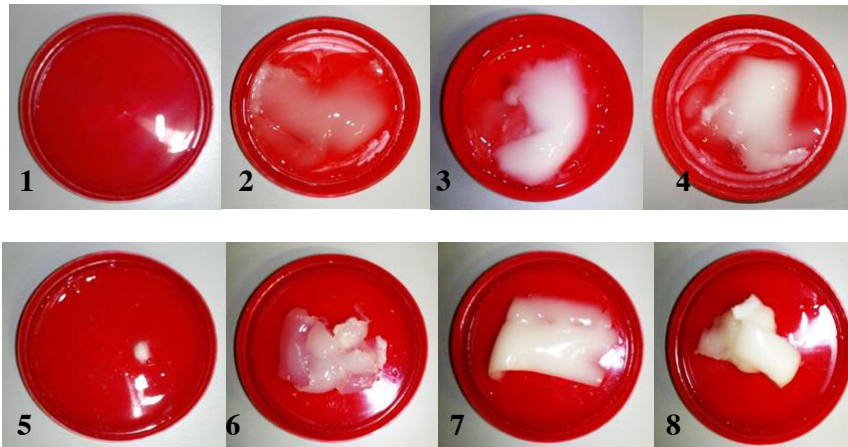


Figure 4.3. Pictures of the BSA protein samples suspended in sodium phosphate buffer (50 mM, pH:8) treated at different pressures, treatment times and concentrations. Processing conditions: 1) $P=600$ MPa, $t=15$ min, $c_p=50$ mg/mL; 2) $P=700$ MPa, $t=15$ min, $c_p=50$ mg/mL; 3) $P=800$ MPa, $t=15$ min, $c_p=50$ mg/mL; 4) $P=900$ MPa, $t=15$ min, $c_p=50$ mg/mL; 5) $P=600$ MPa, $t=25$ min, $c_p=100$ mg/mL; 6) $P=700$ MPa, $t=25$ min, $c_p=100$ mg/mL; 7) $P=800$ MPa, $t=25$ min, $c_p=100$ mg/mL; 8) $P=900$ MPa, $t=25$ min, $c_p=100$ mg/mL.

4.3.2. Effect of concentration and operating conditions

The results of strain sweep tests carried out on HHP treated BSA samples were used to evaluate the limits of the viscoelasticity region in which the rheological parameters, namely viscosity or loss and storage moduli, are independent from the strain. These limits may be influenced by the processing parameters (namely pressure level and treatment time) and protein concentration.

The storage or elastic modulus, G' , and the loss or viscous modulus, G'' , can be only defined in the linear viscoelasticity region, while outside it they have no physical meaning. When the sample is subjected to high strains, the stress response of a viscoelastic material is no longer sinusoidal and, consequently, the stress-strain relationship cannot be described in terms of

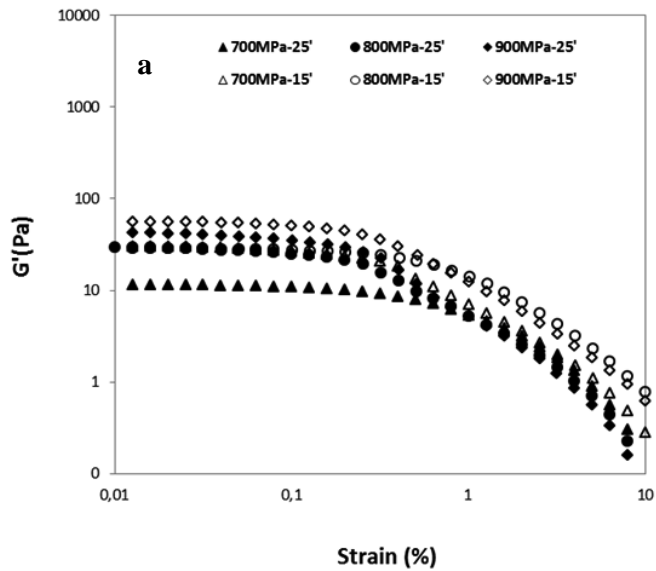
the strain-independent storage and loss moduli, due to higher harmonic contributions (Dealy et al. 1990).

Rheological tests in the dynamic regime were carried out on BSA samples dispersed in sodium phosphate buffer at different concentrations of 50 and 100 mg/mL, processed at pressure levels between 600 and 900 MPa and two different holding times: 15 and 25 min. The experimental results were reported in terms of G' and G'' moduli values as a function of the strain (%), as shown in Figure 4.4 (a) (b) and Figure 4.5 (a) (b). Both storage (Figure 4.4 (a)) and loss moduli (Figure 4.4 (b)) increase with increasing the pressure applied, independently of the treatment times. This behaviour can be attributed to aggregation and coagulation phenomena of proteins taking place as already observed by Apichartsrangkoon et al., (2003) for soy protein gels and Aguilar et al. (2007) for egg yolk gels. As the holding times of the samples treated under high pressure conditions is increased, a slightly higher values of G' and G'' moduli is observed, especially in the samples with the highest protein concentration. In fact, by prolonging the holding time of the protein under high pressure conditions, more drastic denaturation is likely to occur.

Protein concentration mainly influences the values of the storage and loss moduli. Indeed, if the protein concentration increases from 50 mg/mL to 100 mg/mL, the values of G' modulus of samples processed at 900 MPa increase of one order of magnitude, passing from values less than 100 (Figure 4.4 (a)) to more than 1000 (Figure 4.5 (a)). A similar trend is observed for G'' modulus, as shown in Figure 4.4 (b) and Figure 4.5 (b). Higher values of protein concentration enhance the formation of both short and long terms bindings, due to the availability of more contact points between neighboring polypeptide side chains (Cheftel et al., 1985). Moreover, the storage modulus is always prevailing with respect to the loss modulus, this indicating that the elastic behaviour of the samples is more relevant than the viscous one (Figure 4.4 (a) and (b), Figure 4.5 (a) and (b)).

The experimental data of the strain sweep tests were worked out in order to individuate the limits of linear viscoelasticity region. Thus, the estimated values of the critical strain (γ_c) and critical stress (τ_c) of the linear region of viscoelasticity at different pressures are listed in Table 4.1. At strain values lower than about 0.3%, a linear region is observed, for all pressures and concentrations tested, while at higher strain the nonlinear behaviour is shown. The results obtained so far are in agreement with those reported by Won Song et al. (2006), who carried out oscillating rheological tests on concentrated xanthan gum solutions. The authors obtained loss modulus curves with similar shape, and this behaviour has been attributed to the occurrence of a strain-overshoot phenomena. When the protein undergoes a stress deformation, the structure shows a resistance up to a certain strain amplitude, leading to an increase of the G'' modulus. At higher values of the strain, the protein structure starts to be over-stressed, which results in a

decrease of the loss modulus. As reported in Table 4.1, the values of γ_c are independent of the operating pressure while depend on the holding time only at higher protein concentrations. Higher values of the critical strain can be attributed to a higher resistance of the gels which, indeed, is observed at higher holding times for the storage modulus. On the contrary, critical stress increases linearly with the pressure applied at low protein concentration (50 mg/mL), showing a sudden exponential increase with pressure at higher protein concentration. Our results are in agreement with those of Aguilar et al. (2007) who reported that while the critical stress increases exponentially with the pressure applied, the critical strain is independent on it. Our experimental data demonstrate the relevance of the protein concentration and processing time in defining the limits of the viscoelasticity region, which can be related to the occurrence of protein denaturation.



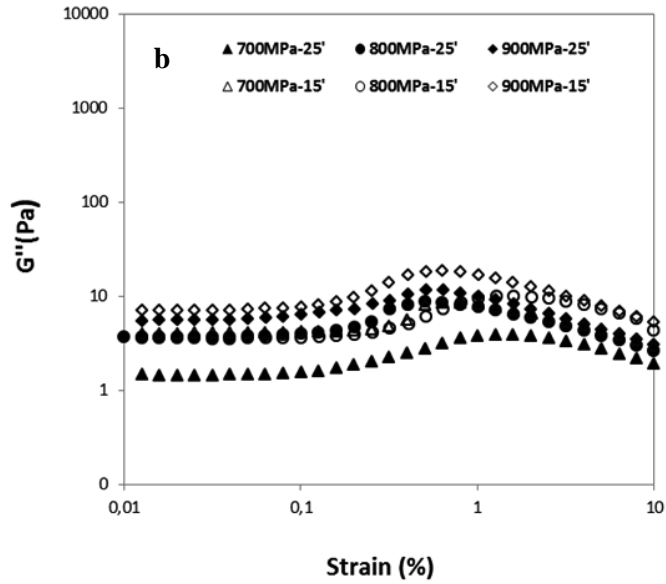
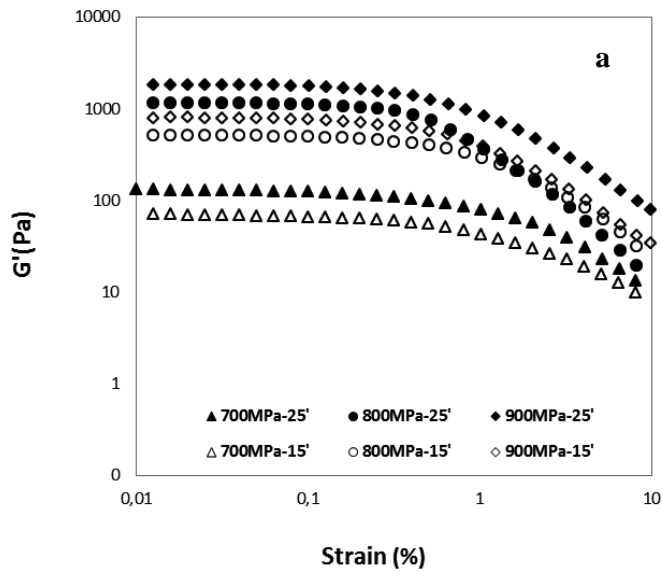


Figure 4.4. Strain sweep tests: storage, G' (a) and loss, G'' (b) moduli as a function of strain. BSA protein samples suspended in sodium phosphate buffer (pH: 8, 50 mg/mL) treated at different pressure levels (700 MPa, 800 MPa, 900 MPa) and treatment times (15, 25 min).



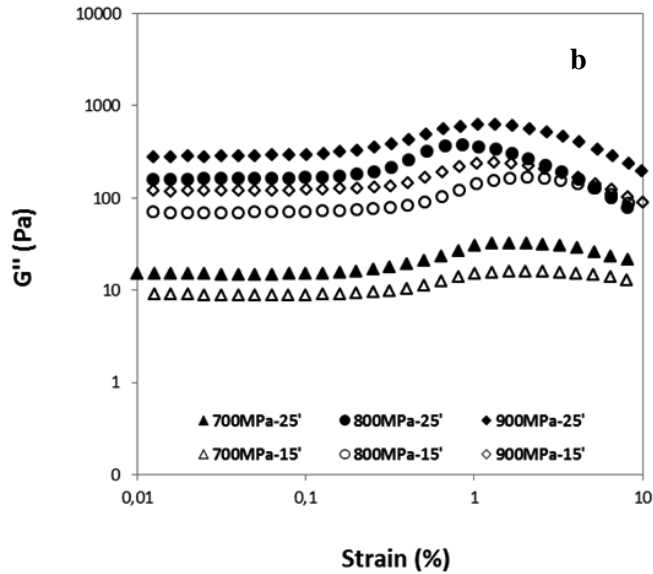


Figure 4.5. Strain sweep tests: storage, $G'(a)$ and loss, $G''(b)$ moduli as a function of strain. BSA protein samples suspended in sodium phosphate buffer (pH: 8, 100 mg/mL) treated at different pressure levels (700 MPa, 800 MPa, 900 MPa) and treatment times (15, 25 min).

Table 4.1. Critical storage modulus (τ_c) and strain (γ_c) measured for BSA (a: 50 mg/mL; b: 100 mg/mL) in sodium phosphate after treatments at different pressure levels (700 – 900 MPa), and treatment times (15min and 25 min) at 25°C

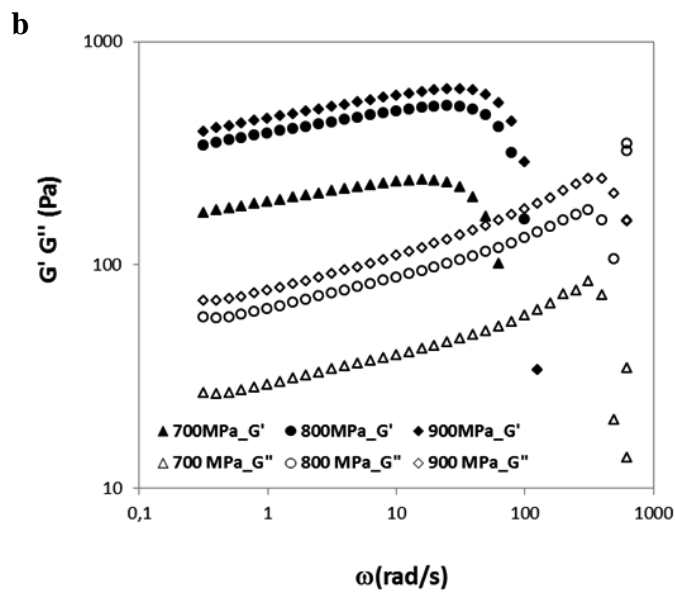
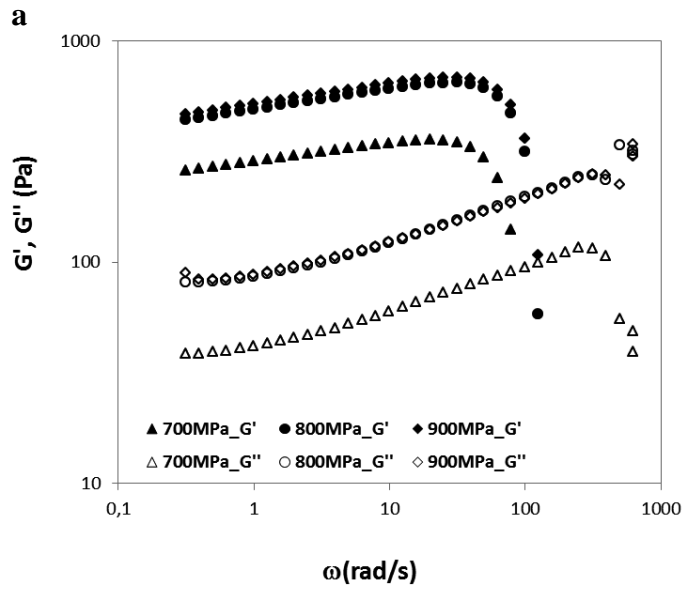
| a) | 15 min | | 25 min | | |
|----|----------------|----------------|---------------|----------------|---------------|
| | Pressure (MPa) | γ_c (%) | τ_c (Pa) | γ_c (%) | τ_c (Pa) |
| | 700 | 0.255 | 13.2 | 0.255 | 9.60 |
| | 800 | 0.202 | 25.8 | 0.255 | 19.1 |
| | 900 | 0.201 | 44.2 | 0.316 | 29.3 |

| b) | 15 min | | 25 min | | |
|----|----------------|----------------|---------------|----------------|---------------|
| | Pressure (MPa) | γ_c (%) | τ_c (Pa) | γ_c (%) | τ_c (Pa) |
| | 700 | 0.320 | 60.0 | 0.200 | 116.9 |
| | 800 | 0.404 | 420.7 | 0.200 | 859.0 |
| | 900 | 0.400 | 652.3 | 0.160 | 1760.5 |

4.3.3. Effect of the pH

Frequency sweep tests were carried out utilizing small deformation values, belonging to the linear viscoelasticity region (at strain lower than the critical one) in order to evaluate the effect of the pH on HHP induced denaturation of BSA samples. Protein solutions with a concentration of 100 mg/mL and different pH values (6, 7 and 8), were processed at four pressure levels, 600, 700, 800, 900 MPa, respectively, for 25 min and immediately analysed. BSA gels produced under HHP show a viscoelastic behaviour with both G' and G'' moduli increasing with frequency. The higher value of the storage modulus with respect to the loss modulus indicates that the elastic component is predominant on the viscous one in the frequency range tested. At higher pressures both G' and G'' moduli are increased thus indicating that the HHP induced gels exhibit more resistance at higher pressure levels (Figure 4.6 (a), (b) and (c)).

Also pH strongly influences the consistence of the gels: at pH closer to IP higher dependence of both moduli is observed indicating that the resistance of the gels against the deformation applied during rheological characterization is higher. In fact, the pH changes the balance between the electrostatic and the hydrophobic interactions leading to a modified reactivity of the protein towards the effects of the high pressure processes (Speroni et al. 2005). Furthermore, at the isoelectric point the net electric charge of the protein is null, while, when the pH of the sample is close to the isoelectric point of the protein, the hydrophobic interactions induced by the HHP treatment are not counter-balanced by the repulsive forces and, thus, protein aggregation occurs. At pH values far from the isoelectric point of the protein, repulsive forces increase and, consequently, the aggregation phenomena are limited. The results are in agreement with those of Aguilar et al. (2007) who carried out tests on egg yolk and observed that the increase of the pH caused an increase of loss and storage moduli and, consequently, that in HHP induced albumin gels the denaturation and subsequent aggregation increased. Similarly, Van Camp and Huyghebaert (1995) studied the effects of pH on high pressure induced whey protein gels (Van Camp, 1995). The authors found that an increase of the pH above the IP (isoelectric point), where whey proteins are negatively charged, results in an increase of the strength of the gel network and in a reduction of the liquid phase not incorporated in the gel forming network.



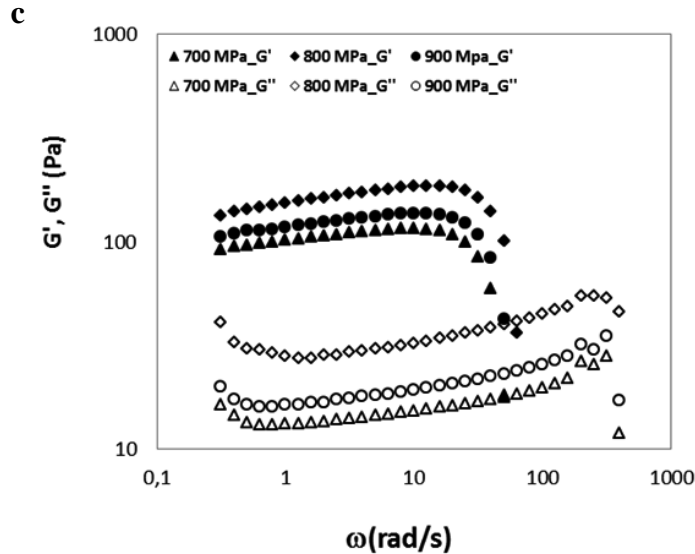


Figure 4.6. Frequency sweep tests: storage, G' , and loss, G'' , moduli as a function of frequency. BSA protein (pH: 8, 100 mg/mL) in sodium phosphate buffer with different pH (a: pH=6; b: pH=7; c: pH=8), treated at different pressure levels (700 MPa, 800 MPa, 900 MPa) for 25 min.

4.3.4. Effect of HHP on thermal denaturation

In order to verify if a HHP treatment might induce a partial protein denaturation, the influence of treatment pressure and protein concentration on the transition phase temperature was also investigated. In the first case, samples of BSA dissolved in sodium phosphate buffer (50 mg/mL) were treated at three different pressures of 500, 600 and 700 MPa for 15 min, while different amount of albumin were dispersed in the phosphate buffer in order to assess the effect of protein concentration, namely 12, 25, 50 and 100 mg/mL, and treated under pressure at 500 MPa for 15 min. These mild treatment conditions were set in order to avoid the gelation of samples. At the end of the treatment, the samples were subjected to temperature ramp rheological tests in dynamic regime and the values of G' and G'' were determined as a function of temperature. The transition phase temperature is defined as the temperature at which the G' modulus starts to increase. Figure 5 reports the curves of the G' of BSA samples treated at different pressures, while Figure 4.7 shows the curves of the G' of BSA samples with different protein concentration processed at 500 MPa for 15 min.

From the experimental curves the transition phase temperatures were determined and the values are reported in Table 4.2 and Tables 4.3.

It can be observed that the higher the pressure applied, the lower the value of the coagulation temperature. At a fixed concentration level of 50 mg/mL, native protein denaturation occurs at 65.8 °C, while in protein treated at 500 MPa, 600 MPa and 700 MPa denaturation takes place at 59.2 °C, 53.6 °C and 41.9 °C, respectively, as shown in Figure 4.7 and in Table 4.2. Thus, it can be concluded that the main effect of the pressure treatment is to accelerate thermal denaturation, the higher the applied pressure the lower the transition phase temperature. Experimental data demonstrate that, although the processing conditions are unable to induce protein gelation, high pressure treatments cause a significant modification of the BSA protein structure.

This effect can be explained by considering that high pressure induces the reversible protein unfolding, which, in turn, can contribute to modify the protein response to other processing methods, including thermal treatments. A similar behaviour is observed with increasing the protein concentration. In fact, as reported in Table 4.3 the transition phase temperature decreases with increasing the protein concentration.

