Scientific Bulletin. Series F. Biotechnologies, Vol. XXV, No. 1, 2021
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

PROTEIN AND AMINO ACID PROFILE ANALYSIS OF AGRI-FOOD WASTE BIOMASSES

Eleonora CALZONI[1], Alessio CESARETTI[1, 2], Nicolò MONTEGIOVE[1], Roberto Maria PELLEGRINO[1], Leonardo LEONARDI[3], Carla EMILIANI[1, 2]

[1]Department of Chemistry, Biology and Biotechnology, Biochemistry and Molecular Biology Section, University of Perugia, Via del Giochetto, 06123 Perugia, Italy
[2]Centro di Eccellenza sui Materiali Innovativi Nanostrutturati (CEMIN), University of Perugia, Via del Giochetto, 06123 Perugia, Italy
[3]Department of Veterinary Medicine, University of Perugia, Via San Costanzo 4, 06126 Perugia, Italy

Corresponding author email: nicolo.montegiove@gmail.com

Abstract

The degradation of biomasses derived from agriculture and food industry presents the double advantage of both eliminating often polluting wastes and giving the possibility of introducing novel bio-derived products into the market. The recovery of the protein component and its transformation into protein hydrolysates is generally carried out chemically or enzymatically and brings great added value to waste biomasses. The recovery of the protein component from waste products is extremely advantageous inasmuch as bio-based products can be reintroduced into the market by virtue of their high added value in full compliance with the perspective of the Circular Economy. The products obtained can be used as food supplements both in human and animal nutrition, as growth stimulants in agriculture, or as biofertilizers. The peculiar characteristics of the obtained protein hydrolysates allow their multiple applications in many industrial fields. Therefore, the aim of this work is to assess the protein and amino acid (AA) profiles of agri-food biomass, i.e. soy wastes, previously subjected to an enzymatic hydrolysis process. The protein profile of the initial biomass and the corresponding hydrolysate was analyzed by SDS-PAGE followed by Coomassie Blue staining, while the AA profile was evaluated by quadrupole time-of-flight liquid chromatography/mass spectrometry (Q-TOF LC/MS). The results indicate how the enzymatic hydrolysis process breaks down the peptide bonds of the protein component in the biomass, leading to the formation of hydrolysates rich in small peptides and free AAs.

Key words*: Circular Economy, enzymatic hydrolysis, enzyme immobilization, protein hydrolysates, waste biomasses.*

INTRODUCTION

The use of biomasses for industrial purposes represents an extremely alluring tool for the recovery of the waste products coming from the agribusiness and food industry.

The recovery of these materials provides environmental and socioeconomic benefits, as the problems concerning their disposal are reduced and, at the same time, they can be used as alternative energetic sources (Calzoni et al., 2019; Calzoni et al., 2020; Cesaretti et al., 2020; Calzoni et al., 2021). The enhancement of waste products deriving from agriculture is also one of the main objectives of *Circular Economy*, which is extremely supported by European Union (EU) policies that promote the use of bioproducts obtained from these types of materials (Stahel, 2016; Tuck et al., 2012; Sherwood, 2020). Biomasses are organic raw materials of natural biological origin that can be used as substrates for the production of bio-based products. Broadly speaking, waste biomasses are classified into different categories according to their chemical composition (Tuck et al., 2012), based on which it is possible to obtain different bio-products: polysaccharides, lignin, triglycerides (from fats and oils), or proteins. All these by-products can thus be exploited to make compost, biogas, or other products with high-added value (Cesaretti et al., 2020; Calzoni et al., 2021). Particularly, the production of high added value protein hydrolysates obtainable from these waste materials is extremely interesting (Martínez-Alvarez et al., 2015). In fact, most of the agricultural waste biomass has a high content of recoverable proteins in the form of hydrolysates, which may find various applications in many industrial and non-

industrial sectors. Furthermore, hydrolysates of vegetable origin are safe products and do not present any toxicity for plants or humans.

