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# PROTEIN HYDROLYSATES: FROM AGRICULTURAL WASTE BIOMASSES TO HIGH ADDED-VALUE PRODUCTS (MINIREVIEW)

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***Abstract***

*The degradation of biomasses originating from agriculture and food processing offers the double benefit of removing polluting waste and introducing new bio-derived products into the market. Protein Hydrolysates (PHs), used as sources of bioactive peptides and amino acids play a fundamental role among biotechnological products deriving from waste biomasses. PHs are bio-based chemicals with high added value that can be produced through different types of hydrolysis: chemical, microbiological, enzymatic or mixed. Depending on the biomass used and the hydrolysis procedure chosen, products endowed with different features, such as high biostimulating, hormonal, fertilizing and nutritive capacities, can be obtained. The production of PHs from vegetable biomasses prove pivotal, as they offer absolute health guarantees and can be marketed in areas that do not abide products derived from animal waste, which currently are often found in the market. Here, the various possible applications of PHs will be discussed, along with the different processes for their production starting from agro-food waste biomasses, paying particular attention to the advantages of enzymatic hydrolysis.*

***Key words***: *Protein Hydrolysates, Waste Biomasses, Circular Economy, Chemical Hydrolysis, Enzymatic Hydrolysis.*

## INTRODUCTION

Industrial processing of agricultural and food products originates large amounts of by-products which need to be disposed of. Although the successful application of the principles of green chemistry is fundamental to reduce the production of waste substances, it is not possible to avoid their generation. Considerable effort is currently going into finding employment and usefulness for the inevitably produced waste, with the aim being to give new value to this waste biomass by converting it into chemicals that can be used in other areas and reintroduced into the market, thus responding to the challenge of Circular Economy (Stahel, 2016; Tuck et al., 2012).

Biomasses can be defined as those organic raw materials, featuring a natural biological origin, which can become substrates for other processes. Depending on its chemical composition, a waste biomass can be classified into four categories (Tuck et al., 2012): polysaccharides, lignin, triglycerides (from fats and oils) and proteins. The large amount of by-products deriving from industrial processing can thus be exploited to make compost, biogas or other low-added value products. Further to this, waste biomasses are also especially interesting for their possible conversion into high-added value hydrolysates (Martínez-Alvarez et al., 2015).

In fact, different types of hydrolysates can be produced, with concentrations in carbohydrates, proteins and lipids that are variable depending on the origin of the initial biomass. In particular, a considerable amount of biomass possesses high protein content, immediately appealing from a nutritional and physiological point of view. The production of food and drinks is the sector most involved in this respect: by-products of the sugar and ethanol industry (*i.e.* vinasse from sugarcane or sugar beet), maize fermentation waste

(Distiller's Dried Grains with Soluble), oil seed press cake, fish silage, tea and coffee grounds and agricultural wastes coming from different crops, are all examples of waste containing a large quantity of proteins (Tuck et al., 2012).

In order to obtain Protein Hydrolysates (PHs) from a biomass, the latter can undergo treatments of different types ranging from chemical (using acids or alkalis), to microbiological and enzymatic, or mixed.

Hydrolysis has traditionally been carried out with extremely low or high pH chemicals, at high temperatures, and, only more recently, through microorganisms or enzymes (Diguta et al., 2007; Bruni et al., 2010) both in their free and immobilized form. The disadvantage of the first approach is the possible development of toxic side-products, whereas the high level of energy required for a purely temperature-based process is negative in terms of costs and carbon dioxide budget. The production of PHs by the enzymatic route instead exploits the ability of these biomolecules to work under mild conditions of temperatures and pH, avoiding the production of toxic contaminants in the final product (Abd et al., 2017).

By performing hydrolysis, the protein fraction of the waste biomass can be broken down into the amino acids that make it up. PHs are produced starting from the hydrolysis of intact proteins, with the formation, by breaking the peptide bonds, of a mix of small peptides of different sizes and free amino acids. They have various applications: PHs can be incorporated in other substances, or used directly as pharmaceutical, cosmetic and nutritional products. It is important that the hydrolysis product is stable in terms of oxidation and has good organoleptic characteristics. For example, if the hydrolysate has to be ingested, it is essential for it to possess a high content of nutrients and to be easily digestible and bioavailable (Canistro et al., 2017; Li-Chan, 2015; Martínez-Alvarez et al., 2015).

The properties of the PHs, and therefore their application, also depend on the degree of hydrolysis of the proteins, that is the number of peptide bonds cleaved, divided by the total number of peptide bonds and multiplied by 100 (Pasupuleti et al., 2008): the average hydrolysed proteins are used for example in sports and clinical nutrition, while the highly hydrolysed ones, thanks to their hypoallergenic nature, are used in the formulation of products for childhood, as alternatives to cow's milk proteins (Kiewiet et al., 2018).

In particular, because of the increasing interest in plant derived products, agricultural wastes have recently aroused considerable attention for the production of PHs. The preference for non-animal derived products is also due to the lack of prion-risk, and consequently because there is no need for strong final chemical treatments. Moreover, it has been observed that these hydrolysates can also be sources of bioactive peptides, with possible clinical applications such as antithrombotic, antihypertensive, antimicrobial, anti-carcinogenic, antioxidant and immune-modulating agents (Kiewiet et al., 2018).

