

Combined Effect of High Hydrostatic Pressure and Pulsed Light on Protein Hydrolysis

Serena De Maria^a; Giovanna Ferrari^{a,b}; Paola Maresca^b, Gianpiero Pataro^a

^a Department of Industrial Engineering, University of Salerno, Via Giovanni Paolo II, 84084 Fisciano (SA), ITALY

^b Prodal Scarl, Via Ponte Don Melillo, 84084 Fisciano (SA), ITALY
p.maresca@prodalricerche.it

Protein hydrolysates are complex mixtures of peptides of different chain length produced from purified protein by enzymatic hydrolysis. The efficiency of the hydrolysis is defined by a global value known as hydrolysis degree (HD), which is the fraction of peptide bonds cleaved in the treated protein. Hydrolysates, showing a similar value of HD, may differ in the composition (free amino acids, dipeptides, tripeptides and/or oligopeptides) and in their absorption kinetics. It is well known that protein hydrolysates containing mostly di- and tripeptides are more rapidly absorbed than those based on longer peptides. Novel methodologies have been investigated in order to control the extent of the enzymatic hydrolysis as well as the quality of the produced hydrolysates. Among them, non-thermal technologies, such as High Hydrostatic Pressure (HHP) and Pulsed light (PL), modify the conformational structure of proteins. Proteolysis can be modulated if it is conducted in combination with these technologies which are able to change the availability of peptide bonds exposed to the enzymatic action. The work aimed at investigating the effects of the combination of these two technologies on the hydrolysis kinetics of a target protein: Bovine Serum Albumin (BSA). BSA protein (5 mg/mL) in sodium phosphate buffer (50 mM, pH =7.5) was treated with PL and HHP at different processing conditions, namely pressure level and treatment time in the case of HHP and treatment time and energy input in the case of PL. The two technologies were applied in different orders: HHP or PL treatment followed by non-thermal assisted hydrolysis; HHP and PL assisted hydrolysis; HHP and PL treatment followed by thermal hydrolysis. Chymotrypsin and trypsin (E/S ratio = 1/10) were used to hydrolyze the BSA protein solutions. The experimental data demonstrate that both HHP and PL treatments are able to cause protein unfolding. Higher pressure level and prolonged times in HHP processes as well as higher number of pulses and lower distances from the lamp in PL treatments enhance protein hydrolysis. The combination of PL and HHP treatments is suitable to increase the extent of BSA proteolysis with chymotrypsin and trypsin. The highest value of the hydrolysis degree is observed when HHP assisted hydrolysis and PL assisted hydrolysis are applied in sequence.

1. Introduction

Protein hydrolysates are specially designed for nutritional support of special patients, food ingredients and/or additives. For those applications, high quality standards of the hydrolysates are necessary, thus requiring an efficient control of the hydrolysis kinetics (Tavano, 2013). Protein hydrolysis, which is based on the cleavage of peptide bonds, can be carried out by chemical or enzymatic processes. The kinetics of the acid hydrolysis, which implies the utilization of strong acids, namely HCl for more than 24 h, at high temperature, are almost complicated to be controlled and tend to modify the produced amino acids. Similarly alkaline hydrolysis can chemically reduce cysteine, arginine, threonine, serine, isoleucine, and/or lysine content, and induce the formation of amino acid crosslinks and isomerization of lysine residues (Provansal et al., 1975). In enzymatic hydrolysis, indeed, milder processing conditions can be used and a higher control on kinetics may be ensured thanks to the substrate specificity of the enzymes. As a consequence the enzymatic hydrolysis allows the protein hydrolysates, with better defined chemical and nutritional characteristics, to be produced (Castro et al., 2011). The extent of enzymatic hydrolysis mainly depends on the accessibility of the peptide bonds, which