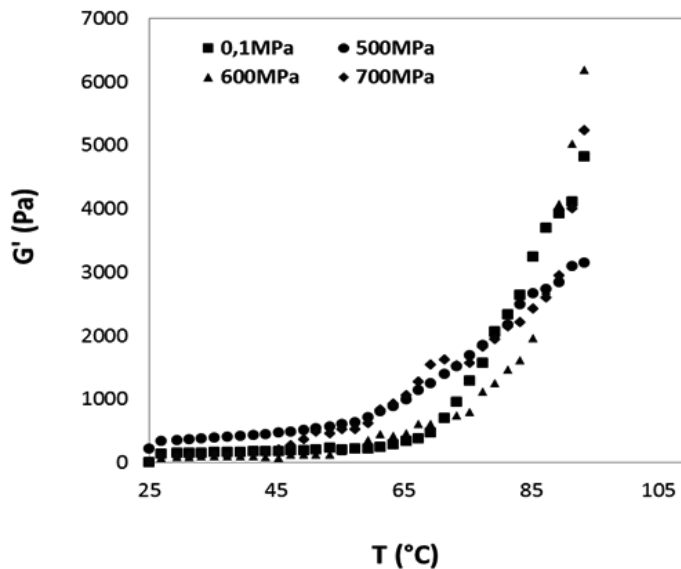


Figure 4.7. Temperature ramp tests: storage modulus, G' as a function of temperature. BSA protein samples, suspended with a concentration of 50 mg/mL in sodium phosphate buffer (pH: 8), treated at different pressure levels (500 MPa, 600 MPa, 700 MPa) for 15 min.

Table 4.2. Effect of pressure (0.1, 500, 600 and 700 MPa) of BSA (50 mg/mL) treated for 15 min on transition phase temperature.

| P (MPa) | T (°C) |
|---------|-------------|
| 0.1 | 65.8 ± 3.29 |
| 500 | 59.2 ± 2.96 |
| 600 | 53.6 ± 2.68 |
| 700 | 41.9 ± 2.10 |

For instance, with processing condition of 500 MPa and 15 min, the transition phase temperature of the BSA sample with a concentration of 12 mg/mL is equal to 83.9 °C, while it falls to 56.1 °C for the BSA sample with a concentration of 100 mg/mL (Figure 4.8 and Table 4.3). Finally, since the interactions among protein macromolecules strongly depend on their concentration in the suspension, the latter may also influence the intensity of the structure modification due to high pressure processing by promoting protein denaturation, as demonstrated by the experimental data.

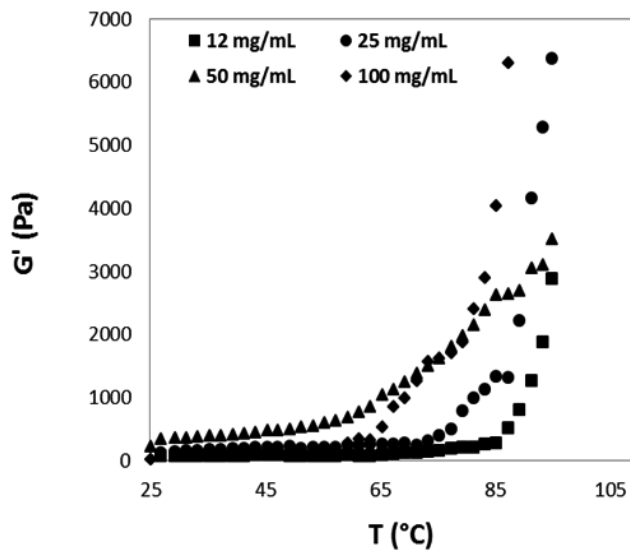


Figure 4.8. Temperature ramp tests: storage modulus, G' , as a function of temperature. BSA protein samples, suspended in sodium phosphate buffer (pH: 8) at different concentrations (12 mg/mL, 25 mg/mL, 50 mg/mL, 100 mg/mL) treated at a pressure of 500 MPa for 15 min.

Table 4.3. Effect of protein concentration (12, 25, 50 and 100 mg/mL) treated at 500 MPa for 15 min on phase transition temperature of BSA.

| C (mg/mL) | T (°C) |
|-----------|-------------|
| 12 | 83.9 ± 4.20 |
| 25 | 75.4 ± 3.77 |
| 50 | 59.7 ± 2.99 |
| 100 | 56.1 ± 2.81 |

4.3.5. Effect of HHP on allergen viscosity

The effect of HHP treatments on structural modifications of BSA was assessed by flow measurements in stationary regime. According to previous results (De Maria et al., 2013), HHP induced gelation of BSA occurs at pressure levels above 600 MPa and this threshold value is strictly related to the pH and concentration of the protein solution. Therefore, in this study HHP treatments were carried out on BSA samples with protein concentrations of 50 and 100 mg/mL at pressure levels above the threshold value, namely 600, 700 and 800 MPa applied for two treatment times of 15 and 25 min. The data obtained are reported in Figure 4.9 and 4.10.

According to the experimental results, HHP treated samples behave as shear thinning fluid, for which the apparent viscosity decreases with increasing the shear rate (Figure 4.9).

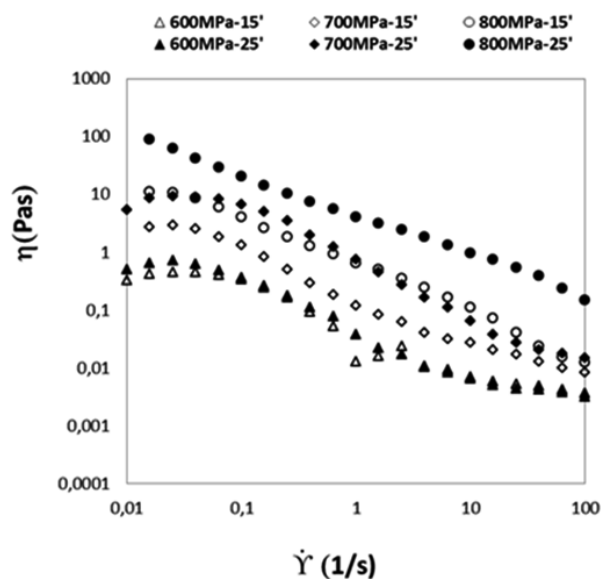


Figure 4.9. Viscosity of BSA samples (50 mg/mL in sodium phosphate buffer) processed under different pressures levels (600 MPa, 700 MPa, 800 MPa) and treatment times (15, 25 min), as a function of the shear rate.

This rheological behavior is typical of disperse materials and polymeric systems, which are characterized by tridimensional aggregates. At lower values of the shear rate, the Newtonian behavior (constant value of the viscosity, η_0 , namely the zero viscosity) observed is due to the Brownian motions of the molecules, which ensure their isotropic distribution, even though there are some initial effects of orientation to the flow. With an increase of the shear rate up to a certain critical point, the molecular orientation induced by the shear forces prevails on the casual orientation due to Brownian motions and, accordingly, the values of the viscosity drastically decrease until reaching a constant value, η_∞ .

According to the results shown in Figure 4.9, the extension of the Newtonian region depends on the applied pressure and treatment time, reducing with increasing the pressure level or prolonging the duration of the treatment. The samples treated at 600 MPa show a constant viscosity up to shear rates of 0.1 Hz. At higher pressure levels or longer processing times the Newtonian region is narrow, while, at a pressure level of 800 MPa and processing time of 25 min, the Newtonian region is not present any more and the viscosity decreases linearly with the shear rate.

Moreover, it can be observed that as the processing pressure increases, the values of the viscosity, measured with the flow tests, increases. The same trend was observed with processing time, the longer the time the higher the

viscosity measured. Therefore, analysing the results of the rheological characterization of processed samples, namely the shape of the curve and the values of the viscosity, it is possible to assess the effects of HHP treatment on protein structure. The effects of the HHP processing on protein rheology may be correlated to the modification of the molecular interactions, which determine the conformation of proteins-ionic and hydrophobic interactions, as well as of the hydrogen bonds (Mozhaev et al., 1996). The HHP treatments of proteins, generally, cause the rupture of the hydrogen bonds and the formation of stretch-conformation (Wu et al., 2009). Moreover, the changes of the conformational structure of the proteins and the networking of protein molecules affect the texture of the gel obtained (Kinsella and Whitehead, 1989). At higher pressure and longer holding time the irreversible unfolding and the consequent aggregation of the BSA protein are enhanced, this inducing a higher resistance to the shear stress and, consequently, an increase of the viscosity.

Figure 4.10 reports the apparent viscosity of BSA samples at a concentration of 100 mg/mL and treated with the same processing conditions (600-800 MPa, 15-25 min).

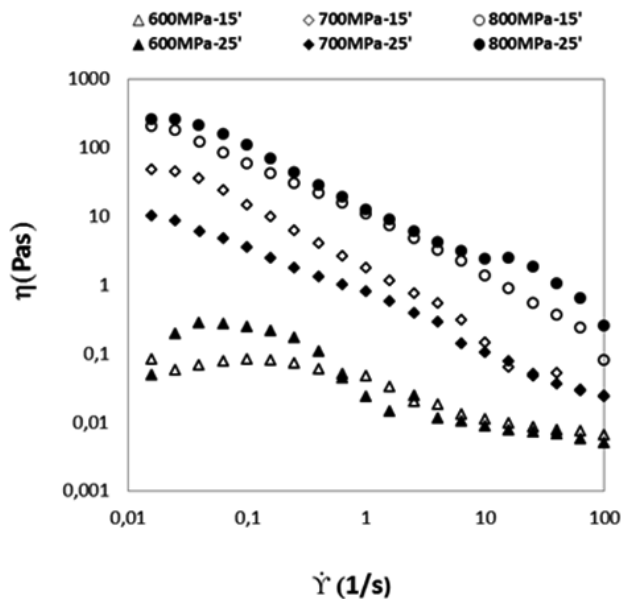


Figure 4.10. Viscosity of BSA samples (100 mg/mL in sodium phosphate buffer) processed under different pressures levels (600 MPa, 700 MPa, 800 MPa) and treatment times (15, 25 min).

In this case, significantly different values of the viscosity were measured. The zero viscosity range was wider with respect to the previous case, namely between 0.1 Pas for the samples processed at 600 and 1000 Pas for the samples processed at 800 MPa.

According to these results, the protein concentration emphasizes the effects of HHP processing parameters on the apparent viscosity. Indeed, with higher protein concentration protein aggregation is promoted and the interactions among the peptide of adjacent chains are more likely to occur.

These findings are in agreement with the experimental results reported by De Maria et al. (2013). The authors demonstrated that BSA protein gelation occurred at a pressure level above 600 MPa and the texture of the gels obtained is controlled by the protein concentration and pH.

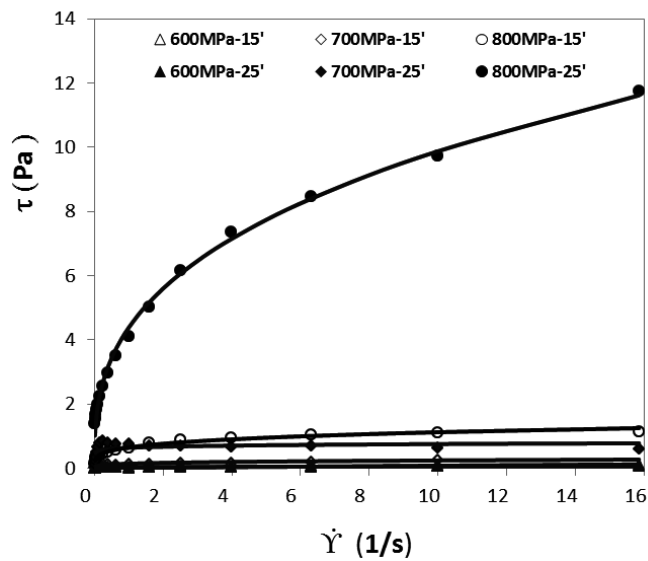
Similar conclusions were reported by Velèz-Ruiz (1998) for concentrated milk, Shibauchi et al. (1992) for casein, and Wu (2009) for soy-proteins. These latest authors investigated the effects of HHP treatments on WPI (Whey Protein Isolates) and observed that the HHP treatment caused the loss of the protein tertiary structure and the increase of its volume, due to the formation of high molecular weight aggregates and to swelling, typical of HHP treated globular proteins. As a consequence, the authors observed an increase of protein hydrodynamic radius and resistance to flow (in terms of viscosity). Similarly, Marjanović et al. (2011) applied HHP cycles (0.1-600 MPa) on β -lactoglobulin and noticed that the treatments with pressure below 100 MPa did not affect the viscosity of β -lactoglobulin solution, suggesting that structural changes of the protein did not occur. HHP treatments in the pressure range between 100-300 MPa, instead, promoted the dissociation of protein dimers into monomers, the penetration of water inside the protein structure and the exposure of hydrophobic zones to the aqueous solvent, causing an increase of the viscosity and hydration of β -lactoglobulin molecules. Moreover, the authors reported a partial unfolding only at pressure levels above 300 MPa, this inducing the formation of intermolecular disulfide bonds through sulphhydryl/disulfide interchange reactions, followed by the formation of β -lactoglobulin aggregates.

HHP treatments of proteins, generally, cause the rupture of the hydrogen bonds and the formation of stretch-conformation (Wu et al., 2009). Moreover, the changes of the conformational structure of the proteins and the networking of protein molecules affect the texture of the gel obtained (Kinsella and Whitehead, 1989). At higher pressure and longer holding time the irreversible unfolding and the consequent aggregation of the BSA protein are enhanced, this inducing a higher resistance to the shear stress and, consequently, an increase of the viscosity.

4.3.6. Modelling of the rheological data

The experimental data of the shear stress (τ) as a function of the shear rate ($\dot{\gamma}$) measured for the BSA samples with concentration between 50 and 100 mg/mL treated with HHP processes at pressure level of 600 to 800 MPa and processing time between 15 and 25 min were fitted with the *Ostwald de Waele* model equation (Eq. 1). As already described, this model assumes a power-law dependence of the shear stress on the shear rate. Figure 4.11 (a) and Figure 4.11 (B) report the model curves and the experimental data for the BSA samples with the two concentration of 50 mg/mL and 100 mg/mL, respectively, while in Table 4.4 and Table 4.5 the values of the model parameters, K and n , as well as the parameters of the statistical analysis, R^2 and the standard deviation (SD), for the two samples are listed.

A)



B)

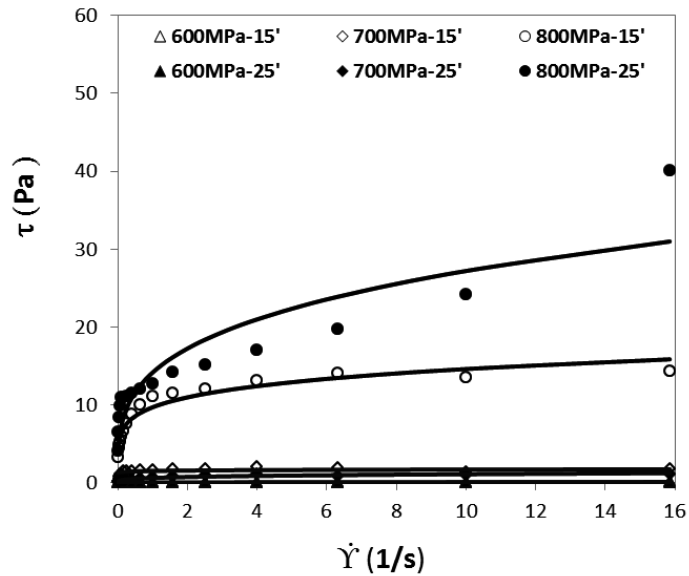


Figure 4.11. Rheograms of BSA samples (A: 50 mg/mL and B: 100 mg/mL in sodium phosphate buffer) processed under different pressures levels (600 MPa, 700 MPa, 800 MPa) and treatment times (15, 25 min). Points represent the experimental data and lines the fitting equations.

According to these estimated values, the consistency coefficient (K) of BSA samples increases with increasing pressure levels, treatment times and protein concentration. Thus, the model equation correctly describes the mechanism of action of the processing and product parameters, as already discussed in the previous sections.

According to the values of the parameter n , independently of processing conditions and protein concentration, BSA samples is clearly a non-Newtonian fluid, behaving as a pseudoplastic fluid. With more drastic processing conditions or higher protein concentration, the values of the parameter n change, this demonstrating its clear dependence on process and product variables.

Further studies are necessary to set up a model equation able to describe and account for the dependence of both model parameters on the pressure level, processing time as well as protein concentration. However, the *Ostwald de Waele* model equation utilized in this work is able to correctly represent the mechanism of action of the HHP and the role played by the variables analyzed.

Table 4.4. Values of the model parameters (K and n) and statistical parameters (correlation coefficient and standard deviation) estimated for BSA samples (50 mg/mL in sodium phosphate buffer) treated at different pressure levels (600 MPa, 700 MPa, 800 MPa) and treatment times (15, 25 min).

| Pressure/time | K | n | R ² | SD |
|-----------------------|------|------|----------------|------|
| 600 MPa-15 min | 0.01 | 0.83 | 0.86 | 0.01 |
| 700 MPa-15 min | 0.15 | 0.22 | 0.88 | 0.03 |
| 800 MPa-15 min | 0.66 | 0.24 | 0.99 | 0.05 |
| 600 MPa-25 min | 0.04 | 0.17 | 0.88 | 0.01 |
| 700 MPa-25 min | 0.65 | 0.07 | 0.72 | 0.19 |
| 800 MPa-25 min | 4.39 | 0.35 | 1.00 | 0.21 |

Table 4.5. Values of the model parameters (K and n) and statistical parameters (correlation coefficient and standard deviation) estimated for the BSA samples (100 mg/mL in sodium phosphate buffer) treated at different pressure levels (600 MPa, 700 MPa, 800 MPa) and treatment times (15, 25 min).

| Pressure/time | K | n | R ² | SD |
|-----------------------|-------|------|----------------|------|
| 600 MPa-15 min | 0.03 | 0.56 | 0.99 | 0.00 |
| 700 MPa-15 min | 1.55 | 0.05 | 0.82 | 0.30 |
| 800 MPa-15 min | 9.72 | 0.18 | 0.97 | 0.34 |
| 600 MPa-25 min | 0.04 | 0.39 | 0.92 | 0.01 |
| 700 MPa-25 min | 0.65 | 0.24 | 0.95 | 0.12 |
| 800 MPa-25 min | 14.19 | 0.28 | 0.92 | 0.51 |

4.4. Conclusions

High Hydrostatic Pressure is able to induce protein denaturation, aggregation and/or gelation of globular proteins like Bovine Serum Albumin (BSA). The critical transition determining the gelation phenomena is strictly dependant on protein characteristics such as concentration and pH.

At HHP processing conditions not causing the gelation of BSA protein, i.e. the pressure levels utilized are below the threshold value of 600 MPa, the treatment is able to induce microscopic changes of the protein structure, as demonstrated by the reduction of the denaturation temperature.

In the gelling conditions, the extent of protein gelation is dependent on processing conditions, namely holding time and applied pressure. At more drastical processing conditions, higher percentage of gelling protein can be obtained, as shown by both dynamic and stationary characterization.

In particular, dynamic regime tests revealed that the higher the applied pressure and holding time, the higher the gel compactness and strength. Similarly, viscosity changes induced by High Pressure revealed that the higher the pressure and holding times, the higher is the deviation of the gel from Newtonian behavior.

Reversible denaturation or protein unfolding are favoured at pressure level lower than critical value causing the gelation.

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Chapter V

Effect of High Hydrostatic Pressure on functional properties of allergens

5. Introduction

While in the previous chapter the effects of HHP on globular proteins gelation and aggregation were evaluated, in this chapter the effect of high pressure on allergen displacement were analyzed. It has been shown that 600 MPa represents a critical pressure in BSA gelation being the transition pressure between unfolding and gelation of the protein. To evaluate functional properties of allergens in unfolding conditions, lower pressure range was applied (100-600 MPa).

The functional properties of proteins, namely physicochemical properties (solubility, binding and surfactant properties, water and oil absorption capacity, emulsifying and foaming properties) influence food preparation, processing and storage, and contribute to food quality perception during consumption. The exposure of hydrophobic groups on protein surface controls the interactions with oils (emulsions), air (foam) or other proteins (gels and coagula) (Li-Chan & Nakai, 1989). The hydrophobic amino acids are usually buried inside the globular proteins but the unfolding of the native structure allows these hydrophobic groups to be involved in the intermolecular interactions (Kato et al., 1989).

Food processing can affect the functional properties leading to unfolding and aggregation of proteins, which depend on the technology used, the processing conditions applied and the type of product. The knowledge of the mechanism underlying the structural arrangement of protein molecules may be useful to predict and control protein functionality in food formulations (McClements, 1999). Consequently, the methods able to altering the functional properties of proteins are of interest to the food, chemical and pharmaceutical industries utilizing proteins as functional ingredients in their products.