The production of protein hydrolysates can be obtained by chemical, microbial, or enzymatic routes (Callegaro et al., 2019; Cesaretti et al., 2020). Chemical hydrolysis is carried out under acidic or basic conditions at high temperatures. However, this type of processes leads to the formation of poor-quality products, due to the loss of assimilable amino acids (AAs) such as tryptophan, cysteine, arginine, threonine, serine, and isoleucine, and to the production of modified AAs such as lysinoalanine and lanthionine, as well as secondary products such as chlorides (Tsugita & Scheffler, 1982; Fountoulakis & Lahm, 1998; Tavano, 2013; Corte et al., 2014; Cesaretti et al., 2020). Further to this, high content of acidic or basic residues can be found in the final hydrolysate which limits their applicability, especially in the agricultural sector and in the food industry (Chervan & Deeslie, 1984; Cesaretti et al., 2020). Microbial hydrolysis, on the other hand, is obtained through the use of specific microbial cultures which allow, thanks to their enzymatic pathways, good quality hydrolysates to be obtained; this type of technique is widely used to produce hydrolysates starting from plant biomasses (Lòpez-Barrios et al., 2014; Li-Chan, 2015; Bah et al., 2016). Hydrolysis mediated by microorganisms excludes the use of chemicals or high temperatures, preventing or limiting the loss of essential amino acids (EAAs). However, this type of technique is often expensive and there could be a certain susceptibility of the microbial strain chosen if changes in the environmental conditions occur (Hou et al., 2017).

Protein hydrolysates can also be obtained enzymatically through the use of purified enzymes in their free or immobilized form. This type of process is extremely advantageous compared to the chemical or microbial ones both from an economic and environmental point of view, as it is carried out under mild conditions of temperature and pH; furthermore, the problem of the environmental condition susceptibility of the microbial strains is avoided. Enzymatic hydrolysis does not lead to the formation of unwanted and toxic secondary products, it preserves the structure of AAs and makes the final product more soluble (Fox et al., 1982; Clemente et al., 1999; Clemente, 2000; Cesaretti et al., 2020).

In this regard, proteases constitute the largest group of enzymes used for the production of protein hydrolysates and represent about 60% of the enzymes used in various industrial fields (Zambare et al., 2011; Sawant & Nagendran, 2014; Souza et al., 2015; Salihi et al., 2017; Calzoni et al., 2021). In fact, these enzymes are able to hydrolyze the peptide bonds in proteins, converting them into small peptides and free AAs. There are many sectors in which protein hydrolysates of vegetable origin can find application; they are used in the animal nutrition field by virtue of their nutritional, physiological, and direct regulatory functions, as they promote the growth rate of the animal, and are used as biofertilizers, as the hydrolysates can improve the assimilation of crop nutrients and to mitigate crop stress (Halpern et al., 2015; Cesaretti et al., 2020). Furthermore, those hydrolysates containing bioactive peptides can be used as antihypertensive, antioxidant, and anti-inflammatory agents (Hou et al., 2017; Cesaretti et al., 2020; Zou et al., 2020). It should also be remembered that the vast majority of protein hydrolysates have a hypoallergenic nature, making them suitable ingredients for infant food formulations or as supplements in the diets of children suffering from severe food allergies (Schaafsma, 2009; Cesaretti et al., 2020).

The purpose of this work is to recover protein hydrolysates from agricultural waste biomass, *i.e.* soybean, obtained by the enzymatic method through the use of immobilized proteases extracted from *Aspergillus oryzae*. The degree of hydrolysis of the original biomass was evaluated primarily by SDS-PAGE followed by Coomassie Blue staining, and subsequently by Ninhydrin Assay and Mass Spectrometry analysis to evaluate the amount of AAs and small peptides formed as a result of hydrolysis.

MATERIALS AND METHODS

Protein Solubilization

The biomass used consists of soy wastes resulting from its processing. The soy waste was mechanically shredded to form a

homogeneous powder. The soy powder was suspended in deionized water and incubated for 1 hour at 80°C. During the incubation, the sample was repeatedly shaken to favor the extraction of proteins. At the end of the incubation time, the sample was centrifuged at 16,000 × g at 4°C for 15 minutes and the soluble part was then collected. This solution was further centrifuged at 16,000 x g at 4°C for 15 minutes and the supernatant containing the total extract of solubilized proteins was collected.