stabilize the protein structure. It is well known that proteins are biological polymers made up of polypeptide chains (primary structure) which are stabilized by local interactions to form α -helix and β -pleated sheet structures (secondary structure). Interactions between polar, nonpolar, acidic, and basic R-groups within the polypeptide chains create the complex three-dimensional tertiary structure of a protein. Finally the orientation and arrangement of subunits in a multi-subunit protein determine the quaternary structure of the proteins. Several interactions stabilize the four levels of protein structures: Van der Waals forces, hydrogen bonds, salt bridges, ionic interactions, loop tension, helix dipole interactions, and disulphide bridges. In order to increase the hydrolysis efficiency, besides making the correct choice of the enzyme, the enzyme sensitivity can be increased by inducing protein unfolding, which improves the exposure of the binding sites. In the recent years novel methodologies have been investigated in order to control the extent of the enzymatic hydrolysis as well as the quality of the produced hydrolysates. Among them, non-thermal technologies, such as High Hydrostatic Pressure (HHP) and Pulsed light (PL), affect protein inducing modifications in protein conformational structure. HHP process is able to induce functional and structural modifications of food biomolecules and, in the case of proteins, the structural changes induced by the HHP treatments depend on several factors: protein species, pH, ionic strength, type of buffer, pressure and temperature levels, and processing time (Messens et al., 1997). HHP processes modify the quaternary structure, which is stabilized by hydrophobic interactions, the tertiary structure (causing the reversible unfolding), and secondary structure (causing the irreversible unfolding). In the case of globular proteins, such as albumin, HHP processes promote the dissociation and the following assembly of systems with higher complexity on the one hand, and the unfolding and misassembly on the other one (Jaenicke, 1987). Similarly Pulsed Light technology is able to induce structural modifications/denaturation of proteins due to the photothermal, photophysical and photochemical effects of the UV-NIR radiations (Shriver et al., 2011). These effects result in changes in the conformational structure, the loss of conformational epitopes as well as aggregation of proteins. Specifically, Pulsed Light can ionize the molecules due to the energy released in each pulse while the VIS and NIR radiations are responsible respectively of vibrations and rotations of the molecules (Yang et al., 2012).

The work investigates the effects of HHP and PL technologies on the hydrolysis kinetics of a target protein. Bovine Serum Albumin (BSA), a globular protein of bovine meat and whey proteins and responsible for several allergic cross-reactions, was selected for the experiments. The two technologies were applied in different orders: HHP or PL treatment followed by non-thermal assisted hydrolysis; HHP and PL assisted hydrolysis; HHP and PL treatment followed by thermal hydrolysis. The aim is to define the optimal combination of the processing conditions and/or technologies to be applied in order to increase the hydrolysis degree and to produce protein hydrolysates containing mostly di- and tripeptides, which can be more rapidly absorbed.

2. Materials and Methods

2.1 Preparation of the samples

BSA (CAS NUMBER: 9048-46-8, Sigma-Aldrich, Italy) was dissolved at a temperature of 25 °C in Sodium Phosphate Buffer (50 mM, pH=7.5), according to the protocols reported by Penãs et al. (2006), at a fixed concentration (5 mg/mL) and gently mixed 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 before the hydrolysis treatments. Two different enzymes, chymotrypsin and trypsin (Sigma-Aldrich, Italy) were tested in the experimental plan. The enzymatic solutions were prepared by dissolving the enzymes (50 mg/mL) at a temperature of 25 °C in Sodium Phosphate Buffer (50 mM, pH=7.5) and stored at refrigerated conditions (4°C) until the utilization. For each test, the enzymatic solutions were added to BSA solution in order to achieve an enzyme/substrate ratio equal to 1/10.

2.2 Experimental apparatus

The high pressure multi-vessel system U111 (Unipress, Warsaw (Poland)) was used for the HHP treatments. The system can operate at pressures up to 700 MPa and temperatures from -40 °C to 100 °C and includes five high pressure reactors, made from Cu-Be alloy and working in parallel, immersed in a thermostatic bath. Each reactor is equipped with a manual cut-off valve and a K-type thermocouple, placed at the bottom of each reactor and able to measure the temperature of the liquid in contact with the samples. The pressurization system consists of a hydraulic low-pressure pump connected to a high pressure intensifier, while a high pressure manual valve is used to release the pressure. The compression rate can be changed in the range of 2,5-25 MPa/s and for the experimental campaign was set at 10,5 MPa/s. A silicon oil was used as pressurization fluid and heating fluid of the thermostatic bath. Due to the technical features of the high pressure system, the pressurization causes an average temperature increase of 4°C/100 MPa, while during