HHP processes are able to modify the protein quaternary structure by destroying the hydrophobic interactions, the tertiary structure by reversible

unfolding and the secondary structure via irreversible unfolding (Tauscher, 1995). Protein unfolding induced by HHP treatments in the pressure range between 100 and 500 MPa allows the inaccessible SH groups to be exposed. Consequently, the number of the free sulphhydryl (SH) groups and disulphide (SS) bonds also undergo changes during HHP treatments. Under severe processing conditions (above 500-600 MPa, depending on the protein and the temperature applied), the number of free SH group, instead, decreases, probably due to the formation of disulphide bonds by oxidation, especially at alkaline pH where the thiolate anion is more reactive. For instance, the SH-SS interchange reactions induced by HHP treatments were observed for α -lactoglobulin by Funtenberg, (1995). The high degree of exposure of sulphhydryl groups, and the subsequent oxidation and sulphhydryl-disulphide bond exchange reactions result in insoluble and/or soluble aggregates and gel formation. Tang & Ma (2009), who investigated the occurrence of aggregates in soy protein isolate (SPI) treated by HHP processes at 200–600 MPa, observed the formation of combined insoluble (IA) and soluble (SA) aggregates. The relative ratio of IA and SA aggregates depended on the pressure levels. At pressure level above 200 MPa, more soluble aggregate were formed at the expense of insoluble aggregates (Tang & Ma, 2009). Soluble aggregates were also observed for β -conglycinin and glycinin treated at pressure levels between 300-600 MPa (Speroni et al. 2009). Similarly HHP promoted the aggregation and significantly increased the surface hydrophobicity of Rapeseed Protein Isolates (RPI) (Rong et al., 2013).

Moreover, the number of the disulphide bonds, determines the texture of the HHP induced gels, since the storage modulus, related to the elasticity of a solid, is proportional to the cross-linking density in the gel network (Qin et al., 2012).

Moreover, HHP treatments affect the proteins functional properties: the viscosity and the surface tension increased, as observed for egg white (Yang et al., 2009) and the foaming capability increased, as demonstrated for ovalbumin (Denda & Hayashi, 1992) and egg white (Knorr et al., 1992; Yang et al., 2009). Yang et al. (2009) observed a positive correlation between the egg white foaming ability of and the number of free SH groups. When the hydrophobicity of egg white was the lowest, the foaming capacity and foam stability of egg white reached the maximum value (Yang et al., 2009).

The changes in the functional properties as well as the variation of the conformational structure may be particularly relevant in case of allergens, which are mainly specific glycoproteins able to activate the reaction of the human immune systems (Gross, M. & Jaenicke, 1994; Penãs, et al., 2006; Shriver et al., 2010). The effect of process conditions, namely the pressure level and treatment times, on the conformational structure and the functional properties of a globular water soluble protein, Bovine Serum Albumin (BSA), with different concentration in buffer solution was analyzed. The

experimental data were worked out in order to define the role of the process parameters in the transition from reversible unfolding to irreversible unfolding/aggregation. The comprehension of this mechanism is very helpful in designing products with desired function properties.

5.1. Materials and methods

5.1.1. Preparation of the samples

BSA (Sigma-Aldrich, Italy) was dissolved at a temperature of 25 °C in Sodium Phosphate Buffer (50 mM, pH=8), according to the protocols reported by Penãs et al. (2006), at different concentrations (50 and 100 mg/mL) under a gentle mixing until a homogenous solution was obtained. The pH of the protein solution was measured with a pH-meter (S400 SevenExcellence, Mettler Toledo International Inc.). The protein solutions were stored under refrigerated conditions until HHP treatments.

5.1.2 Experimental plan

5.1.2.1 Experimental protocols

For the experimental campaign BSA samples (5 mL) were sealed in flexible pouches made of a multilayer film (Polyethylene-Aluminium-Polypropylene). The pouches were introduced into the U22 vessel and the pressure cycle set at the desired experimental conditions. All tests were carried out at ambient temperature (25 °C), while the operating pressure was varied in the range between 100 and 500 MPa, and the operating time between 15 and 25 min. At the end of the pressure release, the pouches were collected from the vessel, dried with blotting paper and stored at 4 °C before undergoing chemical characterization. All experiments were conducted in triplicate.

The determination of the SH free groups and of the foaming properties, and the FT-IR spectra were carried out on the unprocessed and processed samples according to the protocols reported in the following paragraphs.

5.1.2.2 Determination of free SH groups

Free sulphhydryl (SH) groups of the BSA samples were determined according to the protocols reported by Beveridge et al. (1974) and by Hardham (1981). Ellman's reagent was prepared by dissolving 4 mg of 5,5-dithio-bis 2-nitrobenzoic acid (DTNB) (Sigma-Aldrich, Italy) in 1 mL of Tris-Glycine buffer (0.1 M Tris-(hydroxymethyl)-aminomethane (Tris), 0.1 M glycine, and 4 mM ethylenediamine-tetraacetic acid disodium salt, pH=8.0; Sigma-Aldrich, Italy)). BSA (10 mg), dissolved in 5 mL of 8 M

urea in Tris–Glycine buffer, was added to the Ellman’s reagent (40 μL) and incubated for 30 min at room temperature (25 $^{\circ}\text{C}$). The absorbance was measured at 412 nm on a UV/VIS spectrophotometer (V-650, Jasco Europe Srl, Italy), using Tris–Glycine buffer as blank. Free sulphhydryl groups were evaluated according to the equation 1:

$$\text{SH}(\mu\text{M/g}) = 73.53 \cdot A_{412} \cdot D/C \quad (1)$$

where A_{412} is the absorbance at 412 nm, C the sample concentration in mg solid/mL and D the dilution factor. Results were expressed as mean value of three measurements.

5.1.2.3 Determination of FT-IR spectra

The secondary structure of the BSA samples was determined with a FT-IR spectrophotometer (FT-IR-4000, Jasco Europe Srl, Italy) at ambient temperature (25 $^{\circ}\text{C}$). BSA samples (50-100 mg/mL) were dissolved in Sodium Phosphate Buffer (pH= 8) and treated under HHP (pressure level=100-600 MPa; treatment time=15, 25 min).

For each test the transitions among different vibrational energetic levels were recorded in terms of absorbance spectra as a function of the measurement time.

The Fourier Transform was applied to the recorded spectra in order to observe the behavior of the absorbance values as a function of the frequency (wavelength factor, cm^{-1}). The second derivative and Fourier self-deconvolution were applied to the infrared spectra.

5.1.2.4 Determination of the foaming properties

The foaming properties of BSA solutions treated under HHP (P = 100-600 MPa; t=15, 25 min) were determined according to the protocol of Hammershoj and Larsen (1999) reported by Lomakina et al. (2006). A volume of 5 mL of the sample was placed in a glass beaker of 100 mL and stirred at 18000 rpm for 5 minutes at ambient temperature (25 $^{\circ}\text{C}$). The produced foam was gently transferred into a graduated glass cylinder (diameter = 2 cm, height = 16 cm, graduated volume = 25 mL) to determine the foam volume (V_f). The Foaming Capacity (FC) was calculated as the ratio between the measured foam volume (V_f) and the initial volume of the liquid solutions (V_0), according to the equation 2:

$$FC = V_f/V_0\% \quad (2)$$

The results were expressed as the mean value of three independent measurements.

5.1.2.5 Statistical analysis

The experiments were carried out in triplicate, with the standard deviations of the results being calculated and reported. Experimental data were statistically analyzed performing an analysis of variance (ANOVA). A $p < 0.05$ was used as the criterion value to determine significant differences within treatments.

5.2. Results and discussion

5.2.1 Effect of HHP treatments on free SH groups

In the native globular proteins, most of the free SH groups are masked to the attack by Ellman's reagents, due to their location in poorly accessible regions of the polypeptide chain. The application of a denaturing agent, such as temperature and/or pressure, modifies the protein conformational structure with the consequent unmasking and activation of SH groups, which can be detected according to the protocol of the Ellman's reaction (Beveridge et al., 1974; Hardham, 1981). Therefore the analysis of the free SH-groups may be considered as an index of protein unfolding and/or denaturation.

Accordingly, the concentration of free SH groups was estimated for the BSA samples (50 and 100 mg/mL) treated at pressure levels from 100 to 600 MPa for 15 and 25 min. Figure 5.1 shows the experimental data obtained. The native protein solutions show a concentration of SH group of 0.27 $\mu\text{mol SH/g}$ about.

The effect of HHP treatments on the concentration of free SH groups depends on the processing conditions (pressure level and holding time) as well as on the BSA concentration in the processed protein solutions.

BSA samples with a concentration of 50 mg/mL show a number of free thiol groups increasing with the pressure level up to a threshold value, which depends on treatment time and protein concentration in the solution. According to the experimental data plotted in Figure 5.1, the value of the threshold pressure is 400 MPa for a treatment time of 15 minutes and 300 MPa about for a treatment time of 30 minutes. The number of SH groups estimated for the samples treated at the same pressure level decreases with prolonging the processing times, which, similarly to pressure level, contribute to anticipate aggregation phenomena in protein solutions.

Above the defined value of the threshold pressure, the dependence of the number of free SH groups on pressure level follows an opposite trend. The experimental data demonstrate that, similarly to temperature, high hydrostatic pressure is able to induce protein unfolding, which allows the masked SH groups to be exposed and consequently detected. However, under pressure the unmasked SH groups are more reactive and above the threshold pressure level the decrease in total SH content could be due to the sulfhydryl-disulfide bond exchange reactions, which are especially promoted at alkaline pH. Similarly Qin et al. (2012) observed an increase of SH content in the pressure range between 300-400 MPa and a significant decrease in the pressure range of 400-500 MPa probably due to the formation of aggregates (Qin et al. 2012). Van der Plancken et al. (2005), who studied the effect of heating and high pressure treatments on egg white proteins, observed the exposure of buried SH groups and the formation of aggregates at pressure levels of 500-600 MPa with a consequent decrease in SH content due to the oxidation of the thiols and the formation of disulphide bonds.

The experimental data reported in Figure 5.1 and Figure 5.2 demonstrate the relevant role of protein concentration in determining the effect of HHP treatment on the conformational structure of BSA. As already observed for the BSA samples with protein concentrations of 50 mg/mL, the number of free SH groups is higher in the HHP treated samples than in the native protein solution. However, if the data of BSA samples with protein concentrations of 50 mg/mL and 100 mg/mL are compared, higher protein concentrations reduce the number of free SH groups detectable.

This result demonstrates a different mechanism determining the protein behavior under pressure rather than a reduced efficacy of the HHP treatment in unmasking the buried SH groups. The availability of free SH groups unmasked under pressure and of more contact points due the high protein concentration in solution enhance the protein-protein interaction and the formation of both short and long terms bindings between neighbouring polypeptide side chains (Cheftel et al., 1985). Accordingly, the samples with a concentration of 100 mg/mL show a lower number of free SH groups, due to the aggregation phenomena which already occur at pressure level of 300 MPa. In this case prolonged holding time do not have any significant effect on this parameter, while pressure levels above 300 MPa determine a slight increase of the number of free SH groups, thus demonstrating a progressive modification of the conformational structure of the pressure-induced aggregates.

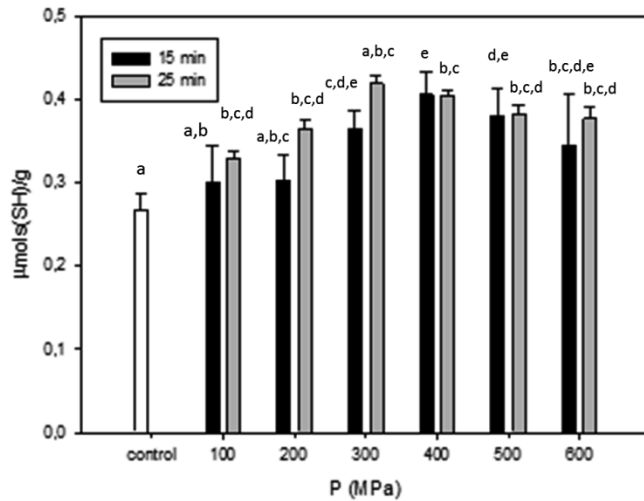


Figure 5.1. Free SH-groups in BSA samples processed in HHP treatments at different pressure levels (100-600 MPa) and processing times (15 min, 25 min). Protein concentration of 50 mg/mL.

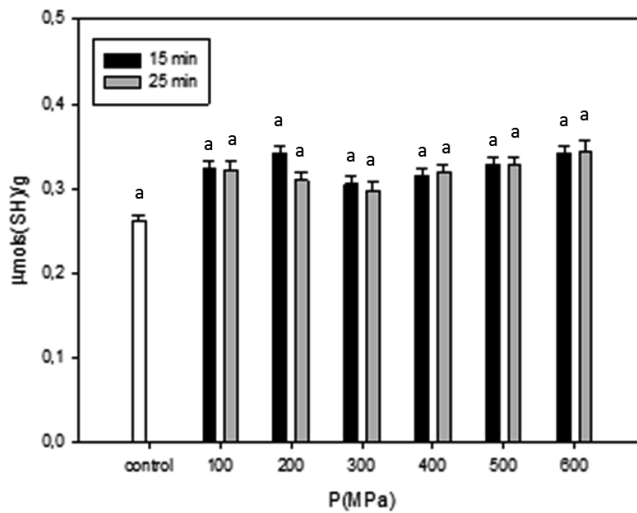


Figure 5.2. Free SH-groups in BSA samples processed in HHP treatments at different pressure levels (100-600 MPa) and processing times (15 min, 25 min). Protein concentration of 100 mg/mL.

5.2.2 Effect of HPP on secondary structure of BSA

5.2.2.1 FTIR spectrum of BSA at ambient temperature

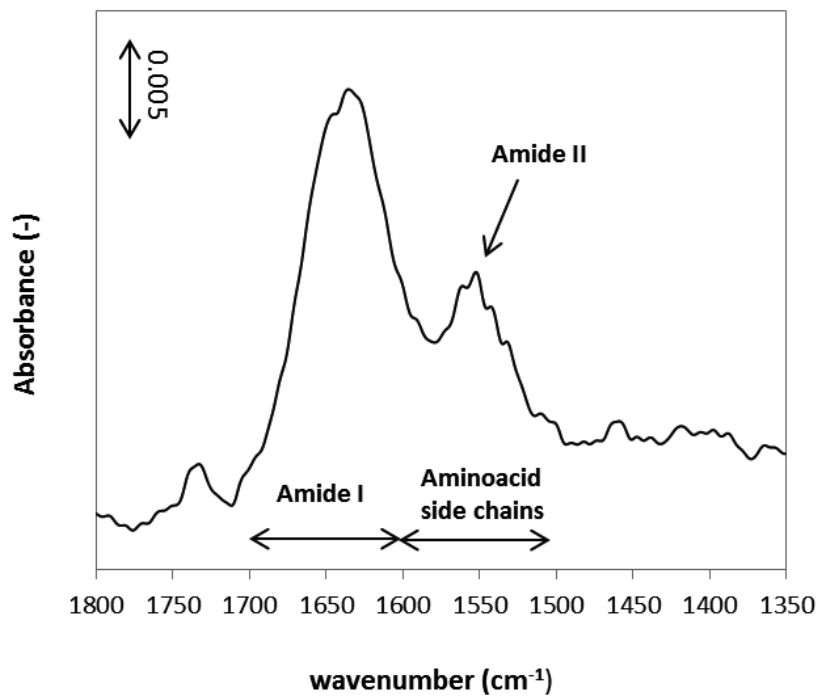
FT-IR spectroscopy, unlike X-ray crystallography and NMR spectroscopy, provides information about the secondary structure content of proteins in water solutions as well as in deuterated forms and dried states (Susi et al., 1986).

FT-IR spectrum of native BSA (50 mg/mL) in water solution at ambient temperature is shown in Figure 5.3A, in which the absorbance of IR radiation as a function of wavenumber (in cm^{-1}) is reported. The protein structural units are able to absorb specific wavelengths of radiation in the infrared region, giving rise to a characteristic set of nine absorption bands, namely Amide A, B and Amide I-VII. Among them the amide I and II represent the vibrational bands of the protein backbone (Krimm et al., 1986). In particular, the absorption associated with the Amide I band takes into account the stretching vibrations of the C=O bond (approximately 80%) of the amide groups, while the Amide II band leads primarily to bending vibrations of the N—H bond (40–60% of the potential energy) and CN stretching vibration (18–40%). Both the C=O and the N—H bonds are involved in the hydrogen bonding between the different units of secondary structure, and, accordingly, the locations of the Amide I and Amide II bands are sensitive to the secondary structure content of a protein. Generally the analysis of FTIR spectra is restricted to the region of the Amide I bands, because, according to several studies reported in the literature, Amide II band is not as good a predictor for quantifying the secondary structure of proteins (Fabian et al. 2002; Kong & Yu, 2007; Krimm et al., 1986; Murayama and Tomida, 2004; Surewicz & Mantsch, 1988). According to the FT-IR spectrum shown in Figure 5.3A, the Amide I region is located in the range between 1700 and 1600 cm^{-1} in agreement with the data reported in the literature (Kong & Yu, 2007). The database, available in the literature and containing the association between FT-IR peaks and secondary structure types, allows the secondary structure of BSA to be defined through the analysis of the FT-IR spectra of native protein and HHP treated samples. In order to narrow and separate the intrinsically broad bands of the BSA spectrum, the second derivative analysis was selected among the mathematical data analysis methods suitable to enhance the resolution of the FT-IR spectrum.

The second derivative spectrum of BSA (50 mg/mL) at ambient pressure, shown in Figure 5.3B, is dominated by four peaks at 1654, 1674, 1630, and 1610 cm^{-1} in the Amide I region, in agreement with the studies of Murayama and Tomida (2004). The structure of BSA protein is composed of 76% helix, 10% turns and 23% extended chain and no beta (β) sheets (Fabian et al. 2002). The peak at 1654 cm^{-1} corresponds to α -helical structures while the peak at 1674 cm^{-1} is associated with turn structures. Furthermore the band at

1630 cm^{-1} is assigned to short-segment chains connecting to α -helix segments of BSA and the one at 1610 cm^{-1} is due to the vibrations of glutamyl (Gln) residues and the aromatic side chain, as already observed by Fabian et al. (2002).

A



B

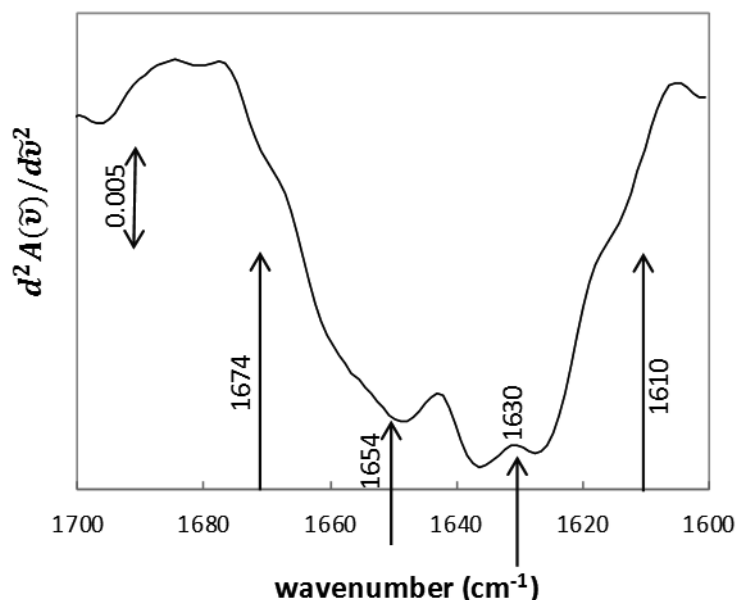


Figure 5.3. FT-IR Spectra of BSA (50 mg/mL) native protein at 25 °C. A: FT-IR spectrum; B: Second derivative spectrum of the Amide I.

5.2.2.2 FT-IR spectra of HHP treated BSA

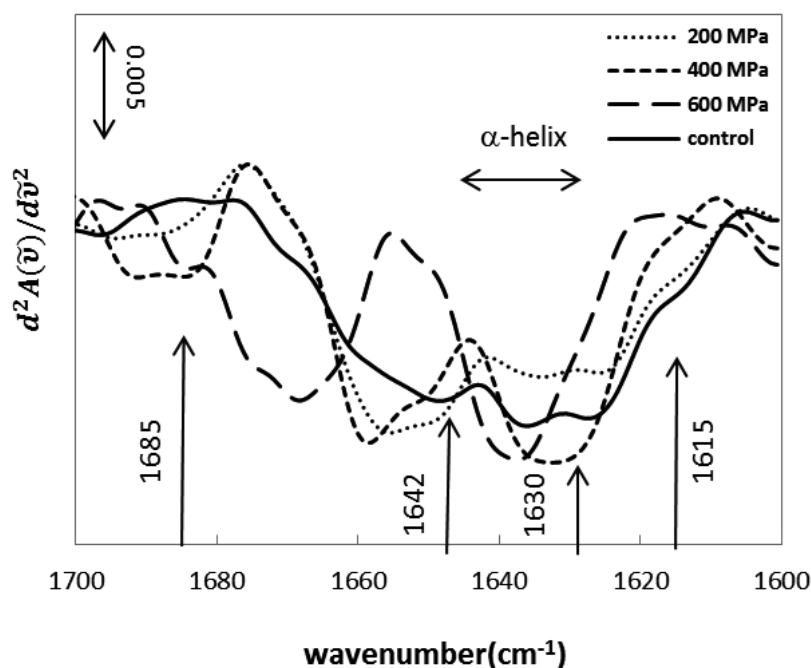
Figure 5.4 (A) and (B) and Figure 5.5 (A) and (B) show the second derivative of the FT-IR spectra of untreated BSA samples (50 and 100 mg/mL respectively) and treated at different pressure levels (100-600 MPa) and treatment times (15-25 min) in the region of the Amide I (1600-1700 cm^{-1}). As already observed for the samples of the native BSA, the second derivative analysis clearly resolves the peaks of FT-IR spectra, which can be associated to the secondary structure types, namely α -helix, β -sheets, random coils and turns.