Protein Content Analysis

Protein content in the samples was determined with the Bradford assay (Bradford, 1976) using Quick Start™ Bradford 1× Dye Reagent (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions for one-step determination of protein concentration. The quantitative determination was carried out using the Coomassie Brilliant Blue G-250 dye (Bio-Rad, Hercules, CA, USA), which in the protein-bound form has an absorption peak at 595 nm. The absorbance at 595 nm was measured using a Shimadzu UV-160A UV-Visible Recording Spectrophotometer (Shimadzu Scientific Instruments, Kyoto, Japan). The concentration of the proteins of the samples was obtained from their absorbance using a calibration curve prepared with known concentrations of bovine serum albumin (BSA; Sigma-Aldrich, Saint Louis, MO, USA).

Enzymatic Hydrolysis

In order to obtain soybean protein hydrolysates, proteases from *Aspergillus oryzae*, purchased from Sigma-Aldrich (Saint Louis, MO, USA) were used without any further purification. These fungal proteases consist of a cocktail of different enzymes exhibiting both endoprotease and exopeptidase activities. 200 μg/cm^2 of proteases were immobilized by adsorption on nitrocellulose membranes of the size of 1 cm^2. The soy extract was treated with immobilized enzymes for 4 hours at 37°C and the hydrolysate obtained was collected.

SDS-PAGE and Coomassie Blue Staining Method

The electrophoretic profile of the samples was assessed through SDS-PAGE according to the Laemmli protocol (Laemmli, 1970). An exact quantity of soluble protein extract was mixed with sample buffer 5× (0.5 M Tris-HCl pH 6.8, 10% (w/v) SDS, 50% (v/v) glycerol, 0.01% (w/v) bromophenol blue, and 125 mM dithiothreitol; Sigma-Aldrich, Saint Louis, MO, USA) at a concentration ratio of 4:1 (v/v). Samples were then boiled for 5 minutes and electrophoresed on 15% acrylamide gel (Mini-PROTEAN® 3 Cell, Bio-Rad, Hercules, CA, USA) at 40 mA. Gels were later stained with Coomassie Blue R-250 (Bio-Rad, Hercules, CA, USA).

Hydrolysis Degree Evaluation by Ninhydrin Assay

The degree of hydrolysis was assessed by estimating the concentration of free AAs in solution through the Ninhydrin assay (Rosen, 1957) and by making a comparison with the concentration of free AAs found in the starting extract. The test was carried out using 2,2-dihydroxy-1,3-dioxyhydrindene (Ninhydrin) supplied by Sigma-Aldrich (Saint Louis, MO, USA), which, added to the protein hydrolysate, interacts with the primary amines giving a blue-violet color with maximum absorption at 570 nm. The absorbance at 570 nm was measured using a Shimadzu UV-160A UV-Visible Recording Spectrophotometer (Shimadzu Scientific Instruments, Kyoto, Japan).

Peptides analysis by Q-TOF LC/MS

The raw extract of soybean waste and the hydrolysates produced after 4-hour hydrolysis were analyzed for peptide quantification with Q-TOF LC/MS. Protein hydrolysates obtained from the hydrolysis of the soy waste biomass were diluted with methanol (up to 90% of the final volume) to allow both peptide extraction and protein precipitation. After a centrifugation step, an aliquot of the Supernatant was subject to Q-TOF LC/MS analysis. LC separation was performed on an Agilent 1260 Infinity LC System (Agilent Technologies, Inc., Santa Clara, CA, USA) with a 10 minutes gradient time on a reverse phase column (Ascentis Express Peptide ES-C18 75 x 2.1 mm, 2.7 μm, Supelco Inc., Bellefonte, PA, USA) at 50°C and 0.5 mL/min flow. The mobile phase consisted of water and acetonitrile both containing 0.1% formic acid. Positive polarity

data were acquired on the Agilent 6530 LC/QTOF (Agilent Technologies, Inc., Santa Clara, CA, USA) using an Agilent JetStream source in the range 50-1700 m/z and in Auto MSMS modality, at 5 spectra/sec and 3 spectra/sec for MS and MS/MS, respectively. The acquired raw data were processed with Agilent MassHunter Bioconfirm Software (B.09.00) (Agilent Technologies, Inc., Santa Clara, CA, USA) and searched for the identification of small peptides using MassHunter Metlin Peptides AM PCD library (Agilent Technologies, Inc., Santa Clara, CA, USA).