the holding time under pressure, the temperature quickly reduces to the initial setup value. The multi-vessel system is also equipped with a control unit to set the pressure level, set and control the pressurization ramp, start the filling of the intensifier and reactors, as well as measure and control the pressure and temperature levels in the reactors. A data acquisition system, U111-DAS, records the pressure measured in the reactors and that of the feeding line as well as the temperature measured in the reactors and that of the thermostatic bath. PL treatments were carried out using a bench-top RS-3000C SteriPulse-XL system (Xenon Corp., Wilmington, Mass., USA) which included a power/control module, a treatment chamber and lamp housing with a linear 16" xenon flash lamp. The system generates 3 pulse/s (360 μ s width) of a polychromatic light in the wavelength range between 200 and 1100 nm. An adjustable 15.75 x 40.64 cm stainless steel tray in the treatment chamber allowed changing the vertical distance (n) from the quartz window surface from 1.93 to 16.46 cm. Consequently, the intensity of the flashes of light (F_p) that reach the target can be changed from 1.21 (in correspondence of the smaller distance) to 0.22 J/cm²/pulse (in correspondence of the higher distance). A forced air system with filter was used to remove ozone and heat from both the housing lamp and treatment zone. Thermal hydrolysis was carried out in an agitated incubator under gentle mixing.

2.3 Experimental plan

BSA samples were hydrolysed at different combinations of the processing parameters (pressure (P), temperature (T) and time (t) in HHP treatments; distance from the lamp (n) and time (t) in PL treatments) as well as at different combinations of HHP and PL technologies. The Table 1 summarizes the experimental plan.

Table 1: Experimental plan

Test	HHP	PL	Thermal Hydrolysis	Enzyme	Note
(HHP)	P=0.1- 400 MPa T= 37 °C t=20-30 min	-	-	Chymotrypsin; Trypsin	HHP assisted hydrolysis
(PL)	-	n=7.01-10.82 cm t=0-120 s	-	Chymotrypsin; Trypsin	PL assisted hydrolysis
(H)	-	-	T=37 °C t= 25 min	Chymotrypsin; Trypsin	Thermal hydrolysis
HHP+(H)	P=400 MPa T=25 °C t=30 min	-	T=37 °C t= 25 min	Chymotrypsin; Trypsin	HHP pre-treatment followed by a thermal hydrolysis
PL+(H)	-	n=7.01 cm T=25 °C t=120 s	T=37 °C t= 25 min	Chymotrypsin; Trypsin	PL pre-treatment followed by a thermal hydrolysis
HHP+(PL)	P=400 MPa T=25 °C t=30 min	n=7.01 cm T=37 °C t=120 s	-	Chymotrypsin; Trypsin	HHP pre-treatment followed by a PL assisted hydrolysis
PL+(HHP)	P=200 MPa T=37 °C t=30 min	n=7.01 cm T=25 °C t=120 s	-	Chymotrypsin; Trypsin	PL pre-treatment followed by a HHP assisted hydrolysis
HHP+PL+(H)	P=400 MPa T=25 °C t=30 min	n=7.01 cm T=25 °C t=120 s	T=37 °C t= 25 min	Chymotrypsin; Trypsin	HHP and PL pre-treatment followed by a thermal hydrolysis

Different protocols were used to carry out the HHP and PL experiments. For HHP tests, BSA samples (5 mL) were sealed in flexible pouches made of a multilayer film (Polyethylene-Aluminium-Polypropylene). The pouches were introduced into the U111 vessel and the pressure cycle set at the desired experimental conditions. At the end of the pressure release, the pouches were collected from the vessel and stored at 4 °C before the chemical characterization. In PL experiments, BSA samples (5 mL) were placed in plastic Petri dishes (diameter=35 mm, height=10 mm) and treated at different distances from the lamp and different treatment times according to the experimental plan reported in Table 1. In thermal hydrolysis, BSA samples (5 mL) were placed in plastic tube (15 mL) and incubated at 37 °C under gentle mixing for 25 min. At the end of each hydrolysis test, the hydrolytic reaction was stopped by heating the samples at 90 °C for 5 min and then

quickly cooled (25 °C). The hydrolysed proteins were immediately used for the determination of the degree of hydrolysis.

2.4 Hydrolysis degree by OPA reaction

Hydrolysis degree was measured according to the protocols described by Nielsen et al. (2001). The method is based on the reaction of primary amino nitrogen with ortho-phthalaldehyde (OPA), which form a colored compound detectable at 340 nm in a UV-Vis spectrophotometer (V-650, Jasco Europe, Italy). OPA reagent was prepared by dissolving Natetraborate decahydrate, Na-dodecyl-sulfate (SDS), o-phthalaldehyde 97% (OPA) and dithiothreitol 99% (DTT) in deionized water solution. A Serine solution (0.1 g/L) in deionized water was used as standard. In each measurement 3 mL of OPA reagent were added to 400 μ L of deionized water (blank), serine solution (standard) or sample. All the measurements were performed in triplicate and carried out at 25°C using deionized water as a control after 2 minutes of reaction. The absorbance measurements were worked out in order to determine the hydrolysis degree (HD), which is estimated as the increase of the free amino groups in protein hydrolysates per mg of protein (ΔC (μ mol/mg)).