The second derivative spectrum of BSA samples (50 mg/mL) treated at high pressure (200-600 MPa) for 15 minutes (Figure 5.4A) changes with the pressure level applied and the holding time under pressure. BSA samples treated at 200 and 400 MPa show two higher and broader peaks at 1645 and 1630 cm^{-1} , thus clearly indicating that high pressure treatments are able to modify the secondary structure. In particular, in the case of protein in water solution, the band between 1642 and 1624 cm^{-1} are assigned to α -helix structure. At higher pressure levels, the changes in the secondary structure of

BSA become more relevant. Accordingly, the spectrum of BSA treated at 600 MPa for 15 minutes is characterized by a new peak at 1685 cm^{-1} , which can be associated with the formation of intermolecular β -sheet aggregates.

Prolonged treatment times (25 min) enhance the effect of pressure, thus determining higher differences among the spectra of the samples treated at different pressure level (200, 400 and 600 MPa), as shown in Figure 5.4B. Furthermore, a more evident occurrence of the peak at 1685 cm^{-1} is observed together with the occurrence of a new peak at 1615 cm^{-1} associated with the formation of β -sheet intermolecular aggregates (Lefevre et al., 2000). In particular, the 1685 cm^{-1} band correspond to antiparallel β -sheet while the 1615 cm^{-1} band is due to the intermolecular β -sheets resulting from the formation of molecular aggregates.

A



B

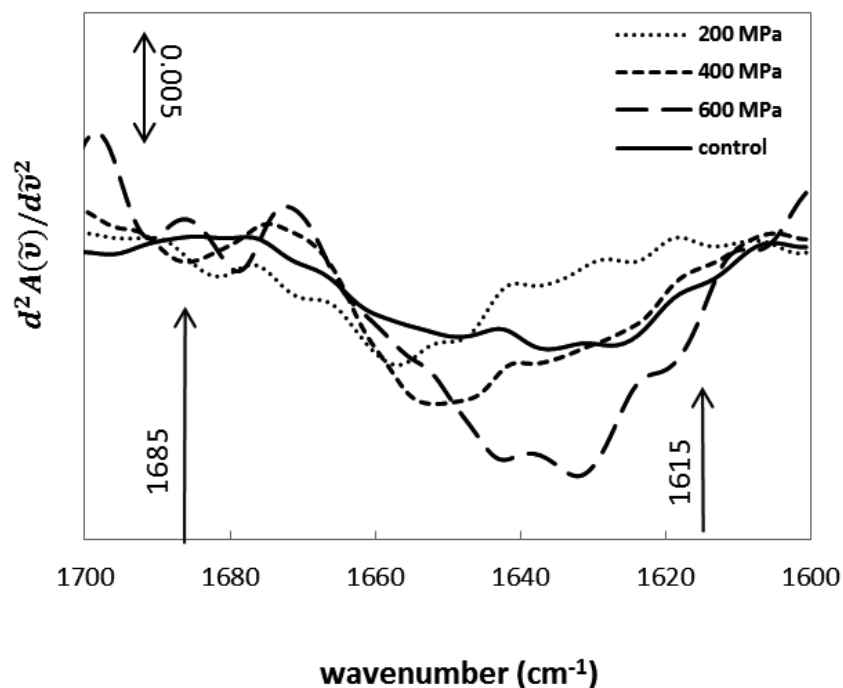


Figure 5.4. FT-IR Spectrum of BSA (100 mg/mL) samples processed in HHP treatments at different pressure levels (100-600 MPa). **A:** treatment time=15 min; **B:** treatment time=25 min.

In more concentrated samples (Figure 5.5 A and B), the spectra result more amplified in the region between 1660-1630 cm^{-1} and a new peak at 1654 cm^{-1} associated with α -helix structure is formed. At longer treatment times (Figure 4 B) the α -helix structures (1645 cm^{-1}) unfold into the disordered structure and turns, as demonstrated by the peak at 1654 cm^{-1} . Also in this set of experiments, prolonged processing times enhance the extent of molecular aggregation in protein secondary structure.

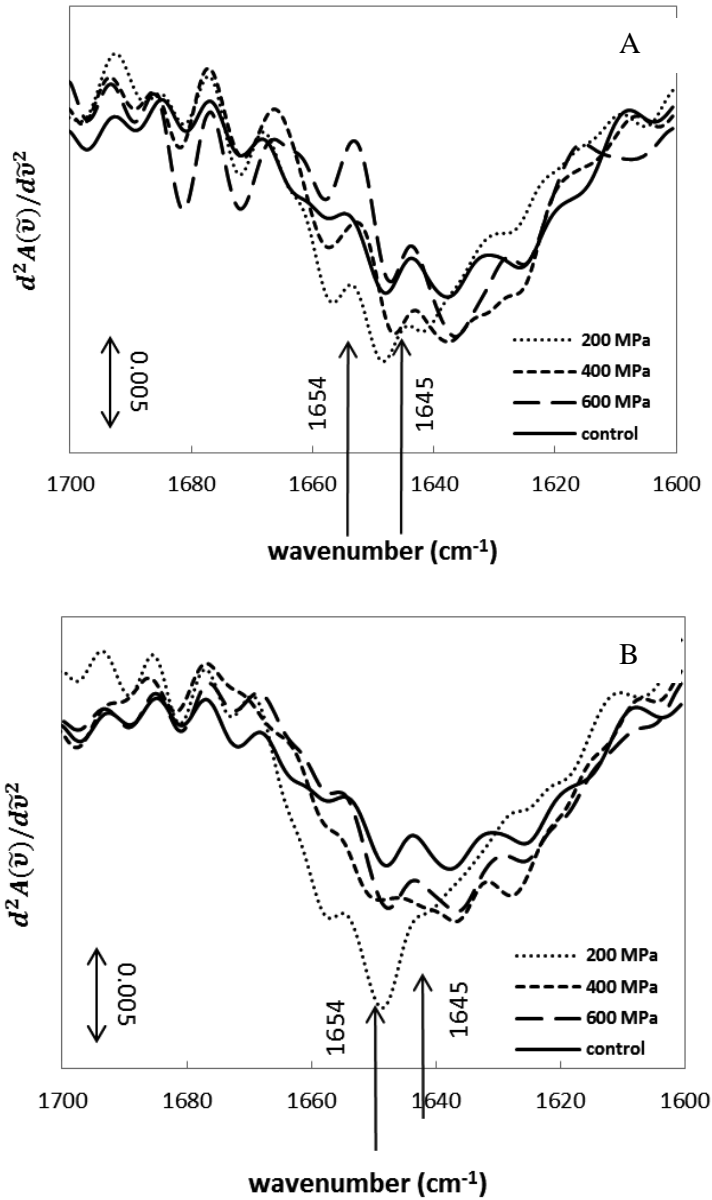


Figure 5.5. FT-IR Spectrum of BSA (100 mg/mL) samples processed in HHP treatments at different pressure levels (100-600 MPa). A: treatment time=15 min; B: treatment time=25 min.

5.2.2.3. Foaming properties

Foaming capacity (FC %) of BSA samples with different concentration (50-100 mg/mL) processed in high pressure treatments (100-600 MPa, 15-25 min) was evaluated and compared to the foaming ability of the untreated samples. The experimental data are shown in Figure 5.6A and 5.6B for the BSA sample with the concentration of 50 mg/mL and 100 mg/mL, respectively.

According to the data shown in Figure 5.6A, HHP treatment is able to enhance the foaming capacity of BSA, independently of the processing conditions. The values of this functional properties increases with increasing the pressure level in the range between 100 and 400 MPa, while a decreasing trend is detected at pressure level above 400 MPa. The dependence of the foaming ability on the pressure level, therefore, follows the same trend already observed for the free SH groups, thus confirming BSA partial unfolding in the pressure range between 100 and 400 MPa and the progressive loss of the tertiary structure and the consequent polymerization of BSA protein in dimers, trimers and higher oligomers held together by disulfide bonds.

The foaming properties, in fact, are mainly related to the protein capability to form film at the air-water interface. The interfacial behavior of the protein, therefore, is strictly controlled by the electrostatic and hydrophobic interactions as well as by the stability of the disulphide bonds, which stabilize its secondary and tertiary structures. High pressure disrupts hydrophobic interactions and ionic bonds resulting in protein molecules being more suitable to adsorb air bubbles at air-water interface (Dickinson, 1989). This effect is enhanced for the albumin proteins, which are amphiphilic molecules and are easy to extend on the water-air interface (Li & Zhang, 2005; Yang et al., 2009). Similar results are shown by Yang et al. (2009), who observed an increased foaming capability and stability of egg white processed at pressure levels of 100-400 MPa. The authors explained that HHP treatment reduced the hydrophobicity and solubility of the protein and observed a positive correlation between the foaming capability and the surface sulphhydryl content. The egg white samples exhibited greater foaming properties after a HHP treatment at 350 MPa for 10 min.

The reduced foaming capacity observed for the protein samples treated with pressure level above 400 MPa may be related to the irreversible unfolding, the SH-SS interchange reactions and the formation of aggregates. This hypothesis is confirmed by the results reported by Galaska et al. (1997), who observed a reduction in surface hydrophobicity of pure BSA in solution (pH=7) processed with pressure level between 400 and 800 MPa. The authors attributed the loss of surface hydrophobicity to the partial unfolding and the intermolecular association of BSA protein after pressure release.

Moreover, as the increase of the pressure levels enhances the formation of aggregates the foaming ability of the samples decreases.

Similarly, Krešić et al. (2006) observed that high-pressure treatments (300 MPa and 600 MPa, for 5 and 10 min) significantly improved the foaming capacity of WPI and WPC due to the protein unfolding. A decrease of the foaming volume (FV) was detected if processing pressure was higher than 600 MPa because of the loss in protein solubility occurring at very high pressures. A significant reduction of the solubility of whey proteins after pressurization was also observed by Pittia et al. (1996). Van der Plancken et al. (2006) investigated the effect of a combined process of high pressure (400-700 MPa) and heating (50-85 °C) on foaming properties of egg white solutions. The authors observed an increase in foaming ability of those samples with high extent of unfolding and residual solubility with the best stability and volume foam reached at pH 8.8 above 500 MPa. The authors reported the association between foaming ability and protein flexibility/solubility and noticed an increase in foaming ability at higher exposed SH content (Van der Plancken et al., 2006).

If the effect of the processing time is considered, foaming ability was clearly higher in samples treated for a shorter processing time (15 min). Prolonged processing times (25 min) promotes a larger extent of aggregation which prevent the formation of protein foams (Figure 5.6 A and B). A similar mechanism can explain the effect of protein concentration in the processed samples. If the results shown in Figure 5.6A and 5.6B are compared, the foaming ability decreases with the protein concentration in the processed samples. Protein concentration in solution controls the extent of protein-protein interaction, this allowing the formation of small aggregates at lower pressure level.

Effect of HHP on functional properties of allergens

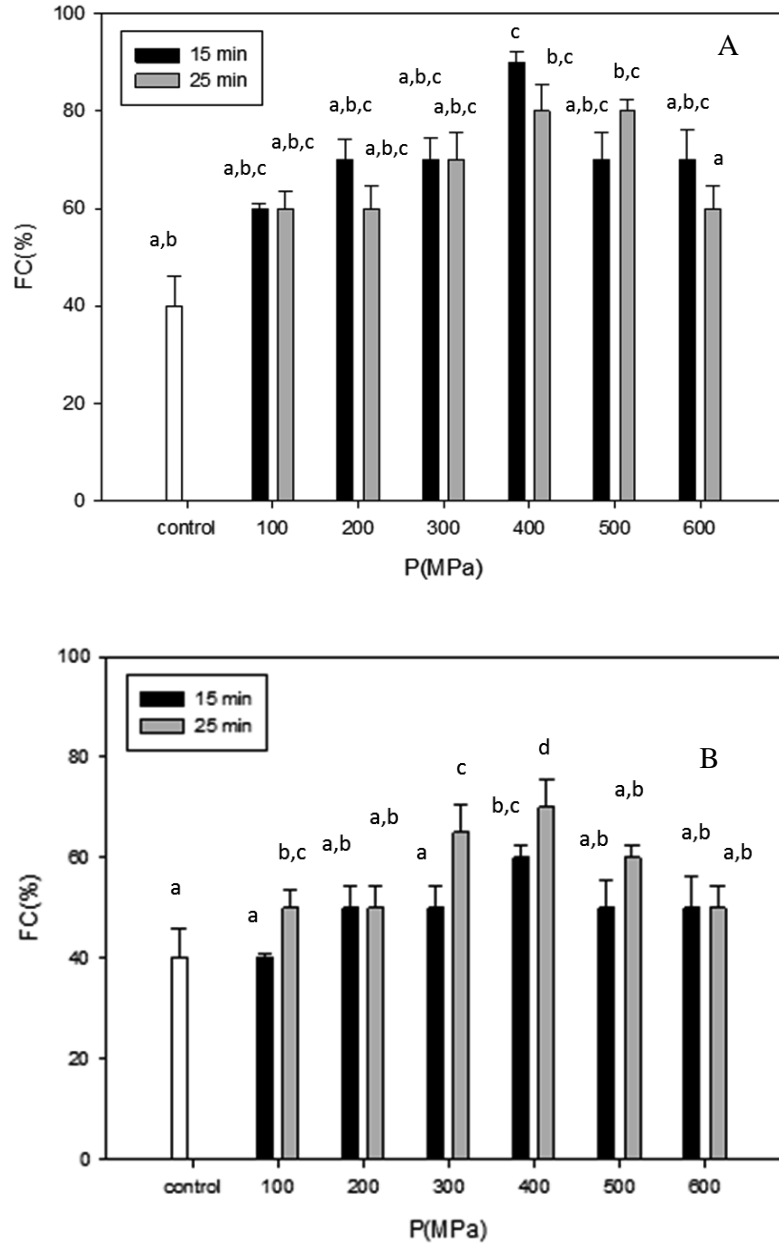


Figure 5.6. Foaming ability of BSA samples processed in HHP treatments at different pressure levels (100-600 MPa) and processing times (15 min, 25 min). A: protein concentration of 50 mg/mL; B: protein concentration of 100 mg/mL.

5.3. Conclusions

High Hydrostatic Pressure resulted in protein unfolding and/or aggregation of proteins showing the potential to induce changes in BSA structure as well as in its functional properties. Protein unfolding occurs in the pressure range, which depends on protein concentration and treatments time. In the unfolded state, BSA proteins show improved foaming properties, expose a higher number of SH free groups and are characterized by negligible modifications of the secondary structure. Pressure levels above the threshold valued, prolonged processing times and/or high protein concentration allow the polymerization of BSA protein in dimers, trimers and higher oligomers held together by disulfide bonds, as demonstrated by the reduction of foaming properties and the significant changes in secondary structure detected.

The experimental results demonstrated so far that the effect of HHP treatments on protein structure may be predicted through a proper selection of both protein concentration and process conditions. The availability and reactivity of free SH groups represent the key factor controlling the mechanism of HHP action on proteins and determining the functional properties as well as the stability of the proteins in solution.

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Chapter VI

Effects of High Hydrostatic Pressure on allergen proteolysis

6. Introduction

Protein peptides have lower allergenic power and also higher digestibility than the native protein, and therefore they are used as ingredients of several products designed for special diets, such as dietetic or hypoallergenic foods but also geriatric products, high energy supplements and many clinical products used to cure cancer, trauma, encephalopathy and burns (Fisher et al., 1976; Adachi et al., 1991, Frokjaer et al., 1994). Furthermore the hypoallergenic foodstuffs match the need of people who are not able to digest the proteins because of some specific disorders of digestion, absorption or amino acid metabolism diseases (Milla et al., 1983).

The most popular methodology applied for the production of hypoallergenic hydrolysates is the enzymatic hydrolysis which is the most convenient process not only from an economical point of view but also because it is able to produce good quality products (Clemente et al., 1999).

Usually the enzymatic treatment is applied together with an additional treatment with the scope to enhance the hydrolysis degree reached during enzymatic reaction by inducing protein modification thus facilitating the enzymatic attack.

Similarly to heating, non-thermal technologies are able to modify the conformational structure of the proteins causing their denaturation or aggregation/gelation as a function of both product (pH, ionic strength, protein concentration) and process parameters.

As shown in the previous chapter, HHP processes are able to induce, in certain conditions of protein concentration and pH as well as at specific operating conditions, the displacement of the protein. This effect of HHP on allergen structure suggests that this process may be used to modify protein resistance to the enzymatic attack, thus promoting protein hydrolysis.

In this chapter the effects of High Hydrostatic Pressure on the enzymatic hydrolysis of two different allergens, BSA and caseins, was described. The hydrolysis degree achieved in assisted hydrolysis or hydrolysis induced by high hydrostatic pressure was evaluated for both proteins by using two different proteolytic enzymes (chymotrypsin and trypsin).

In addition a complete characterization of the high pressure treated peptides was made in terms of structure (FT-IR, HPLC peptides profiles) and dimensions (Particle Size Distribution by Light scattering analysis and Molecular weight Distribution by SDS-Page Electrophoresis).

6.1. Materials and methods

6.1.1. Preparation of the samples

Bovine serum albumin (BSA, from Sigma-Aldrich, Italy) samples were prepared by dissolving protein in a 50 mM Sodium Phosphate Buffer at a concentration of 5 mg/mL under gentle mixing until complete solubilisation at 25 °C.

Similarly casein (from Sigma-Aldrich, Italy) samples were prepared by dissolving protein in the same buffer at a concentration of 10 mg/mL under gentle mixing until complete solubilisation at 25 °C.

Buffer solution was kept at pH=8 with a laboratory pH-meter which is the pH value corresponding to the maximum enzyme activity. Two different enzymes, chymotrypsin and trypsin were added to protein solution just before treatment with a concentration corresponding to an enzyme/substrate ratio equal to 1/20 for BSA solution and 1/10 for caseins.

6.1.2. Experimental protocol

Protein samples (5 mL) were sealed in bags of flexible multilayer film (Polyethylene-Aluminium-Polypropylene). All the experiments were carried out at 37 °C and a pressure level in the range between 100 and 500 MPa was applied for several holding times between 5 and 30 minutes. At the end of the treatment, the sample pouches were stored at 4 °C and enzyme inactivation was conducted by heating the samples at 95 °C for 5 minutes.

Treated samples were stored at 4°C before undergoing the physicochemical characterization in terms of hydrolysis degree, SDS-Page Pattern and FT-IR.

6.1.3. Hydrolysis degree by OPA reaction

Hydrolysis degree was measured in terms of primary amino groups which are formed as the result of hydrolysis reaction between protein and enzymes. As reported by Nielsen et al. (2001), a spectrophotometric method, was used to determine the hydrolysis degree.

The reaction among the OPA reagent, primary amino and SH groups forms a compound absorbing at 340 nm. OPA reagent is composed by Na-tetraborate decahydrate, Na-dodecyl-sulfate (SDS), o-phthaldialdehyde 97% (OPA) and dithiothreitol 99% (DTT) in deionized water solution. Blank,

standard and sample were measured by the addition of 3 mL of OPA reagent with 400 μ L of blank, standard and sample. The serine standard was prepared as follows: 50 mg serine was diluted in 500 mL deionized water (0.9516 meqv/L). All the measurements were performed in triplicate and carried out at 25°C using deionized water as a reference after 2 minutes of reaction.

6.1.4.SDS-PAGE profiles of hydrolysates

HHP treated and non-treated peptides produced together with the control constituted by not hydrolyzed BSA were lyophilized and dissolved (with a concentration of 25 μ g/mL) in a sample buffer containing Tris-Cl pH 6.8 0.125 M, SDS 2%, glycerol 10%, Blue of Bromophenol 0.02% and β -mercaptoetanol 5% as reducing agent. Samples were heated at 95°C for 5 minutes before being loaded in polyacrilammide gels composed by a separating and a stacking gel. Analysis of the images was conducted after fixing, staining and detaining phases.

6.1.5.FT-IR spectra of hydrolysates

Differences in BSA hydrolysates at ambient pressure and produced under pressure were determined by means of Fourier Transform-infrared spectrophotometer (Model FT/IR-4000, Jasco) at ambient temperature. BSA samples (5 mg/mL) in enzymatic solutions of chymotrypsin and trypsin (E/S = 1/20) were dissolved in Sodium Phosphate Buffer and deposed in the form of a droplet on the diamond surface of the FT-IR spectrophotometer. The transitions between vibrational energetic levels occurring were recorded in terms of absorbance spectra in the dominium of the time. The application of Fourier Transform algorithm was used to convert the time dominium spectra into frequency (wavenumber, cm^{-1}) dominium ones. Finally the results of the FTIR analysis of HPP treated and non-treated BSA hydrolysates were expressed in terms of 2nd derivative and deconvolved spectra.