Statistical analysis

Data shown in this study are reported as mean values of three analyzed samples ± standard error of the mean (SEM). The Student's t-test was used to analyze the significance of the differences between the means of control values (soy extract) and the sample values after the hydrolysis process. The level of significance for the data was set at $p < 0.05$. All statistical tests were done using GraphPad Prism 6.00 for Windows (GraphPad Software, San Diego, CA, USA).

RESULTS AND DISCUSSIONS

In this study, waste resulting from the processing of soy, biomass extremely rich in proteins, was used. The process that led to the production of the protein hydrolysate from the waste is shown in Figure 1. The hydrolysis was carried out using a pool of proteases extracted from *Aspergillus oryzae* immobilized on nitrocellulose membranes. The advantage deriving from the immobilization of enzymes on solid supports is represented by the possibility of reusing the same biocatalysts for several reaction cycles (Husain, 2016; Husain, 2018; Calzoni et al., 2021).

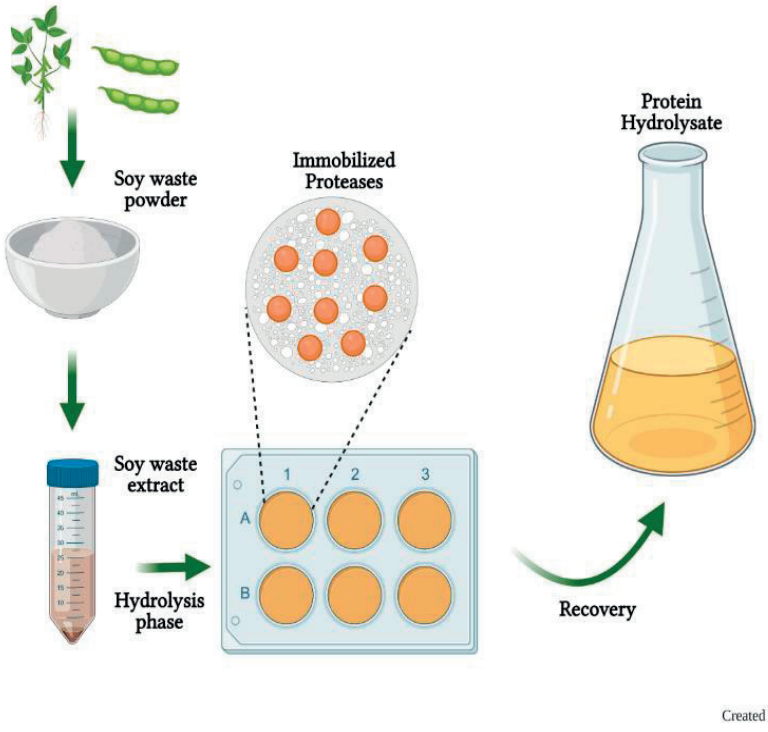

Figure 1. Experimental plan to obtain protein hydrolysates from soybean waste by immobilized enzymes

In this regard, the enzyme-mediated hydrolysis was carried out at 37°C for 4 hours and repeated for three operating cycles.
The degree of hydrolysis of the starting biomass was then evaluated by SDS-PAGE followed by Coomassie Blue staining (Figure 2).

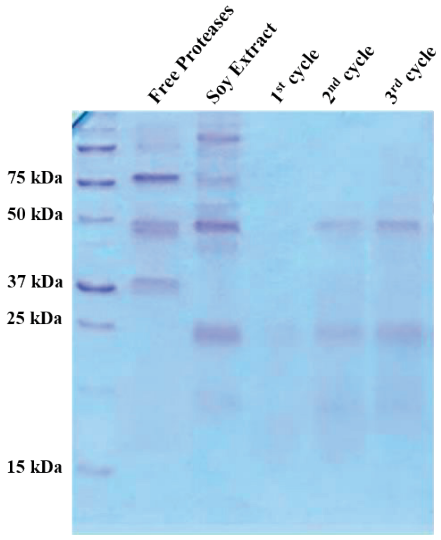

Figure 2. Protein profile of free proteases, soy extract, and soy hydrolysates obtained after three operating cycles at 37 °C for 4 hours with immobilized proteases, evaluated by SDS-PAGE, followed by Coomassie Blue staining method.