3. Results and discussion

Preliminary experiments were carried out in order to determine the effect of the processing conditions on the hydrolysis of BSA samples treated in HHP assisted hydrolysis as well as PL assisted hydrolysis cycles with chymotrypsin and trypsin. The results, expressed as increase of the free amino groups content in protein hydrolysates (ΔC (μ mol/mg)), are shown in Figure 1.

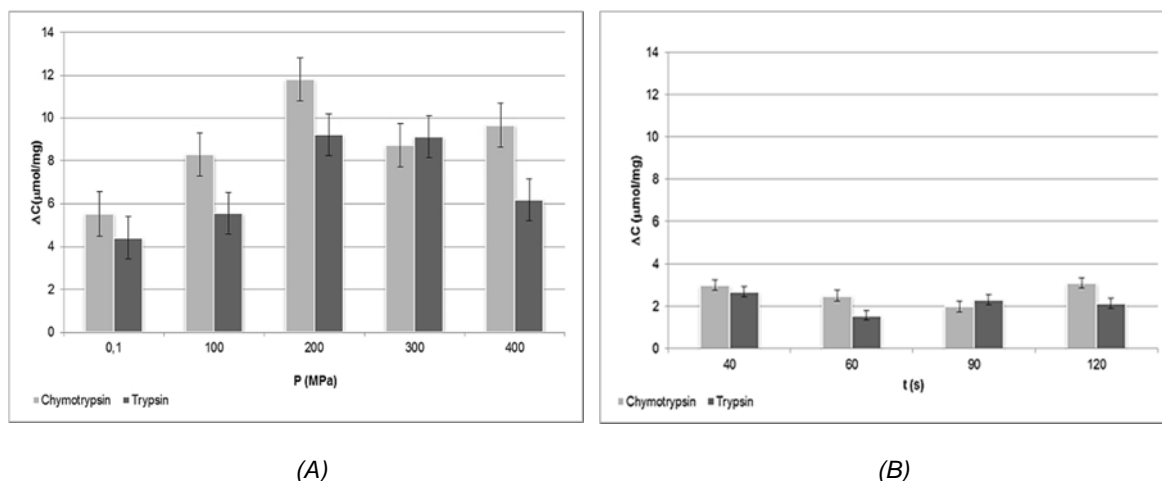


Figure 1: Extent of the hydrolysis degree evaluated for BSA samples processed in HHP assisted hydrolysis treatments (A) carried out at different pressure levels and fixed operating temperature and time (37 °C, 30 min) and in PL assisted hydrolysis treatments (B) carried out at different operating times and fixed operating temperature and distance from the lamp (37 °C, 7.01 cm)

In HHP assisted hydrolysis treatments, the values of the hydrolysis degree (HD) depends on the pressure level and the type of enzyme used for the hydrolysis. The concentration of the detectable free amino groups increases with the pressure level up to 200 MPa, while at higher pressure level lower values of HD are estimated. In a previous experimental campaign, our research group demonstrated that HHP process is able to induce protein unfolding in the pressure range between 100 and 300 MPa, which allows the masked peptide bonds to be exposed and consequently attacked by the proteolytic enzymes (De Maria et al., 2014). Moreover at higher pressure levels protein peptides tended to form small aggregates, thus reducing the efficiency of the proteolysis. PL assisted hydrolysis shows to be less effective than HHP assisted hydrolysis, as demonstrated by the lower HD values estimated for the BSA samples treated at different processing times. The extent of the hydrolysis, moreover, does not depend linearly on the treatment time, this demonstrating that the prolonged exposure to PL may modify BSA conformational structure. Among the tested enzymes, chymotrypsin shows a higher efficiency in hydrolysing BSA samples. The experimental data, shown in Figure 1, were worked out in order to select the optimal processing conditions to be tested in the combined treatments. Figure 2 reports the HD values estimated for the BSA samples treated in single HHP or PL

assisted hydrolysis processes as well as in combined HHP – PL assisted hydrolysis applied in two different orders.