6.1.6.Particle size distribution by Light Dynamic Scattering (LDS)

Particle Size Distribution (PSD) of casein samples processed by HHP assisted hydrolysis in a pressure range between 100 and 400 MPa for 25 min (the holding time leading to the highest hydrolysis degree) was evaluated by means of photon correlation spectroscopy (PCS) at 25 °C (HPPS, Malvern Instruments, UK).

For each measurement, the samples were diluted with bidistilled water to a suitable concentration. The mean droplet size (Z-diameter) was evaluated by

Cumulant analysis of the intensity-intensity autocorrelation function, $G(q,t)$ (Stepanek, 1993). Polydispersity Index (PI) was used to define the extent of particle size uniformity in the samples.

6.1.7.HPLC peptides profiles analysis

Casein peptides samples were also analyzed by reverse-phase high performance liquid chromatography (RP-HPLC). The HPLC system consists of a Consta metric 4100 pump, Spectra System AS 1000 auto sampler and Spectrophotomonitor 5000 diode array detector (Thermo Separation Product, Riviera Beach, FL, USA) fitted with a Purospher Star column (5 mm particle size, 259x4.0 mm; Merck, Darmstadt, Germany).

Operative conditions were the following: column temperature, 25 °C; flow rate: 0.8 mL/min⁻¹; solvent A: 0.1% (v/v) trifluoroacetic acid (TFA) (sequential grade, Sigma, St Louis, MO, USA) in aceto nitrile in Milli-Q water (1:9); solvent B: 0.07% (v/v) trifluoroacetic in acetonitrile-MilliQ (9:1). Elution was made by applying 100% A for 5 min and to 70% B over the next 5 min. Absorbance was recorded at 214 nm. Samples were diluted in water at a ratio of 1:1 (v/v).

6.2.Results and discussion 1th study case: BSA

6.2.1.Hydrolysis degree of Bovine Serum Albumin

The values of the Hydrolysis Degree (HD) of BSA samples processed by HPP assisted hydrolysis by chymotrypsin and trypsin are reported in Figure 6.1 and Figure 6.2 and are expressed as the concentration of primary ammino groups (which represents the typical product formed during the hydrolysis reaction) formed per unit of grams of protein. Results show that there is no significance difference in hydrolysis degree between samples hydrolysates at different times at ambient pressure which indicates that hydrolysis reaction is slow in the first 25 minutes in *non-treated samples*.

On the contrary, when the reaction is carried out *under pressure* (applying a pressure level between 100-500 MPa), the reaction rate of hydrolysis increases and relevant differences between control and treated samples can be observed already after 5 minutes of hydrolysis. In fact when proteolysis takes place under pressure, it results to be strongly enhanced reaching a maximum value after a treatment at 400 MPa for 25 minutes. Chymotrypsin (Figure 6.1) shows to be more efficient in HHP assisted hydrolysis than trypsin (Figure 6.2).

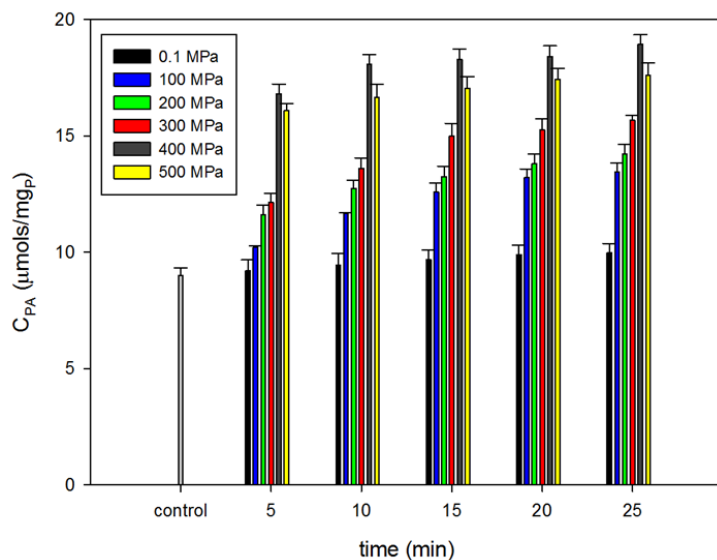


Figure 6.1. Hydrolysis degree (primary amino groups) of untreated Bovine Serum Albumin (37°C) and treated by high pressure (100, 200, 300, 400 and 500 MPa) for 0, 5, 10, 15, 20, 25 min with chymotrypsin at 37°C.

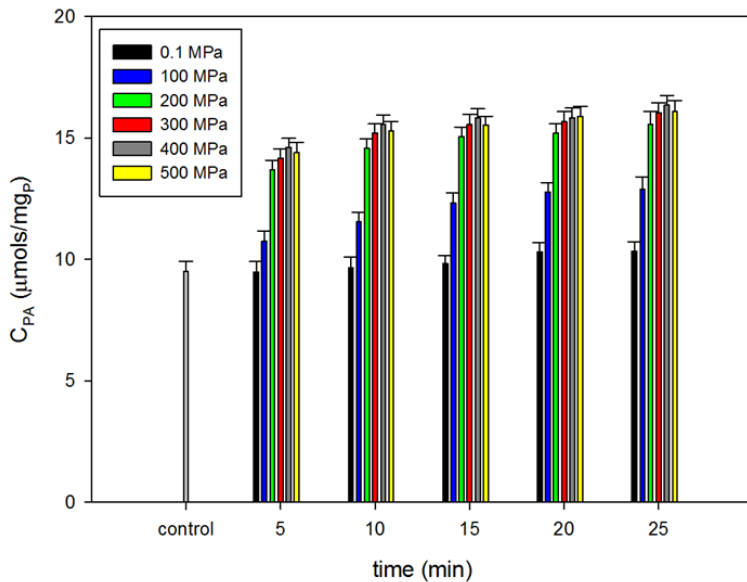


Figure 6.2. Hydrolysis degree (primary amino groups) of untreated Bovine Serum Albumin (37°C) and treated by high pressure (100, 200, 300, 400 and 500 MPa) for 0, 5, 10, 15, 20, 25 min with trypsin at 37°C.

This synergic effect of High Hydrostatic Pressure and enzymatic reactions can be explained by the higher exposure of the peptide bonds to the proteolytic attack due to protein unfolding. As already reported in the literature proteins may be more easily hydrolysed in their denatured form than in their native one (Fukushima, 1969 and Dumay, Kalichevsky and Cheftel, 1994).

Thus, the modifications induced by High Hydrostatic Pressure in proteins can be efficiently used in combination with the reaction of protein hydrolysis by the aim to increase the rate of enzymatic reactions. In fact when the hydrolysis is carried out under high pressure processing or by applying HHP as a pre-treatment before hydrolysis, the values of the hydrolysis degree increase as shown by several authors for whey proteins (López-Expósito et al., 2008), dairy whey proteins (Peñas et al., 2006) treated in a pressure range between 100-300 MPa with proteolytic enzymes.

Similar results were obtained by Peñas et al. (2006) who studied the effect of HPP processes (100-300 MPa for 15 min) before the hydrolysis of dairy whey proteins by three different enzymes: chymotrypsin, trypsin and pepsin. The authors observed that HPP process improved the degree of hydrolysis and chymotrypsin, and trypsin showed the highest proteolytic activity at 100 and 200 MPa, followed by pepsin at 300 MPa. Kleber et al. (2004) and Svenning et al. (2000) confirmed that unfolded β -lactoglobulin was more digestible and more susceptible to enzymatic hydrolysis. Similarly, Vilela et al. (2006) observed that the HPP process could enhance in vitro enzymatic digestion of whey proteins by pepsin and pancreatic. They observed that native proteins were more resistant to pepsin: after 30 min of hydrolysis a decrease of 30.9% in protein concentration occurred while a reduction of 68% in proteins was observed if hydrolysis was carried out under high pressure (single cycle of 500 MPa or triple cycles of 400 MPa).

Chicón et al. (2008) treated WPI with porcine pepsin under high pressure (200 and 400 MPa) and they observed a higher digestibility of high pressure treated proteins than non-treated ones. An increase in hydrolysis degree between 10% and 30% was also observed by Alëman et al. (2011) on fish skin gelatin hydrolyzed with alcalase, collagenase, trypsin and pepsin both at atmospheric pressure and under high pressure (100-300 MPa) as well as by Zhang et al. (2012) on chickpea protein isolates.

The advantage in combining the two processes is not only to be searched in the improvement of hydrolysis degree but also in the effect on allergen immunoresponse (Bonomi et al., 2003). Bonomi et al. (2003) carried out similar experiments producing β -lactoglobulin hydrolysates with trypsin and chymotrypsin under pressure (600 MPa, 10 min) at different temperatures (30, 37, 44 °C). The authors observed that the value of the hydrolysis degree was higher under high pressure than at atmospherical pressure. The

conformational changes of allergens induced by thermal as well as by non-thermal technologies allow digestibility of the allergen to be increased. However the immunoresponse is not affected because no modifications in proteins primary structure can be obtained. In fact many epitopes, which are the part of the allergen reacting against an antibody produced by immunosystem, are not conformational but linear. Therefore the changes of the conformational structure induced by HHP are not able to guarantee a reduction in allergenicity, which can be affected at very high pressures levels and long holding time (Hereldova et al., 2009; Husband et al., 2011; Kleber et al. 2007).

On the other side, at pressures level of 500 MPa, HHP treatments cause protein aggregation. Irreversible denaturation promotes the formation of molecular aggregates, which can reduce the accessibility of the enzymes to the peptide bonds Funtenberger, Dumay, and Cheftel, 1995). In addition a reduction of enzymatic activity can occur, reducing the efficiency of the proteolysis.

In conclusion, the combination of High Hydrostatic Pressure with enzymatic hydrolysis can be very useful to produce hypoallergenic peptides with novel characteristics and properties.

6.2.2.SDS-PAGE profiles of Bovine Serum Albumin hydrolysates

In Figure 6.3 and Figure 6.4 the SDS Page Profiles of untreated (0.1 MPa) and treated (100-500 MPa for 15-25 min) BSA hydrolysates produced by chymotrypsin and trypsin are reported. The pattern of native BSA and two markers (a mix of proteins and myoglobin) were included in order to define the molecular weight range in which the samples are located. Native BSA shows a major band at 66 kDa with very few other bands at lower molecular weight. On the contrary, the profiles of HHP treated samples are characterized by different bands corresponding to peptides with low molecular weight.

In particular samples treated at 100 and 300 MPa show very similar profile with bands at molecular weights (MW) ranging between 45 and 66 kDa, and lower than 17 kDa. BSA hydrolysates produced at 200 MPa show more peptides with high molecular weight (45-66 kDa) and less peptides with low molecular weight (<17 kDa). Hydrolysates produced at 400 MPa show also the presence of very short peptides (additional band at molecular weight lower than 17 kDa).

Finally, in samples treated at higher pressures (500 MPa), many bands corresponding to the lowest molecular weight peptides disappear maybe because at this very high pressures, protein aggregation occurs n. Furthermore, the inactivation of the enzyme occurring at very high pressure can reduce the efficiency of hydrolysis assisted by high hydrostatic pressure.

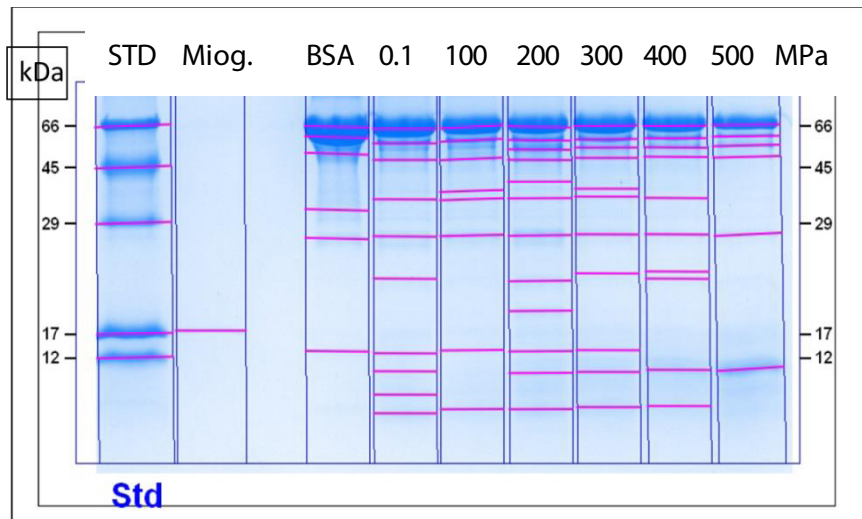


Figure 6.3. SDS Page of hydrolysates of BSA (00) by chymotrypsin, non-treated (A0) and treated by HHP by high pressure: 100 (A1), 200 (A2), 300 (A3), 400 (A4) and 500 (A5) MPa for 25 min at 37°C.

Similar results can be observed in the SDS-Page Patterns of BSA hydrolysates produced by high pressure (in the range 100-500 MPa for 25 minutes) assisted hydrolysis by trypsin (Figure 6.4). An increase in the number of peptides with molecular weight lower than native BSA (66 kDa) can be observed. Samples treated at pressure levels of 200 and 300 MPa are the ones showing more bands associated with lower molecular weight peptides (MW<17 kDa).

Similarly to the case of the hydrolysis by chymotrypsin, at pressure of 500 MPa the smallest peptides disappear for the effect of both protein aggregation and enzymatic activity reduction. Moreover an increase in molecular weight distribution can be observed when protein hydrolysates are produced under high pressure because high pressure induced denaturation increases hydrolysis efficiency as shown by results of hydrolysis degree.

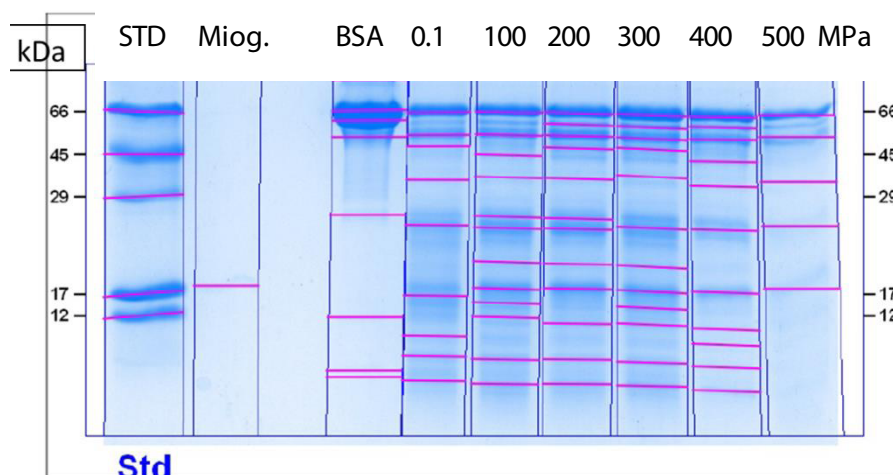


Figure 6.4. SDS Page of hydrolysates of BSA (00) by trypsin, non-treated (A0) and treated by HHP by high pressure: 100 (A1), 200 (A2), 300 (A3), 400 (A4) and 500 (A5) MPa for 25 min at 37°C.

6.2.3 FT-IR spectra of Bovine Serum Albumin hydrolysates

FT-IR spectroscopy was used to characterize BSA hydrolysates produced under high pressure by chymotrypsin and trypsin. In Figure 6.5 and Figure 6.6 BSA hydrolysates produced by chymotrypsin and trypsin under pressures treatment between 100 and 500 MPa for 15 minutes are reported respectively.

FT-IR 2nd derivative spectra are reported in the region between 1600 and 1700 cm^{-1} (amide I) associated to the C=O stretching vibration (approximately 80%) of the amide groups coupled with little in-plane NH bending (<20%) (Krimm et al. 1986).

Results show that a deviation from native BSA spectra can be observed in both hydrolysates at atmospheric pressure and under high pressure. The occurrence of a peak at 1650 cm^{-1} can be observed in spectra of both hydrolysates produced at 0.1 MPa and at 100 and 300 MPa. Another peak in hydrolysates spectra can be observed at 1680 cm^{-1} about, being this peak emphasized for the native BSA. The peak at 1685 cm^{-1} band corresponds to the formation of antiparallel β -sheet aggregates. Finally a peak at 1630 cm^{-1} can be observed in hydrolysates produced at very high pressure (500 MPa) this peak being related to a modifications in α -helix secondary structure.

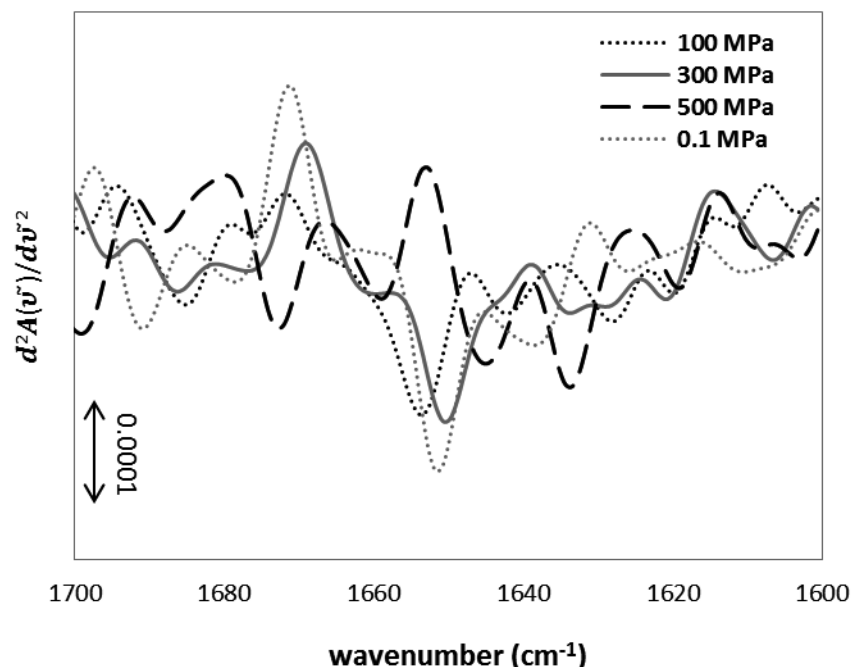


Figure 6.5. FT-IR 2nd derivative spectra of hydrolysates of BSA by chymotrypsin, non-treated and treated by HHP by high pressure: 100, 300, 500 MPa for 25 min at 37°C.

Similar observations can be made for hydrolysates produced under the same pressure range (100-500 MPa) by trypsin (Figure 6.6). A higher deviation of the FTIR spectra can be observed in hydrolysates under pressure: at a pressure level of 100 MPa, higher differences can be detected between treated and non-treated samples. Similarly to hydrolysates produced by chymotrypsin, higher differences in the peaks are shown at wavenumber of 1630 cm^{-1} and 1680 cm^{-1} . Hydrolysates produced at ambient pressure (0.1 MPa) showed an additional peak at 1650 cm^{-1} confirming a difference in proteolytic profile of hydrolysates produced at ambient pressure if compared with high pressure produced ones (100-500 MPa).

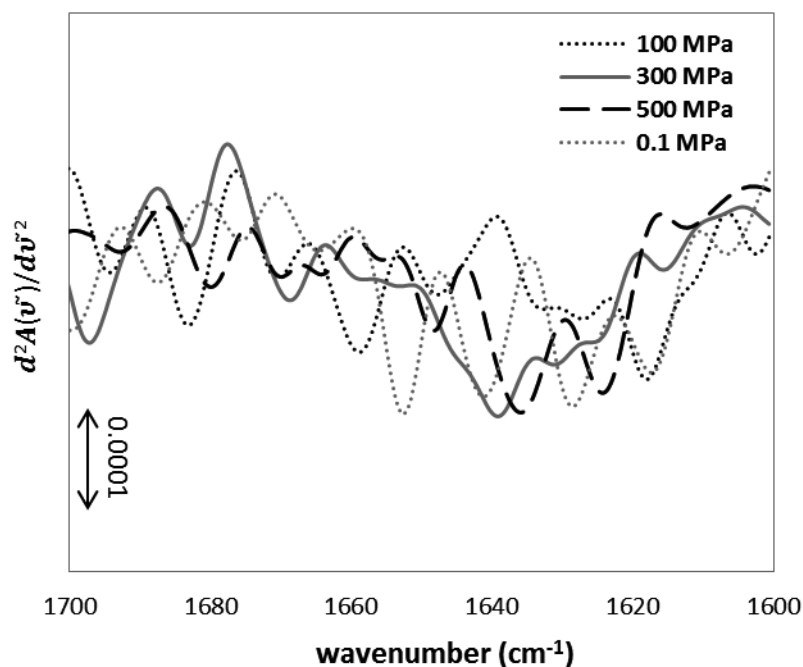


Figure 6.6. FT-IR 2nd derivative spectra of hydrolysates of BSA by trypsin, non-treated and treated by HHP by high pressure: 100, 300, 500 MPa for 25 min at 37°C.