The protein profile obtained by SDS-PAGE shows how the soy extract is almost completely hydrolyzed after the first operating cycle; in fact, the disappearance of the main bands that characterize the biomass protein profile is evident. This is due to the probable production of small peptides and free AAs following the hydrolysis process. With the following reaction cycles, a slight loss of efficiency of the membrane-bound proteases can be noted, but nevertheless, a satisfactory level of hydrolysis is again obtained after the third reaction.
The same samples were also analyzed employing the Ninhydrin assay, to obtain an estimate of the free AAs in solution before and after the enzymatic hydrolysis. In fact, Ninhydrin is a reagent that interacts with the primary amines of AAs leading to the formation of a complex that absorbs at 570 nm.
The results shown in Figure 3 confirm those previously obtained by SDS-PAGE analysis.

In fact, following the first reaction cycle, the enzymatic hydrolysis process produces a concentration of free AAs about 14 times higher than that found in the starting biomass; while in the two subsequent hydrolysis cycles a slight decrease is observed relatively to the first cycle but the concentration of free AAs is still significantly higher than in the control.
The soybean protein hydrolysate obtained after the hydrolysis performed by immobilized proteases was then analyzed by Q-TOF LC/MS. The control, consisting of a raw extract of soybean waste, and the mix of hydrolysates produced after 3 cycles of hydrolysis were searched for tri- and tetrapeptides with the aid of the MassHunter Metlin Peptides AM PCD database.

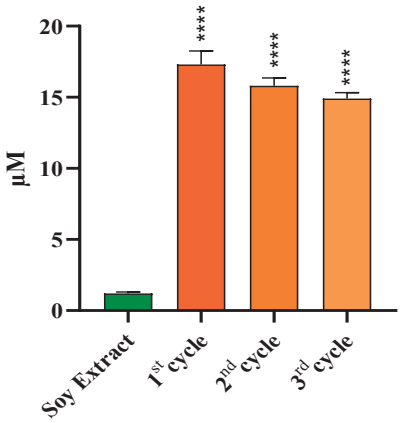

Figure 3. A concentration obtained using the Ninhydrin assay after enzymatic hydrolysis at 37°C for 4 hours in three consecutive operating cycles. Data are reported as mean ± SEM, n = 3. **** $p < 0.0001$

Figure 4 reports the peptide abundances, measured as the area under the chromatogram peaks, of the total peptides, detected in the mix of hydrolysates after three operating cycles, compared with the control. The presence of some peptides already in the control extract may be due to the thermal treatment of the initial soybean powder, carried at 80°C as described in the Materials and Methods section. However, in the hydrolysate mix, a massive increase of total peptides is detected, thus corroborating the results acquired with the SDS-PAGE and Ninhydrin assay analyses, and confirming the efficient functioning of immobilized proteases on a nitrocellulose membrane support.

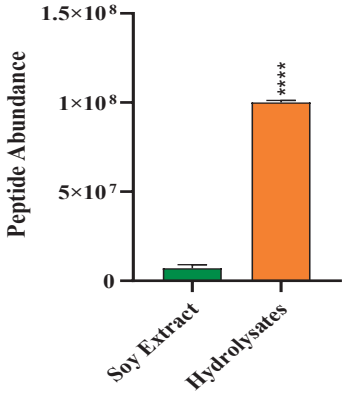

Figure 4. The abundance of total peptides in soy extracts and hydrolysate mix evaluated by Q-TOF LC/MS analysis. Data are reported as mean ± SEM, n = 3. **** $p < 0.0001$

The most abundant tri- and tetrapeptides detected in the hydrolysate are reported in Table 1, the abundance of these peptides represents about 15% of the total peptides detected.

Table 1. Peptide characterization in soy hydrolysate mix.

Peptides characterization	
Tetrapeptides	Tripeptides
Arg-Ile-Lys-Pro	Lys-Pro-Val
Gln-Lys-Lys-Lys	Arg-Gly-Ser

The most abundant species found are the tetrapeptides Arg-Ile-Lys-Pro and Gln-Lys-Lys-Lys, followed by two other tripeptides, namely Lys-Pro-Val and Arg-Gly-Ser. As is well-known for protein hydrolysates, these kinds of peptides arouse interest in the industrial field for their several potential biological activities and applications in the food and agricultural sector (Karami & Akbari-Adergani, 2019; Calzoni et al., 2021).