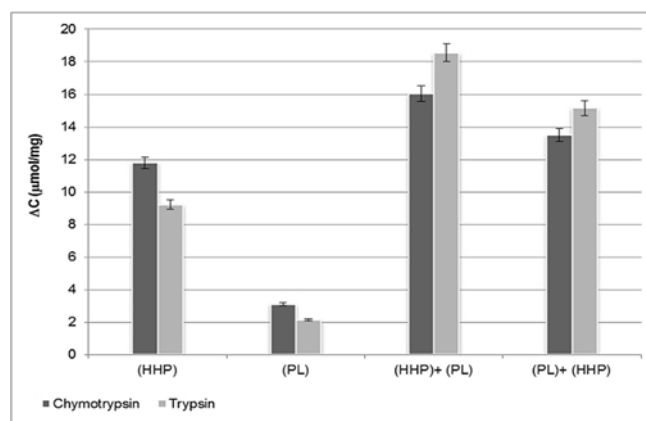


Figure 2: Extent of the hydrolysis degree evaluated for BSA samples processed in HHP assisted hydrolysis (200 MPa, 37 °C, 30 min), in PL assisted hydrolysis treatments (120 s, 7.01 cm) and in combined HHP –PL assisted hydrolysis treatments (200 MPa, 37 °C, 30 min; 120 s, 7.01 cm) with chymotrypsin and trypsin

The experimental data demonstrate that the efficiency of the combined HHP – PL assisted hydrolysis depends on the order of application of the tested technologies and the type of enzyme. If the HHP assisted hydrolysis is applied before the PL assisted hydrolysis, the extent of the hydrolysis is higher than in the opposite order. If the HD values of the combined treatment are compared to the ones of the single treatment, as shown in Figure 2, a synergistic effect of the combined innovative treatments can be observed. The opposite combination, indeed, gives a less than additive effect on the extent of hydrolysis with chymotrypsin and still a synergistic effect in the case of hydrolysis with trypsin. Moreover in the combined treatments trypsin shows to be more effective on BSA samples than chymotrypsin. This result demonstrates that the first step of hydrolysis, independently on the technology applied, is able to unmask the peptide sequences which trypsin can hydrolyse more efficiently than chymotrypsin. Finally the combination of innovative technologies and thermal hydrolysis was tested to verify the applicability of this hurdle approach with the aim of reducing the processing time and improving the quality of the thermal hydrolysates. The processing conditions of the HHP and PL treatments, which were able to maximize the modification of the BSA conformational structure (data not shown) were selected for the experiments. The results of the different combinations tested are reported in Figure 3 in terms of increase of the free amino acids content detected by OPA method.

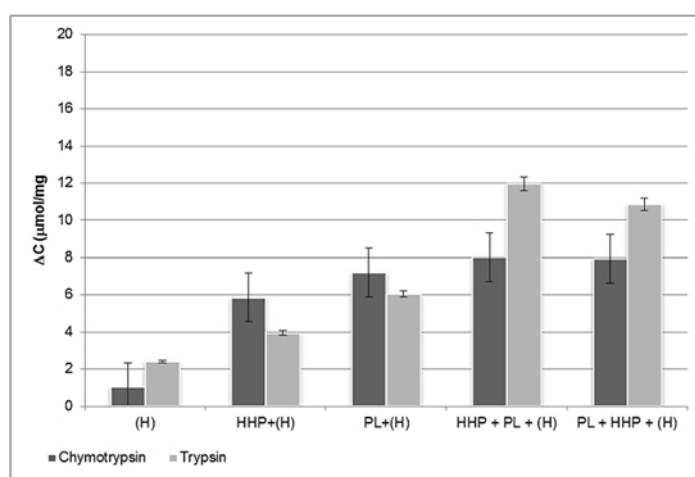


Figure 3: Extent of the hydrolysis degree evaluated for BSA samples processed in thermal hydrolysis treatments ((H)=37 °C, 25 min) carried out after HHP treatments (HHP= 400 MPa, 25 °C, 30 min) and/or PL treatments (PL=120 s, 7.01 cm, 25 °C)

According to the data shown in Figure 3, low level of hydrolysis are achieved by the thermal hydrolysis in the testing conditions, ranging the HD values between 1.03 (with chymotrypsin) and 2.41 μmol/mg (with trypsin).