6.2.4. Effect of High Pressure pretreatment on BSA hydrolysis

The previous results that HHP assisted hydrolysis may increase the values of the hydrolysis degree. On the other side, the formation of aggregates and the reduction of enzyme activity, occurring at pressure level above 400 MPa, could limit this effect. For this reason, the application of high pressure as a pretreatment of protein to be hydrolyzed at atmospheric pressure was investigated.

The effect of HHP treatments at pressure levels between 100 and 400 MPa and a defined treatment time (15 minutes) on the hydrolysis reaction at atmospheric conditions (P=0.1 MPa, 25 min) was investigated.

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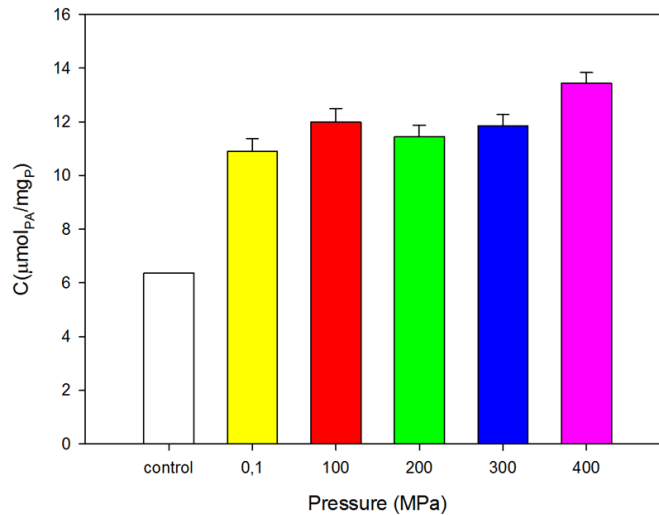


Figure 6.7. Hydrolysis degree (primary amino groups) by chymotrypsin of untreated Bovine Serum Albumin (37°C) and pre-treated by high pressure (100, 200, 300, 400 and 500 MPa, 15 minutes) for 25 min at 37°C.

At parity of applied pressure and holding reaction time, that higher hydrolysis degree value is obtained when the enzymatic reaction is assisted by high hydrostatic pressure than the case of application of HHP treatment as pre-treatment. This effect can be explained considering that, in the case of HHP assisted hydrolysis, the protein unfolding and the production of the peptides occur simultaneously. When HHP treatment is applied before the hydrolysis, the refolding of the protein upon depressurization can occur, thus reducing the efficiency of the hydrolysis.

On the other side, since the hydrolysis is not carried out under high pressure, it is not possible to observe any effect of pressure level on reduction of hydrolysis degree at very high pressure levels (300-400 MPa), since enzyme is not exposed to the HHP treatment.

6.3. Results and discussion 2th study case: Caseins

In order to investigate if high hydrostatic pressure is also able to enhance the proteolysis of not-globular proteins, HHP assisted hydrolysis of caseins was also studied. A different conformational structure might influence the physical mechanism regulating protein modifications and thus hydrolysis reaction.

Caseins are phosphoproteins representing approximately 80% of the total protein content of milk proteins (Brunner, 1977), the most important being α_{s1} -caseins, α_{s2} -caseins, β -caseins (Walstra et al. 1999) and γ -casein in traces

(Swaigood, 1992) as observed by analyzing their DNA sequence. Casein proteins show a micellar structure made of hydrophobic submicelles bonded by calcium phosphate bridges and surrounded by the K-caseins, which represent the hydrophilic fraction of caseins (Figure 6.8). Since caseins have not a tertiary structure or free thiol groups, unfolding or gelation cannot occur and caseins show a structure similar to the one of a globular protein already denatured.

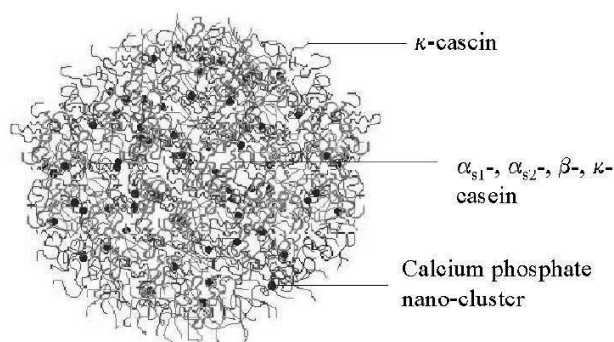


Figure 6.8. Schematic representation of caseins micelle.

The nutritional value of caseins, which represent a very important and common source of nitrogen and essential amino acids, is high. On the other hand, caseins might cause food allergies as well as intolerance. Their fibrillar structure is formed by mainly sequential epitopes able to bind to specific IgG antibodies and β - and κ - caseins are the major antigens. Casein proteins and their hydrolysates represent a very interesting ingredient in food industry. Their hydrodynamic and surface-related properties may be useful in many product formulations and may also modify the color and flavor of food products.

In some cases, casein hydrolysates can be used as an alternative source of amino acids in human diet, since the nutritional value of the native proteins is preserved and the immunogenicity reduced (Cordle et al., 1991). In fact it has been observed that they are able to induce oral tolerance without inducing antibody response (Enomoto et al., 1993). In addition casein hydrolysates can be used as an ingredient in special diet of patients with digestion problems, like cystic fibrosis, malnutrition or for infant formula and medical or dietary products.

The application of caseins hydrolysis assisted by HHP and the characterization of the hydrolysates produced were, therefore, investigated.

6.3.1. Hydrolysis degree of Caseins

The values of the hydrolysis degree of casein samples processed in HHP assisted hydrolysis (pressure levels: 100, 200, 300 and 400 MPa; holding times: 0, 5, 10, 15, 20, 25 and 30 minutes at constant temperature of 37° C) were determined.

The curves of hydrolysis degree with chymotrypsin (Figure 6.9) and trypsin (Figure 6.10) were compared to those of casein hydrolysed at atmospheric pressure (0.1 MPa). The hydrolysis degree values were expressed in terms of concentration of primary amino groups, which are the main products of the cleavage of covalent bonds during hydrolysis reaction, per gram of protein.

The results demonstrate that if the hydrolysis is carried out under pressure, the achieved values of hydrolysis degree are higher than the one detected for the samples hydrolyzed at atmospheric conditions. High Hydrostatic Pressure increases the hydrolysis degree in protein solutions (with the following results: 200 MPa > 400MPa > 300 MPa > 100 MPa > 0.1 MPa) which can be correlated to the protein sensibility to the proteolytic enzyme under pressure.

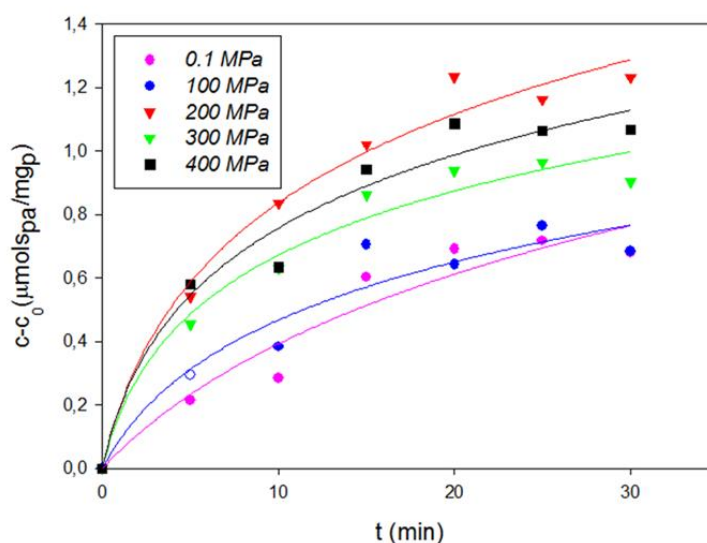


Figure 6.9. Hydrolysis degree (primary amino groups) by chymotrypsin of untreated Caseins (37°C) and treated by high pressure (100, 200, 300, 400 and 500 MPa) for 0, 5, 10, 15, 20, 25 min at 37°C.

Some authors showed that casein proteolysis is reduced in case of a partial exposure of caseins to the enzymes during proteolytic attack. In fact,

despite the most part of β -caseins is digested, a high percentage of both α_{s1} - and α_{s2} - caseins are only partially hydrolysed because of the interaction occurring between colloidal calcium phosphate and phosphoserine residues which explain the weak accessibility of this part of the molecule. This effect suggests that the caseins are only partially accessible to trypsin because they are differently involved in the micellar framework (Gagnaire and Léonil, 1998).

When HHP treatment is applied, hydrophobic and electrostatic interactions are broken (Mozhaev et al. 1996), thus explaining the destruction of the micelles. Furthermore, Calcium phosphate bonds which regulate the equilibrium between electrostatic repulsion and hydrophobic interactions are destabilized, thus casein micelles becoming more susceptible to HHP-induced disruption and more available for proteolytic attack.

On the other side, the application of high pressure above a critical level promotes the reorganization of hydrophobic interactions, thus reducing the hydrolysis degree.

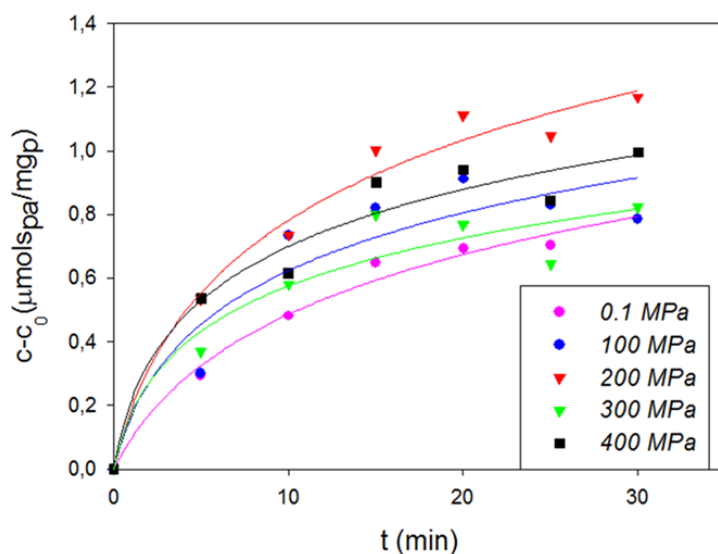


Figure 6.10. Hydrolysis degree (primary amino groups) by trypsin of untreated Caseins (37°C) and treated by high pressure (100, 200, 300, 400 and 500 MPa) for 0, 5, 10, 15, 20, 25 min at 37°C.

6.3.2. SDS-PAGE profiles of Caseins hydrolysates

The characterization of caseins peptides produced by HHP assisted hydrolysis by chymotrypsin (pressure levels between 100 and 400 MPa) for

Effects of HHP on allergen proteolysis

25 minutes (at which the highest hydrolysis degree is detected) was made in order to investigate the molecular distribution of caseins peptides.

The results, reported in Figure 6.11, show that, if compared with non-treated samples (0.1MPa), peptides produced under high pressure treatment, present a different molecular weight distribution made up of peptides having lower dimension (100 MPa).

The optimal operating conditions for the production of peptides with molecular weight lower than the peptides produced at atmospheric pressure are represented by 200 and 300 MPa.

If higher pressure levels are applied (400 MPa) during hydrolysis reaction, the values of the hydrolysis degree reduce. In fact, the presence of higher molecular weight peptides (at around 35 and 66 KDa) demonstrate that the small peptides (dimers and trimmers), which are formed at lower pressure levels, are organized in the form of oligomers due to micelles aggregation and the reorganization of hydrophobic interactions.

In addition, the enzyme activity reduces, carrying out the hydrolysis at these drastical processing conditions.

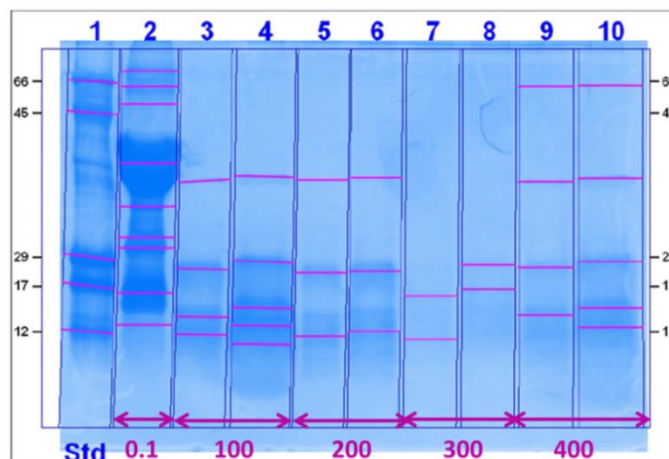


Figure 6.11. SDS Page of caseins hydrolysates by chymotrypsin, non-treated and treated by HHP treatment: 100, 200, 300 and 400 MPa for 25 min at 37°C.

6.3.3. Structure and aggregation of caseins peptides

In order to produce casein hypoallergenic samples, it is very important to minimize the dimension of the peptides produced and the formation of aggregates in order to observe a reduction of the immunoresponse and an increased digestibility.

Because of their typical conformation in forms of micelles, caseins are known to have the tendency to aggregate especially when, as a consequence

of the hydrolysis reaction, the exposure of some functional groups increase their reactivity to interact each other.

For this reason, casein peptides produced under HHP treatment with chymotrypsin and trypsin, were characterized in terms of Particle Size Distribution (PSD) in order to optimize processing conditions allowing lower aggregation (Z-average) and higher samples homogeneity (lower polydispersity index, PI).

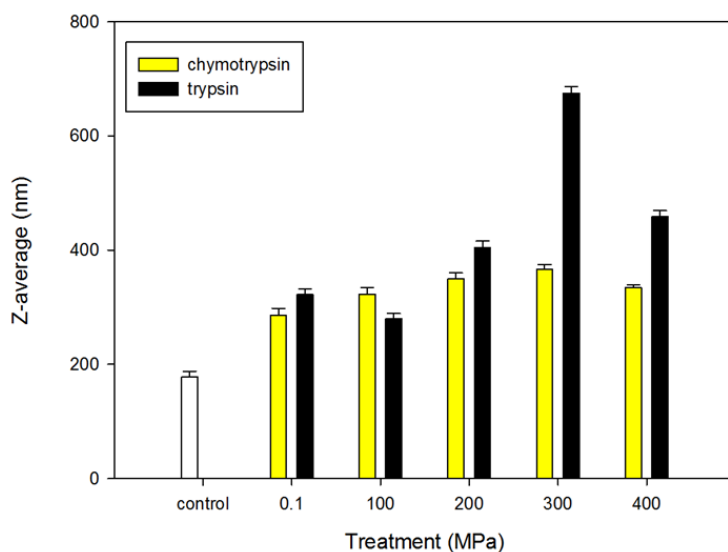


Figure 6.12. Z-average of untreated Caseins (37°C) and treated by high pressure (100, 200, 300, 400 and 500 MPa) for 25 minutes at 37°C.

The results reported in Figure 6.12 show that higher extent of protein aggregation can be observed in hydrolysed samples than in native caseins due to an increased reactivity of the peptides.

When high pressure is applied, peptides produced by hydrolysis by trypsin show more tendency to aggregate, which achieve the maximum value at 300 MPa. On the other side, in the case of hydrolysis by chymotrypsin, the effect of pressure level on the Z-average dimension is not significant and lower extent of aggregation can be observed.

As a measure of peptides dimension homogeneity, PI index was adopted (Figure 6.13). When PI assumes low values, protein peptides show a more similar dimension. On the other side, more polydisperse samples are produced when PI reaches high values.

Results show that, in the case of hydrolysis by trypsin, very high polydispersity can be reached at intermediate pressure (200 MPa), which is the pressure allowing a significant aggregation among the peptides. A higher

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homogeneity is observed if the peptides derive from the hydrolysis by chymotrypsin, decreasing the PI values by increasing the pressure level applied up to 200 MPa. At pressure above 200 MPa a slight increase of this parameter can be detected. Accordingly, the hydrolysis by chymotrypsin at 200 MPa represents the optimal processing conditions for the production of casein peptides with the smallest dimension and highest homogeneity.

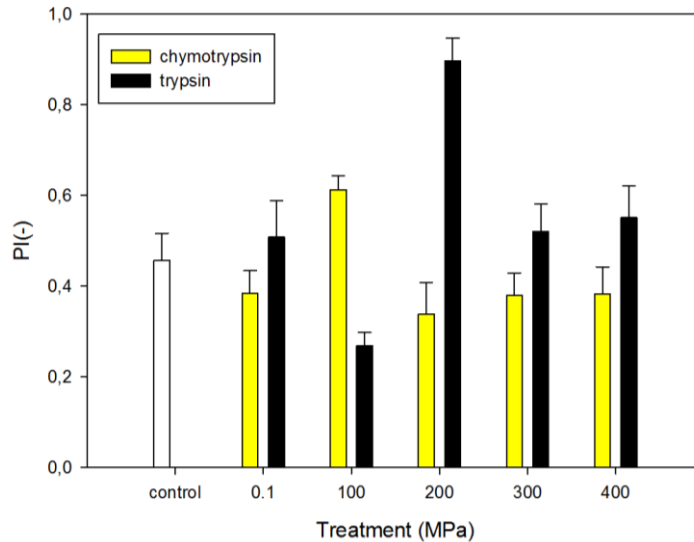
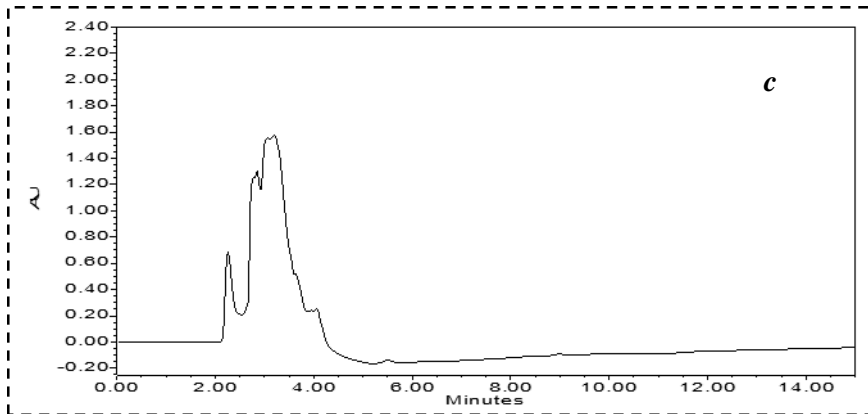
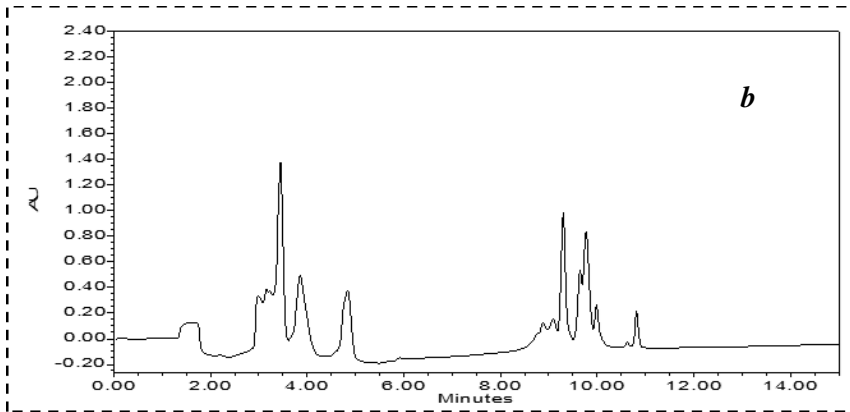
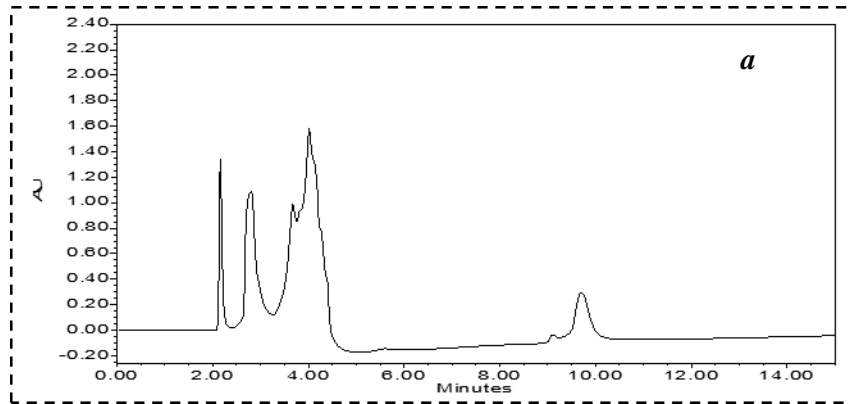


Figure 6.13. Polidispersity index (PI) of untreated (37°C) and high pressure treated Caseins (100, 200, 300, 400 and 500 MPa) for 25 minutes at 37°C.

At the same processing conditions, in order to characterize the casein structure and homogeneity, the peptide profiles by HPLC were measured. The peak distribution of the spectra measured for the high pressure treated peptides are very different from those obtained for the samples processed at atmospheric.