CONCLUSIONS

In this work, the possibility of using immobilized enzymes for biomass degradation has been demonstrated. In particular, the results of the enzymatic hydrolysis of soybean waste extract are very encouraging as demonstrated by the SDS-PAGE, Ninhydrin assay, and Q-TOF LC/MS analysis, which allowed a high degree of hydrolysis of the original biomass to be highlighted. Furthermore, the experiment was repeated for 3 operating cycles on a nitrocellulose membrane support, demonstrating how the system can be reused with only a minimal reduction in its performance. These protein hydrolysates obtained following this hydrolysis process could be used as sources of bioactive peptides and AAs which can be useful both in the food industry as supplements for humans or feed additives for animals, and in the agricultural field as soluble fertilizers or growth factors for plants. The enzymatic way by-passes the problem of toxic side products that are formed with the classic chemical hydrolysis and avoids the susceptibility problematic of the microbial strains to the environmental condition changes. From this perspective, the use of immobilized enzymes expresses great potential and represents a new green technology enabling the disposal of agri-food waste and the production of high-added-value bio-products to be reintroduced into the market.

REFERENCES

Bah, C.S., Carne, A., McConnell, M.A., Mros, S. & Bekhit, A.E.D.A. (2016). Production of bioactive peptide hydrolysates from deer, sheep, pig and cattle red blood cell fractions using plant and fungal protease preparations. *Food chemistry*, 202, 458-466.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry*, 72(1-2), 248-254.

Callegaro, K., Brandelli, A., & Daroit, D. J. (2019). Beyond plucking: Feathers bioprocessing into valuable protein hydrolysates. *Waste Management*, 95, 399-415.

Calzoni, E., Cesaretti, A., & Emiliani, C. (2019). Creating Products and Services in Industrial Biotechnology. *In Introduction to Biotech Entrepreneurship: From Idea to Business* (pp. 129-139). Springer, Cham.

Calzoni, E., Cesaretti, A., Montegiove, N., Pierantoni Casagrande, D., Corte, L., Roscini, L., Emiliani, C., Leonardi, L., Cardinali, G. (2020). Mixed microbial and thermal degradation of agricultural derived plant wastes. *Scientific Bulletin. Series F. Biotechnologies*, 24, 11-16.

Calzoni, E., Cesaretti, A., Tacchi, S., Caponi, S., Pellegrino, R. M., Luzi, F., Cottone, F., Fioretto, D., Emiliani, C., & Di Michele, A. (2021). Covalent Immobilization of Proteases on Polylactic Acid for Proteins Hydrolysis and Waste Biomass Protein Content Valorization. *Catalysts*, 11(2), 167.

Cesaretti, A., Montegiove, N., Calzoni, E., Leonardi, L., & Emiliani, C. (2020). Protein hydrolysates: from agricultural waste biomasses to high added-value products (minireview). *AgroLife Scientific Journal*, 9(1), 79-87.

Chervan, M. & Deeslie, W.D. (1984). U.S. Patent No. 4,443,540. Washington, DC: U.S. Patent and Trademark Office.

Clemente, A. (2000). Enzymatic protein hydrolysates in human nutrition. *Trends in Food Science & Technology*, 11(7), 254-262.

Clemente, A., Vioque, J., Sánchez-Vioque, R., Pedroche, J., Bautista, J. & Millán, F. (1999). Protein quality of chickpea (*Cicer arietinum* L.) protein hydrolysates. *Food Chemistry*, 67(3), 269-274.

Corte, L., Dell'Abate, M. T., Magini, A., Migliore, M., Felici, B., Roscini, L., ... & Benedetti, A. (2014). Assessment of safety and efficiency of nitrogen organic fertilizers from animal based protein hydrolysates - a laboratory multidisciplinary approach. *Journal of the Science of Food and Agricultur*e, 94(2), 235-245.

Fountoulakis, M. & Lahm, H.W. (1998). Hydrolysis and amino acid composition analysis of proteins. *Journal of chromatography A*, 826(2), 109-134.

Fox, P.F., Morrissey, P.A. & Mulvihill, D.M. (1982). Chemical and enzymatic modification of food proteins. *Developments in food proteins*.