The pre-treatment of the protein solution by a non-thermal technology (HHP or PL) significantly improves the efficiency of the thermal hydrolysis at a parity of the processing conditions, thus confirming the relevant role of the protein unfolding in the control of the hydrolysis kinetics. The HD values of the combined treatments increase up to 5.86 (HHP pre-treated samples) and 7.19 $\mu\text{mol}/\text{mg}$ (PL pre-treated samples) for the BSA solutions hydrolysed with chymotrypsin and to 3.94 (HHP pre-treated samples) and 6.05 $\mu\text{mol}/\text{mg}$ (PL pre-treated samples) for the BSA solutions hydrolysed with trypsin. The pre-treatment of the BSA solutions by means of HHP and PL technologies in sequence slightly affects the efficiency of the thermal hydrolysis carried out with chymotrypsin, while a further increase of the HD values is detected in case of thermal hydrolysis with trypsin. This latter result confirms that the combination of HHP and PL treatment enhances the activity of trypsin, probably due to the conformational modification of BSA structure. Also in this set of experiments the order of hurdles (HHP and PL treatments) still influences the extent of the hydrolysis, resulting more effective the combined treatment in which HHP process is the first hurdle.

4. Conclusions

The extensive experimental campaign demonstrates that non-thermal technologies, namely HHP and PL treatments, are suitable to control the hydrolysis kinetics of the tested protein. If HHP or PL processes or their combinations are applied as pre-treatment on protein solution before the thermal hydrolysis, irreversible structural modifications of the protein occurs and promote the proteolytic action of the enzymes. The same mechanism can explain the extensive hydrolysis achieved in HHP assisted or PL assisted hydrolysis. However the occurrence of protein unfolding during the treatments allows the buried peptide bonds to be cleaved more efficiently by the proteolytic enzymes, thus increasing significantly the extent of the hydrolysis reactions. Moreover the HHP assisted hydrolysis followed by the PL assisted hydrolysis shows a synergistic effect, demonstrating to be the optimal combination of the technologies tested. The type of the proteolytic enzymes, however, influences the efficiency of the hydrolysis reaction. Further studies are required to define the mechanism of action of both HHP and PL assisted hydrolysis in combined treatments as well as to verify the composition of the novel protein hydrolysates in terms di- and tripeptides and to determine the kinetics of hydrolysates absorption.

References

- Castro H.C., Abreu P.A., Geraldo R.B., Martins R.C.A., Santos R., Loureiro N.I.V., Cabral L.M., Rodrigues C.R., 2011, Looking at the proteases from a simple perspective, *J. Mol. Recognit.* 24, 165–181.
- De Maria S., Maresca P., Ferrari G., Oliveira C., 2014, Study of the effects of High Hydrostatic Pressure (HHP) and Pulsed Light (PL) on BSA structure and hydrolysis, *Proc.1st Conference on food structure design*, 47, ISBN: 978-989-97478-5-2.
- Jaenicke, R., 1987, Folding and associations of proteins, *Prog. Biophys. Mol. Bio.* 49, 117-237.
- Messens W., Van Camp J., Huyghebaert A., 1997, The use of high pressure to modify the functionality of food proteins, *Trends Food Sci.Tech.* 4, 107-112.
- Nielsen P.M., Petersen D., Dambmann C., 2001, Improved method for determining food protein degree of Hydrolysis, *J. Food Sci.* 66(5), 642-646.
- Penãs E., Prestamo G., Baezac M.L., Martinez-oleroc M. I., Gomez R., 2006, Effects of combined high pressure and enzymatic treatments on the hydrolysis and immunoreactivity of dairy whey proteins, *Int. Dairy J.* 16, 831–839.
- Provansal M.M.P., Cuq J.A., Cheftel J.C., 1975, Chemical and nutritional modifications of sunflower proteins due to alkaline processing. Formation of amino acid crosslinks and isomerization of lysine residues, *J. Agric. Food Chem.* 23, 938–943.
- Shriver S., Yang W., Chung S.Y., Percival S., 2011, Pulsed Ultraviolet Light Reduces Immunoglobulin E Binding to Atlantic White Shrimp (*Litopenaeus setiferus*) Extract, *Int. J. Environ Res. Public Health* 8, 2569-2583.
- Tavano O.L., 2013, Protein hydrolysis using proteases: an important tool for food biotechnology, *J. Mol. Catal. B-Enzym.* 90, 1-11.
- Yang W.W, Mwakatage N.R, Goodrich-Schneider R., Krishnamurthy K, Rababah T.M., 2012, Mitigation of major peanut allergens by Pulsed Ultraviolet Light, *Food Bioprocess Tech.* 5(7), 2728 - 2738.