As shown by PSD analysis, when hydrolysis is carried out at lower pressure level (100 MPa), higher numbers of peaks than in non-treated samples (P=0.1 MPa, Figure 6.14 a) can be observed, thus showing a wider variety of peptides in the protein sample (Figure 6.14 b).

On the other side, at higher pressure levels (200-300 MPa) peptide distribution (Figure 6.14c and 6.14d) show a lower number of peaks, thus demonstrating a higher homogeneity of peptides and confirming the results of the Particle size Distribution analysis.



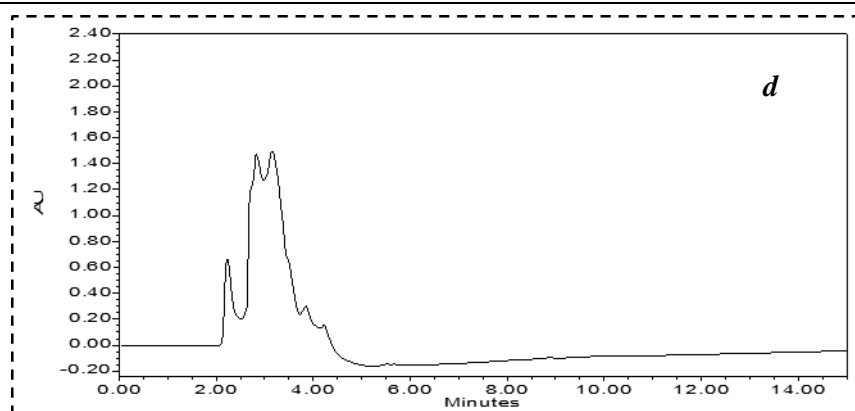


Figure 6.14. HPLC peptides profiles produced by hydrolysis assisted by high hydrostatic pressure (pressure levels: (0.1 MPa (a), 100MPa (b), 200 MPa (c), 300MPa (d)), holding time:25 min).

6.4. Conclusions

The results reported in this chapter demonstrated the potential of High Hydrostatic Pressure (HHP) in enhancing the extent of hydrolysis reaction in both globular and non-globular proteins. HHP assisted hydrolysis showed to be more effective than the HHP pre-treatment followed by a conventional hydrolysis.

In the case of BSA, HHP application increased the values of the hydrolysis degree at the treatment conditions which maximized protein unfolding and thus the protein exposure to the proteolytic enzyme. BSA peptides produced under high pressure showed lower dimensions and a different structure if compared with atmospheric pressure produced peptides.

In the case of caseins, hydrolysis assisted by High Hydrostatic Pressure showed better results in terms of hydrolysis degree than at atmospheric pressure in that treatment conditions allowing the maximum submicelles exposure thus allowing higher protein sensibility to proteolytic attack.

Furthermore, similarly to BSA hydrolysis, casein peptides produced by HHP assisted hydrolysis had lower dimension and higher homogeneity than those produced at atmospheric pressure.

These results suggest the possibility to use HHP assisted hydrolysis by proteolytic enzymes as an efficient method for the production of lower dimension peptides with the potential to be used for hypoallergenic formula.

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Chapter VII

Modelling of hydrolysis kinetics

7. Introduction

Previous results demonstrated that if protein hydrolysis is carried out in combination with high hydrostatic pressure treatment, it is possible to accelerate the hydrolysis reaction and to produce peptides smaller than those produced at atmospheric pressure.

In fact, the structural changes induced by HHP treatment allow an increased accessibility of protein to the action of proteolytic enzyme, thus facilitating the enzymatic attack. To quantify the effect of high hydrostatic pressure on the hydrolysis reaction and to compare the hydrolysis kinetics under high pressure and that at atmospheric pressure, a mathematic model of the hydrolysis kinetics was set up.

A non-linear equation was used to fit the data of the hydrolysis degree, shown in the previous chapter, as a function of the hydrolysis time. A zero - order hydrolysis reaction in competition with a simultaneous second order enzyme inactivation reaction were considered to represent the hydrolysis under pressure.

From the correlation between modeling parameters and the correspondent physical dimensions, the reaction rate constant and enzyme inactivation constant for BSA hydrolysis with chymotrypsin assisted by high pressure (at 400 MPa).

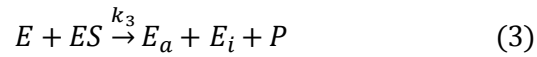
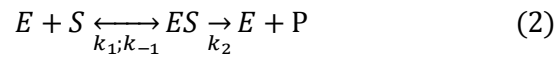
7.1. Modeling hydrolysis kinetics

The values of the hydrolysis degree (expressed as $HD = \Delta C / C_0$, where C_0 and C are respectively the content of primary amino groups before and after hydrolysis) measured for BSA samples processed with HHP assisted hydrolysis (100-600 MPa) at 37°C and different hydrolysis times ($t=0, 5, 10, 15, 20, 25, 30$ minutes) were fitted using Eq.1:

$$\frac{d(HD)}{dt} = a * [\exp(-b * HD)] \quad (1)$$

Where a and b are two modeling parameters with different values for different experimental tests (Marquez et al. 1999).

A fitting model as described in Eq.1 can be used by considering that the chemical reactions occurring during the HHP assisted hydrolysis are constituted by a set of reactions, namely a zero order hydrolysis reaction (Eq. 2) and a simultaneous second order enzyme inactivation reaction (Eq.3), can be written:



The hydrolysis reaction rates of the two reactions can be defined as follows:

$$r = s_0 * \frac{d(DH)}{dt} = k_2 |ES| \quad (4)$$

$$-\frac{de}{dt} = k_3 |E| |ES| \quad (5)$$

Combining the Eq.4 and Eq.5, the Eq. 6 can be derived:

$$-s_0 \frac{d(HD)}{dt} = \frac{k_2}{k_3 E} \quad (6)$$

The total quantity of the enzyme is given by the sum of the enzyme in free form and the enzyme in the complex Enzyme/Substrate (ES) form, as given in Eq.7:

$$e = |E| + |ES| \quad (7)$$

From the mass balance in steady state condition, the concentration of ES is given by Eq.8:

$$|ES| = \frac{|E||S|}{k_M} \quad (8)$$

Where k_M is the Michaelis-Menten constant. Combining Eq. 8 and Eq. 7 and considering S equal to s_0 , the concentration of the enzyme in free form can be obtained (Eq.9):

$$E = \frac{k_M * e}{k_M + s_0} \quad (9)$$

If $k_M \ll s_0$, Eq.10 is obtained:

$$|E| = \frac{k_m * e}{s_0} \quad (10)$$

Combination Eq.10 and Eq.6, Eq.11 can be derived:

$$-\frac{d(HD)}{de} = \frac{k_2}{k_3 * k_M e} \quad (11)$$

Integrating Eq.11 with the appropriate boundary conditions, the equation accounting for the free enzymes can be written (Eq.12):

$$e = k_2 e_0 \exp \left[-\frac{k_3 * K_M}{k_2} (HD) \right] \quad (12)$$

Combining Eq.12, Eq.4, Eq.8, and Eq.10 the Eq.12 is obtained:

$$\frac{d(HD)}{dt} = \frac{k_2 e_0}{s_0} \exp \left[-\frac{k_3 K_M}{k_2} (HD) \right] \quad (13)$$

Comparing Eq.1 and Eq.13, the correlation between modeling parameters and physical constants of the hydrolysis reaction and enzyme inactivation reaction is derived as reported in the following:

$$a = \frac{k_2 e_0}{s_0} \quad b = \frac{k_3 K_M}{k_2}$$

7.2. Results and discussion

7.2.1. Fitting model of the hydrolysis curves

The curves of BSA ($C=25$ mg/ml) hydrolysis with chymotrypsin and trypsin at several pressure levels (0.1, 100, 200, 300, 400, 500 and 600 MPa) and hydrolysis times (0, 5, 10, 15, 20, 25 and 30 minutes) at 37°C are reported in Figure 7.1 and Figure 7.2, respectively.

As already discussed in the previous chapter, the data of kinetics of hydrolysis show an initial growth and a plateau for prolonged hydrolysis times.

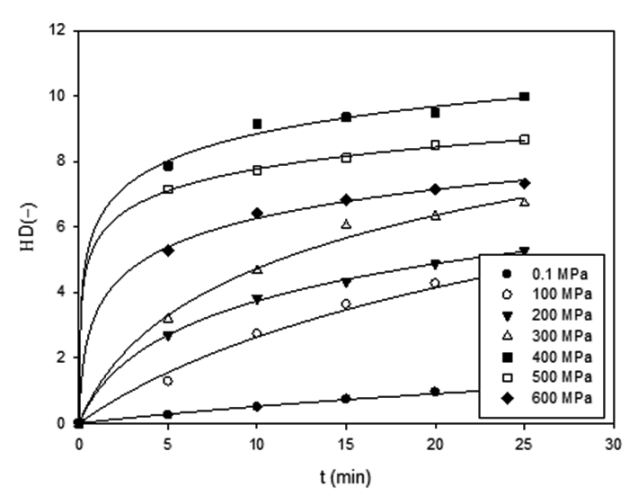


Figure 7.1. Hydrolysis kinetics of BSA with chymotrypsin at atmospheric pressure and at high pressure ($P=100, 200, 300, 400, 500$ and 600 MPa).

The fitting curves derived from the mathematical model allow the analysis of the effect of high pressure on the hydrolysis degree. Higher hydrolysis degrees are reached when the hydrolysis reaction is carried out at higher pressure levels reaching the maximum values at 400 MPa.

Similar results are obtained from the hydrolysis of BSA with trypsin in the same pressure range (100-400 MPa) even though the effect of pressure on hydrolysis is less relevant than in the case of hydrolysis with chymotrypsin.

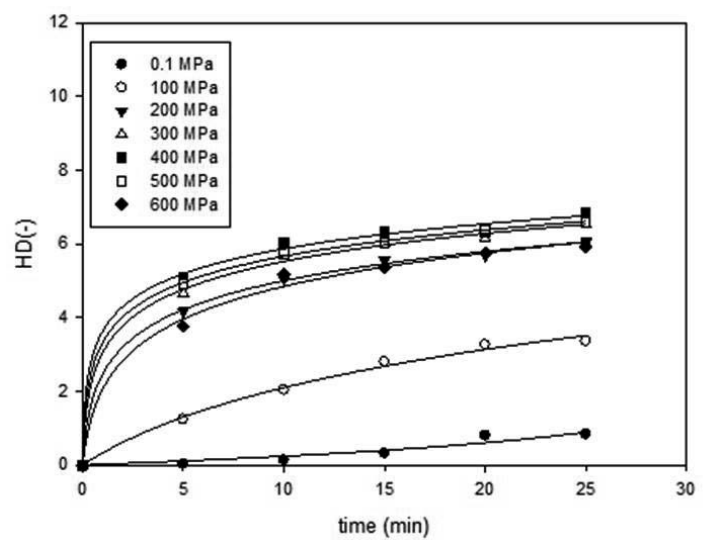


Figure 7.2. Hydrolysis kinetics of BSA with trypsin at atmospheric pressure and at high pressure ($P=100, 200, 300, 400, 500$ and 600 MPa).

From the model of the hydrolysis degree, the parameters a and b can be evaluated by applying the minimum squares methods. The estimated values of a and b parameters are reported in Table 7.1 and Table 7.2.

Eq.1 properly fits the experimental data of the hydrolysis of BSA with both chymotrypsin and trypsin as demonstrated considering the values of the correlation parameters (close to 1) and of standard deviations (close to 0) reported in Table 7.1 and Table 7.2.

These results suggested that the hypotheses made to define the reaction rates (Eq.4, Eq.5) allow to describe the mechanism regulating the hydrolysis reactions carried out at high pressure with good approximation.

Modelling of hydrolysis kinetics

Table 7.1. Kinetic parameters of the hydrolysis degree of BSA with chymotrypsin at atmospheric pressure and at high pressure ($P=100, 200, 300, 400, 500$ and 600 MPa)

| P(MPa) | a (min⁻¹) | b (-) | R² | SD |
|---------------|-----------------------------|--------------|----------------------|-----------|
| 0.1 | 0.0609 | 0.645 | 0.9943 | 0.0338 |
| 100 | 0.3873 | 0.2763 | 0.9895 | 0.2040 |
| 200 | 1.2449 | 0.5528 | 0.9993 | 0.0556 |
| 300 | 1.2979 | 0.3732 | 0.9942 | 0.2182 |
| 400 | 189.4468 | 0.8327 | 0.9980 | 0.1918 |
| 500 | 307.7409 | 1.0389 | 0.9998 | 0.0544 |
| 600 | 17.0030 | 0.7827 | 0.9989 | 0.1037 |

Table 7.2. Kinetic parameters of the hydrolysis degree of BSA with trypsin at atmospheric pressure and at high pressure ($P=100, 200, 300, 400, 500$ and 600 MPa)

| P (MPa) | a (min⁻¹) | b (-) | R² | SD |
|----------------|-----------------------------|--------------|----------------------|-----------|
| 0.1 | 0.0205 | -1.3814 | 0.9054 | 0.1301 |
| 100 | 0.3580 | 0.4695 | 0.9931 | 0.1218 |
| 200 | 8.6738 | 0.8662 | 0.9996 | 0.0746 |
| 300 | 16.5525 | 0.9090 | 0.9979 | 0.1264 |
| 400 | 38.9468 | 1.0206 | 0.9970 | 0.1584 |
| 500 | 24.7595 | 0.9679 | 0.9993 | 0.0735 |
| 600 | 4.9311 | 0.7506 | 0.9935 | 0.2035 |

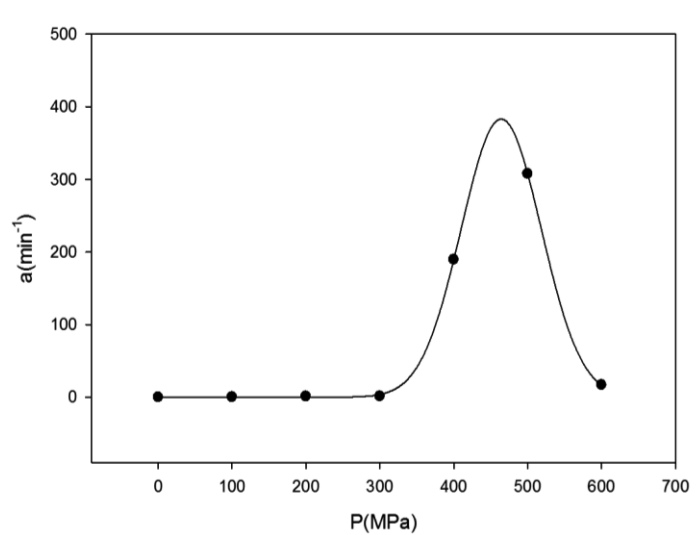


Figure 7.3. Effect of the pressure level on the kinetic parameter a of the hydrolysis of BSA with chymotrypsin.

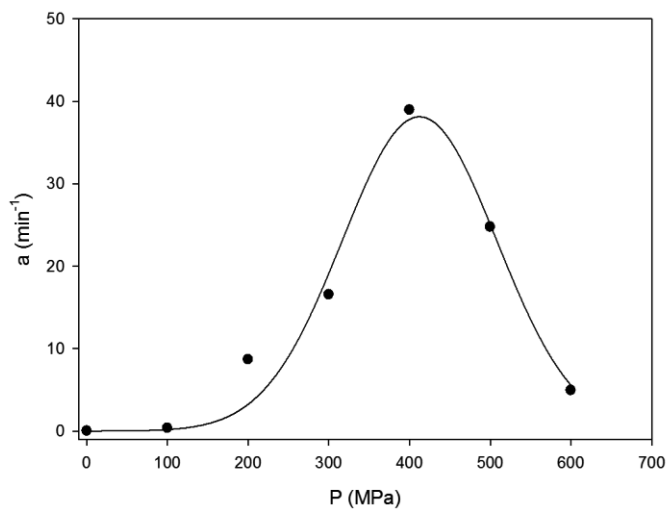


Figure 7.4. Effect of the pressure level on the kinetic parameter a of the hydrolysis of BSA with trypsin.

7.2.2. Evaluation of the reaction rate constants

In order to evaluate the kinetic constants of the hydrolysis reaction of BSA with chymotrypsin, k_2 (reaction rate constant) and k_d (enzyme inactivation constant) were estimated.

Moreover, the influence of both initial enzyme concentration and initial substrate concentration were analyzed. To this purpose, the values of the BSA hydrolysis degree with chymotrypsin at a pressure of 400 MPa and at atmospheric pressure and a temperature of 37°C were considered. In particular, to analyze the effect of the initial substrate concentration, an initial enzyme concentration of 2.5 mg/mL was considered (Figure 7.5 and Figure 7.6):

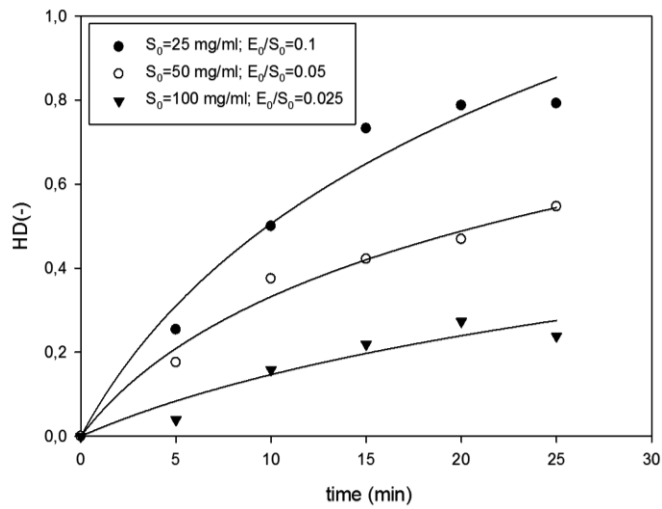


Figure 7.5. Effect of the initial substrate concentration ($S_0=25, 50$ and 100 mg/mL) on the hydrolysis degree of BSA with chymotrypsin ($E_0 = 2.5$ mg/ml) at atmospheric pressure.

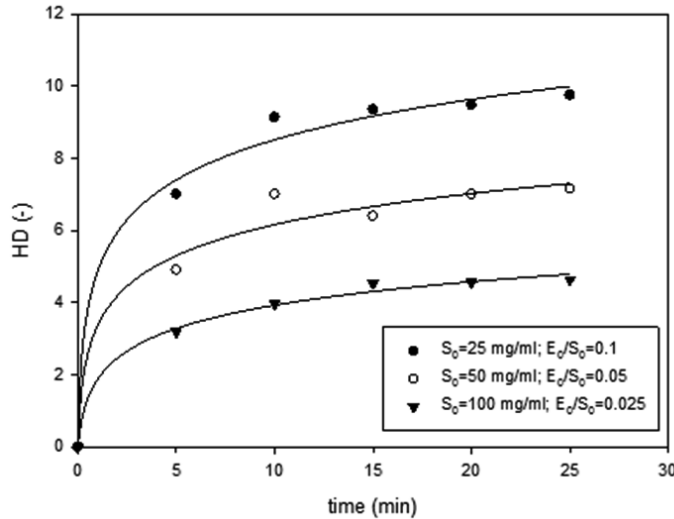


Figure 7.6. Effect of the initial substrate concentration ($S_0=25, 50$ and 100 mg/mL) on the hydrolysis degree of BSA with chymotrypsin ($E_0 = 2.5$ mg/ml) at 400 MPa.

Similarly, to analyze the effect of the initial enzyme concentration, an initial substrate concentration of 25 mg/mL was considered. The results showed that a dependence of the hydrolysis degree on the initial enzyme concentration existed: higher hydrolysis degrees are reached at higher initial substrate concentrations (Figure 7.5 and Figure 7.6) and at lower initial enzyme concentrations (Figure 7.7 and Figure 7.8) and similar observations hold for the samples processed at atmospheric pressure and at high pressure.

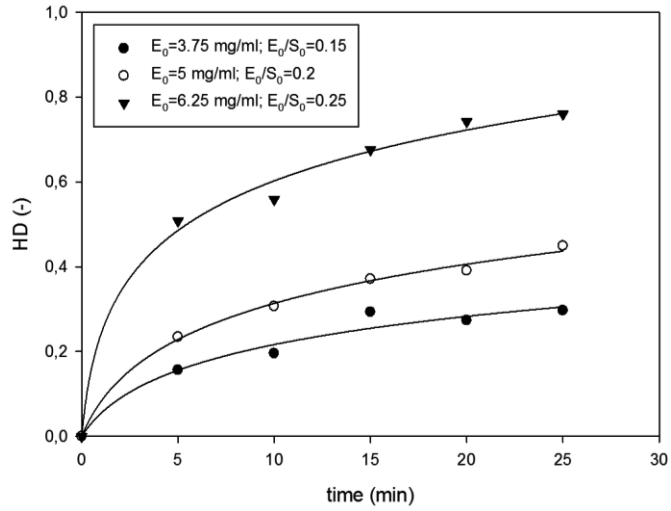


Figure 7.7. Effect of the initial enzyme concentration ($E_0=3.75, 5$ and 6.25 mg/mL) on the hydrolysis degree of BSA with chymotrypsin ($S_0 = 25$ mg/ml) at atmospheric pressure.