Halpern, M., Bar-Tal, A., Ofek, M., Minz, D., Muller, T. & Yermiyahu, U. (2015). The use of biostimulants for enhancing nutrient uptake. *In Advances in agronomy* 130, 141-174. Academic Press.

Hou, Y., Wu, Z., Dai, Z., Wang, G. & Wu, G. (2017). Protein hydrolysates in animal nutrition: Industrial production, bioactive peptides, and functional significance. *Journal of Animal Science and Biotechnology*, 8(1), 24.

Husain, Q. (2016). Magnetic nanoparticles as a tool for the immobilization/stabilization of hydrolases and their applications: An overview. *Biointerface Research in Applied Chemistry*, 6(6).

Husain, Q. (2018). Nanocarriers immobilized proteases and their industrial applications: An overview. *Journal of nanoscience and nanotechnology*, 18(1), 486-499.

Karami, Z., & Akbari-Adergani, B. (2019). Bioactive food derived peptides: A review on correlation between structure of bioactive peptides and their functional properties. *Journal of food science and technology*, 56(2), 535-547.

Laemmli, U. K. (1970). SDS-page Laemmli method. *Nature*, 227, 680-5.

Li-Chan, E.C.Y. (2015). Bioactive peptides and protein hydrolysates: research trends and challenges for application as nutraceuticals and functional food ingredients. *Curr Opin Food Sci*., 1: 28-3.

López-Barrios, L, Gutiérrez-Uribe, J.A., & Serna-Saldívar, S.O. (2014). Bioactive peptides and hydrolysates from pulses and their potential use as functional ingredients. *J Food Sci*., 79: R2 73–83.

Martínez-Alvarez, O., Chamorro, S. & Brenes, A. (2015). Protein hydrolysates from animal processing by-products as a source of bioactive molecules with interest in animal feeding: A review. *Food Research International*, 73, 204-212.

Rosen, H. (1957). A modified ninhydrin colorimetric analysis for amino acids. *Archives of biochemistry and biophysics*, 67(1), 10-15.

Salihi, A., Asoodeh, A., & Aliabadian, M. (2017). Production and biochemical characterization of an alkaline protease from *Aspergillus oryzae* CH93. *International journal of biological macromolecules*, 94, 827-835.

Sawant, R., & Nagendran, S. (2014). Protease: an enzyme with multiple industrial applications. *World J Pharm Sci*, 3, 568-579.

Schaafsma, G. (2009). Safety of protein hydrolysates, fractions thereof and bioactive peptides in human nutrition. *European journal of clinical nutrition*, 63(10), 1161-1168.

Sherwood, J. (2020). The significance of biomass in a circular economy. *Bioresource technology*, 300, 122755

Souza, P. M. D., Bittencourt, M. L. D. A., Caprara, C. C., Freitas, M. D., Almeida, R. P. C. D., Silveira, D., ... & Magalhães, P. O. (2015). A biotechnology perspective of fungal proteases. *Brazilian Journal of Microbiology*, 46(2), 337-346.

Stahel, W.R. (2016). The circular economy. *Nature*, 531(7595), 435-438.

Tavano, O.L. (2013). Protein hydrolysis using proteases: an important tool for food biotechnology. *Journal of Molecular Catalysis B: Enzymatic*, 90, 1-11.

Tsugita, A. & Scheffler, J.J. (1982). A rapid method for acid hydrolysis of protein with a mixture of trifluoroacetic acid and hydrochloric acid. *European Journal of Biochemistry*, 124(3), 585-588.

Tuck, C.O., Pérez, E., Horváth, I.T., Sheldon, R.A. & Poliakoff, M. (2012). Valorization of biomass: deriving more value from waste. *Science,* 337(6095), 695-699.

Zambare, V., Nilegaonkar, S., & Kanekar, P. (2011). A novel extracellular protease from *Pseudomonas aeruginosa* MCM B-327: enzyme production and its partial characterization. *New biotechnology*, 28(2), 173-181.

Zou, Z., Wang, M., Wang, Z., Aluko, R. E. & He, R. (2020). Antihypertensive and antioxidant activities of enzymatic wheat bran protein hydrolysates. *Journal of food biochemistry*, 44(1), e13090.