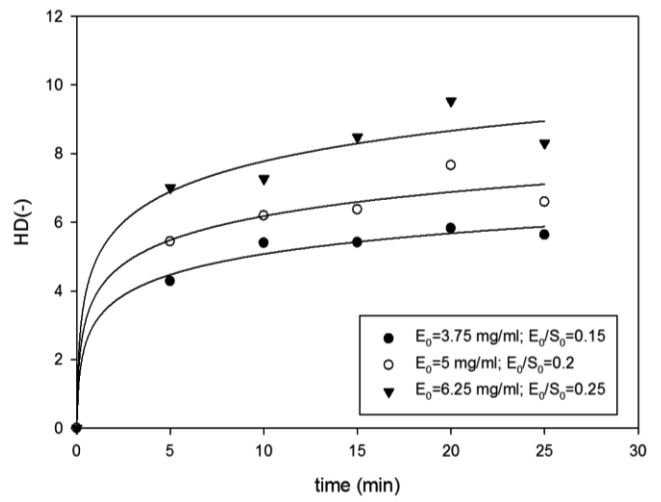


Figure 7.8. Effect of the initial enzyme concentration ($E_0=3.75, 5$ and 6.25 mg/mL) on the hydrolysis degree of BSA with chymotrypsin ($S_0 = 25$ mg/ml) at 400 MPa.

For each hydrolysis curve corresponding to a specific E/S ratio, the correspondent values of the model parameters (**a** and **b**, defined in Eq.10) were evaluated. The parameter **a** was plotted versus the ratio E/S and from the slope of the regression line of the experimental data, the reaction rate constant k_2 (min^{-1}) was evaluated (as shown in Figure 7.9 and 7.10) at both atmospheric and high pressure according to the following definition:

$$a = \frac{k_2 e_0}{s_0}$$

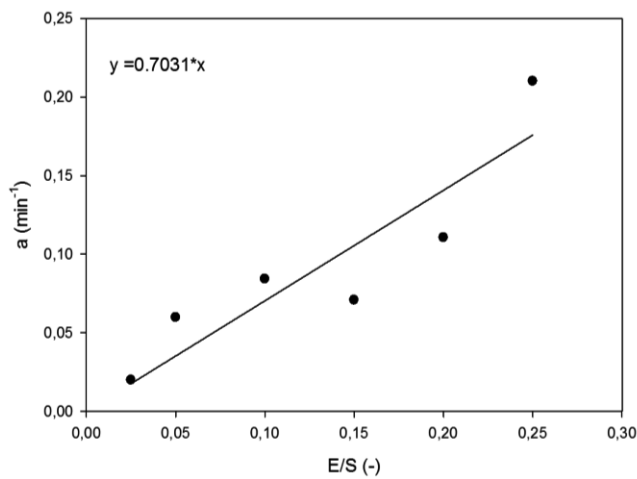


Figure 7.9. Effect of the ratio enzyme/substrate (E/S) on the kinetic parameter **a** of the hydrolysis of BSA with chymotrypsin at atmospheric pressure.

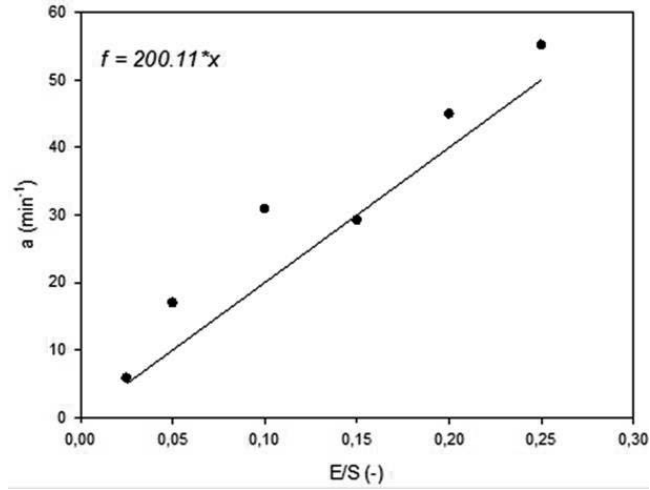


Figure 7.10. Effect of the ratio enzyme/substrate (E/S) on the kinetic parameter *a* of the hydrolysis of BSA with chymotrypsin at 400 MPa.

A similar approach was used to determine the enzyme inactivation constant (k_d) which is defined in the Eq. 14:

$$-\frac{de}{dt} = k_3 K_M \frac{e^2}{s_0} = K_d \frac{e^2}{s_0} \quad (14)$$

As for the reaction rate constant (k_2), the enzyme inactivation constant (k_d) was evaluated from the regression line of the product *ab* values estimated at different enzyme substrate ratio (E/S) versus E/S values (Figure 7.11 and Figure 7.12). In fact, according with Eq.15, the product of *a* and *b* (modeling parameters) *ab* is a function of the kinetic parameter k_d (enzyme inactivation constant) as shown in Eq. 15:

$$ab = \frac{k_2 e_0 k_3 K_M}{s_0 k_2} = k_3 K_M \frac{e_0}{s_0} = k_d \frac{e_0}{s_0} \quad (15)$$

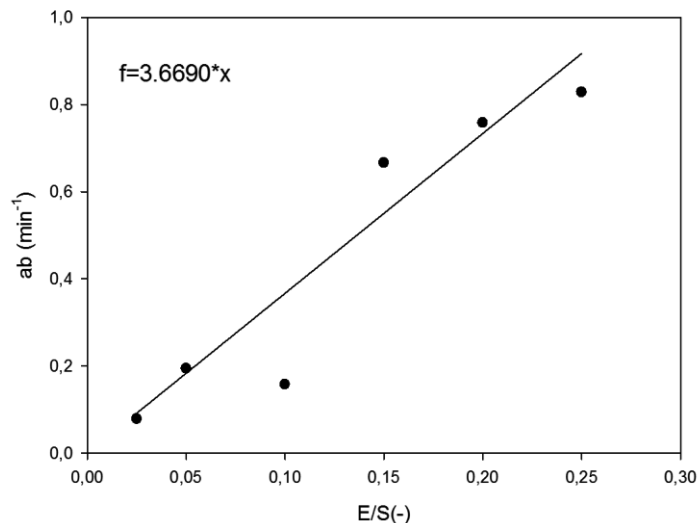


Figure 7.11. Effect of the ratio enzyme/substrate (E/S) on the kinetic parameter ab of the hydrolysis of BSA with chymotrypsin at atmospheric pressure.

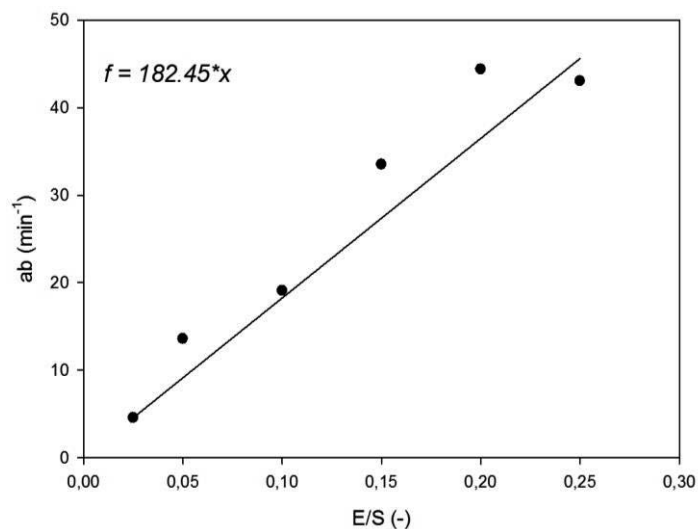


Figure 7.12. Effect of the ratio enzyme/substrate (E/S) on the kinetic parameter ab of the hydrolysis of BSA with chymotrypsin at 400 MPa.

The enzyme inactivation constant k_d was evaluated for both hydrolysis carried out at atmospheric pressure and at 400 MPa and have the following

Modelling of hydrolysis kinetics

values $k_d = 3.6690 \text{ min}^{-1}$ (at 0.1 MPa) and $k_d = 182.45 \text{ min}^{-1}$ (400 MPa). In conclusion, the results suggest that both the reaction rate constant (k_2) and the enzyme inactivation constant (k_d) increase in HHP assisted hydrolysis. The data are reported in Table 7.3.

Table 7.3. Reaction rate constant and enzyme inactivation constant of BSA hydrolysis reaction with chymotrypsin at atmospheric pressure and at 400 MPa (temperature: 37°C).

| <i>P</i> (MPa) | <i>K</i> ₂ (min ⁻¹) | <i>R</i> ² | <i>K</i> _d (min ⁻¹) | <i>R</i> ² |
|----------------|--|-----------------------|--|-----------------------|
| 0.1 | 0.7031 | 0.81 | 3.6690 | 0.90 |
| 400 | 200.11 | 0.92 | 182.45 | 0.93 |

7.3. Conclusions

The results obtained so far demonstrate that the experimental data of the hydrolysis degree may be properly fitted by a non-linear mathematical model based on a zero order kinetic for the hydrolysis reaction and a second order kinetic for the enzyme inactivation reaction.

Furthermore, the kinetic constants, namely the reaction rate constant and enzyme inactivation constant, increase with the pressure level, confirming that high hydrostatic pressure is able to enhance the hydrolysis kinetics.

References

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Chapter VIII

Allergenicity of proteins and protein hydrolysates produced by HHP technology

8. Introduction

In this chapter the results of the characterization of the allergen (casein and BSA) peptides produced by High Hydrostatic Pressure with both the proteolytic enzymes tested (chymotrypsin and trypsin) is reported. Hydrolysis conditions able to produce the lowest dimension peptides were chosen to carry out the *in vivo* tests to detect the allergenicity. The peptides with the lower size were obtained at 300-400 MPa after 25 minutes of hydrolysis for BSA while for casein the conditions that allowed to obtain the peptides with the lower size were 200-300 MPa and 25 min.

As reported in the literature, several *in-vitro* methods can be used to detect protein allergenicity, namely Western Blot or Elisa tests, which imply the use of antibodies from animals, like rabbits. Therefore, these tests can only give an estimate of the allergenicity and not the allergenicity.

For these reasons, *in vivo* tests were chosen to characterize the immunoresponse of the produced peptides in allergic patients.

8.1. Materials and methods

8.1.1. Preparation of the samples

BSA and casein (Sigma-Aldrich, Italy) were dissolved at a temperature of 25 °C in Sodium Phosphate Buffer (50 mM, pH=8), according to the protocols reported by Penãs et al. (2006), at protein concentration of 10 mg/mL under a gentle mixing until a homogenous solution was obtained. The pH of the protein solutions was measured with a pH-meter (S400 SevenExcellence, Mettler Toledo International Inc.). The protein solutions were stored under refrigerated conditions until HHP treatments.

8.1.2. Sample treatment

BSA and casein samples were processed in HHP assisted hydrolysis treatments carried out at the processing conditions maximizing the hydrolysis degree, in other words allowing the production of peptides with the lowest molecular weights. Thus, BSA peptides samples were produced by hydrolysis with chymotrypsin at 300 MPa while casein samples were hydrolyzed with the same enzyme at 400 MPa. The samples were treated at 95 °C for 10 min in order to inactivate the enzyme and stopping the hydrolysis reaction, and the peptide samples were stocked at -20 °C.

8.1.3. In vivo analysis by prick tests

Skin prick test (SPT) is used in the diagnosis of IgE-mediated food allergy. Samples in Sodium Phosphate Buffer were thawed and a small quantity was introduced into the skin of selected patients allergic to caseins and/or BSA. Five patients were selected independently of their sex and age.

A positive SPT formed a wheal (bump) and flare (redness) at the site of testing within 10 to 20 minutes after allergen introduction. The intensity of skin response was expressed in terms of wheal mean diameter, depending on both the inherent allergenicity of the allergen and the level of skin sensitization (IgE). Results were interpreted by comparing the skin response with negative (the buffer) and positive (histamine dihydrochloride 10%) controls.

8.2 Results and discussion

The results of the Prick Test are shown in Table 8.1 for the samples of BSA and in Table 8.2 for those of casein. Non-treated samples are reported together with High Pressure treated and/or hydrolyzed samples. The peptides were produced under pressure or by hydrolysis induced by HHP.

Table 8.1. In vivo analysis of skin response (mean diameter in mm) of BSA samples treated with High Hydrostatic Pressure and/or enzymatic treatment.

| Allergen | Patient 1 | Patient 2 | Patient 3 | Patient 4 | Patient 5* |
|----------|-----------|-----------|-----------|-----------|------------|
| B | 3 | 0 | 0 | 4 | 0 |
| BP | 3 | 2 | 1 | 3 | 0 |
| BP-HDC | 9 | 0 | 2 | 4.5 | 0 |
| BP-HDT | 8 | 0 | 2 | 3 | 0 |
| BP-HAC | 0 | 0 | 0 | 4 | 0 |
| BP-HAT | 3 | 0 | 0 | 3.5 | 0 |
| Buffer | 0 | 0 | 0 | 0 | 0 |

*: Patient only with sensitization to casein and milk globulins; B: BSA; BP: High Pressure processed BSA; BP-HDC: BSA proteolysis assisted by HHP with chymotrypsin; BP-HDT: BSA proteolysis assisted by HHP with trypsin; BP-HAC: BSA proteolysis induced with chymotrypsin after HHP; BP-HAT: Caseins proteolysis induced with trypsin after HHP; HHP: High Hydrostatic Pressure

Table 8.2. *In vivo analysis of skin response (mean diameter in mm) of BSA samples treated with High Hydrostatic Pressure and/or enzymatic treatment.*

| Allergen | Patient 1 | Patient 2 | Patient 3 | Patient 4 | Patient 5 |
|----------|-----------|-----------|-----------|-----------|-----------|
| C | 10 | 0 | 6 | 5 | 2 |
| CP | 11 | 4 | 5 | 4.5 | 0 |
| CP-HDC | 9 | 3 | 7 | 3 | 0 |
| CP-HDT | 6 | 3 | 6 | 6 | 0 |
| CP-HAC | 9 | 2 | 6 | 5 | 0 |
| CP-HAT | 3 | 0 | 0 | 4 | 2 |
| Buffer | 0 | 0 | 0 | 0 | 0 |

C: Caseins; CP: High Pressure processed Caseins; CP-HDC: Caseins proteolysis assisted by HHP with chymotrypsin; CP-HDT: Caseins proteolysis assisted by HHP with trypsin; CP-HAC: Caseins proteolysis induced with chymotrypsin after HHP; CP-HAT: Caseins proteolysis induced with trypsin after HHP; HHP: High Hydrostatic Pressure.

The results show that the allergic response of the patients was different. The prevailing trend of the skin responses suggested that High Hydrostatic Pressure did not affect the allergenicity because HHP treated samples induce skin responses which are comparable to those induced by untreated samples and in some cases the allergenicity increased, this demonstrating that HHP promoted the exposure of some epitopes responsible of allergenic response.

The patient n. 1 had a negative response to BP-HAC (hydrolysis with chymotrypsin induced after HHP), while in case of casein, the sample CP-HAT (Caseins proteolysis induced with trypsin after HHP) induced a lower allergic response in some patients (1, 3 and 4).

8.3. Conclusions

The results demonstrated that, even though the allergenicity of the peptides varied with the specific patient immunoresponse, the application of high hydrostatic pressure treatment or its combination with the hydrolysis reaction can be very effective in controlling the allergic reaction since the treatment was able to modify the position of the epitopes responsible of the allergy symptoms.

In particular, if the HHP treatments did not significantly affect the peptide allergenicity, when HHP was combined with hydrolysis very different results were obtained in terms of wheals diameters if compared with the control.

The high pressure assisted or induced hydrolysis reaction clearly impact on the allergenicity of peptides. In some cases the exposure of some allergenic epitopes can even increase the immunoresponse detected with the *in-vivo* tests but in some operating conditions the combined treatment was demonstrated to have the great potential of reducing or even eliminating the allergenic power of the hydrolysed proteins.

Chapter IX

Conclusions

The utilization of traditional methods, like enzymatic treatments and thermal processes for the treatment of food allergens showed several limits and drawbacks, such as scarce effect on the allergenicity and the reduction of the nutritional value of foods.

This PhD thesis aimed at analyzing the application of a non-thermal technology, namely the High Hydrostatic Pressure process, to reduce the allergenicity of food allergens.

This work investigated the effects of high hydrostatic pressure on the allergens structure and on the reaction of hydrolysis with proteolytic enzymes.

It has been confirmed that the application of high hydrostatic pressure is able to induce protein gelation and/or unfolding, depending on process operating conditions and protein concentration. The transition between the two phenomena seemed to take place at a certain critical pressure level, which depended on protein concentration and isoelectrical point.

At pressure levels and holding times below the critical conditions, protein unfolding can be modulated and for globular proteins reached its maximum value at pressure level of about 300-400 MPa. As a consequence of the structural modifications induced by HHP treatments, both globular and non-globular treated proteins were more prone to the attack of the enzymes during hydrolysis, thus higher hydrolysis degree can be obtained.

The modifications induced by high hydrostatic pressures on food allergens can eventually affect the protein allergenicity. The major conclusions can be summarized as follows:

1) EFFECT OF HHP ON ALLERGENS STRUCTURE:

- ❑ Critical transition of globular proteins, i.e. the beginning of gelation phenomena, depended on operating conditions (pressure level, holding time and temperature) and proteins properties (protein concentration and isoelectric point).
- ❑ The extent of protein gelation is enhanced at higher pressure and longer holding times, as demonstrated by the rheological characterization in both dynamic (oscillating tests) and stationary regimes (flow measurements). In particular, at higher pressure levels

Conclusions

and more prolonged treatment times, BSA gels showed higher storage moduli, that meaning that the hardness of the gels was increased.

- ❑ Similarly, at stationary regime, deviations from the Newtonian behavior were more likely to occur at higher pressure level and the holding times.

2) PROTEIN UNFOLDING INDUCED BY HHP:

- ❑ The unfolding of globular proteins, such as Bovine Serum Albumin, can be maximized at HHP operating conditions below the critical values inducing protein gelation.
- ❑ If the HHP process was carried out at pressure levels lower than the critical value, changes of the secondary structure of the processed proteins can be detected. The structural modifications are maximized at 600 MPa, at which the formation of molecular aggregates occurred and the transition from α -helices to β -sheets anticipated the gelation phenomena.
- ❑ Protein unfolding enhanced proteins functional properties: at pressure levels of 300-400 MPa, the exposure of reactive thiols groups as well as the values of the foaming properties were maximized.

3) EFFECT OF HHP ON ENZYMATIC HYDROLYSIS:

- ❑ At the operating conditions maximizing the unfolding, high hydrostatic pressure treatments accelerated also protein hydrolysis reaction.
- ❑ The values hydrolysis degree obtained were higher in HHP assisted hydrolysis than in HHP induced hydrolysis, i.e. when proteins were pre-treated with HHP process before undergoing the hydrolysis reaction.
- ❑ HHP assisted hydrolysis allowed the production of peptides with low molecular weight. In addition, the structure and the dimensions of the peptides produced under HHP were different from those of the peptide obtained when the hydrolysis was carried out at atmospheric pressure. Peptides with low size, high homogeneity and low tendency to aggregate were formed.
- ❑ High Hydrostatic pressure can also affect the hydrolysis reaction of non-globular proteins, such as caseins. Even though the unfolding of non-globular protein did not occurred, a positive effect of HHP on hydrolysis reaction can be observed, this suggesting the applicability of the combined HHP-Hydrolysis process to a wide range of proteins.

- ❑ The effect of high pressure treatment on the hydrolysis reaction was confirmed by comparing the kinetics of hydrolysis at atmospheric pressure and high pressure. If the hydrolysis was assisted by high hydrostatic pressure, both reaction rate constant and enzyme inactivation constant increased.

4) ALLERGENIC POWER OF THE SAMPLES:

- ❑ High Hydrostatic Pressure did not affect the allergenic reaction. In fact, as observed with in vivo tests, HHP treated samples induced skin responses which were comparable to those induced by untreated samples.
- ❑ High Hydrostatic Pressure assisted hydrolysis may reduce the allergenic power of hydrolyzed and in vivo tests have demonstrated that with some sample a negative response was obtained.

The application of high hydrostatic pressure in combination with enzymatic hydrolysis with proteolytic enzymes induced structural modifications of proteins. In optimized operating conditions, these structural changes could have the great potential to modulate the protein allergenic power by changing the position of the epitopes, representing the reactive part of the allergy reaction.

However, more specific analysis identify the position of the epitopes in the sequence and/or in the structure of the protein will be necessary in order to better understand the mechanism of action of HHP assisted hydrolysis inducing the reduction of the allergenic power of proteins.

Further studies can be also addressed to define the specific cutting points of the enzymes during the enzymatic attack of the proteins in order to maximize the effectiveness of the hydrolysis reaction and individuate the enzymes with higher affinity with the specific protein.

In conclusion, even though further research efforts to scale up the process are necessary, this PhD thesis has demonstrated that high hydrostatic pressure technology has a great potential to be used for the production of hypoallergenic peptides.