Here we report an overview on a series of possible applications for PHs originated from agri-food waste biomasses. Later, we will discuss the differences between chemical hydrolysis and microbiological or enzymatic catalysis, and specifically on the advantages of using the latter approaches over the common chemical route.

## APPLICATIONS

Many are the potential applications of plant-derived PHs. They can serve a major role in animal nutrition for their straightforward nutritional, physiological and regulatory functions, but they can also be used as biofertilizers, in that hydrolysates are able to improve nutrient assimilation of crops and to mitigate crop stress (Halpern et al., 2015). Moreover, those hydrolysates which carry out biological functions other than mere nutritional ones are known as bioactive peptides, *i.e.* those able to act as antihypertensive, antioxidant and anti-inflammatory agents (Hou et al., 2017; Zou et al., 2020).

As for their nutritional functions, PHs boost feed efficiency and can promote growth rate. The hypoallergenic nature of hydrolysates makes them also suitable ingredients for infant food formulations or as supplement in the diets of children suffering from severe food allergies (Schaafsma, 2009). In addition, PHs exhibit interesting rheological properties, like better solubility than intact proteins, and they can be endowed with peculiar physicochemical and functional features for specific applications (Xia et. al., 2012). As an example, rice PHs are employed both as nutritional supplements, and as flavour enhancers in food and as whiteners for coffee. (Fabian & Ju, 2011; Phongthai et al., 2017). To date, not only offers the market hypoallergenic formulations for children, containing widely hydrolysed proteins, but also, PHs are used in the nutrition of sick subjects, as they are easier to digest than intact proteins. Finally, in the nutrition of athletes, PHs are used as sport beverages for recovery after training, in order to reduce inflammation and muscle pain.

An excellent source of PHs is represented by soybean waste. In fact, soybean meals comprise anti-nutritional substances and protein-type allergens, while fermented soybean leads to PHs which can be efficiently used to improve growth-performances in the diet of weaning pigs, young calves, poultry and fish, as well as companion animals (Hou et al., 2017). On the other hand, plant-derived hydrolysates rich in glutamine and glutamate (*i.e.* wheat gluten) can be added to the diet of companion animals to provide savoury flavours, thus enhancing the palatability of the feeding products (Nagodawithana et al., 2010). Broadly speaking, a noticeable health improvement for companion animals can be achieved through the addition of PHs to their diet, especially resulting in good gut health and general well-being (Hou et al., 2017).

A further application of PHs is the one as fertilizer, thanks to their ability to improve nutrient assimilation and to reduce crop stress. PHs are indeed able to guarantee higher yields and productivities in the case of different crops, improving the plant nutritional balance and increasing its endogenous defences against abiotic stresses (like thermal shocks and lack of water). For example, a biostimulant effect on yield performance and nutritional quality has been demonstrated for greenhouse tomato (Colla et al., 2017) treated with 3 $mL \cdot L^{-1}$ legume-derived PHs. As is the case with soybean PHs, the rate of nitrogen, phosphorus and potassium (NPK) release, a key factor for crop growth, can be improved with the addition of hydrolysates in the fertilizer composition, leading to increased crop yields and enhancing the activity of microorganisms in the soil (Liu et al., 2019).

Further to this, PHs can be exploited for their antihypertensive, antioxidant and anti-inflammatory properties as well as for their positive effects on the immune system. If this is the case, hydrolysates with biological functions are called bioactive peptides.

PHs with an immunomodulating role could slow down or prevent the onset of a series of immune-correlated diseases. Lately, progress has been made in defining which characteristics are actually useful for an optimal and reproducible positive effect on the Immune System (IS). A development in this field could improve the effectiveness of existing products that contain hydrolysates, with applications in sports nutrition, clinical nutrition and infant formulas. In this respect, the lymphocytic proliferation and the phagocytic capacity of macrophages are both crucial points to be reckoned with. The increase in lymphocytic proliferation has been observed after stimulation with different types of PHs, such as soy and wheat, but not all hydrolysates necessarily stimulate this proliferation. The effects on phagocytosis also depend on the protein source that is used, for example soy and wheat again promote it, while the hydrolysed rice protein inhibits the phagocytic activity of macrophages (Kiewiet et al., 2018).

In addition, some hydrolysates also have anti-inflammatory properties. This characteristic is mainly attributed to those hydrolysates, *e.g.* rice bran PHs, which involve the reduction of the intestinal damage caused by the inflammatory condition, through a decrease in the production of pro-inflammatory cytokines (Boonloh et al., 2017).

The clinical condition of hypertension is responsible for 45-51% of the total deaths in the modern world. High blood pressure can lead to the development of serious cardiovascular diseases, which can affect the heart, blood vessels and kidneys. The renin-angiotensin-aldosterone system (RAAS) is the one most involved in the control of blood pressure: the renin is synthesized in the kidneys and released into the bloodstream, where it goes to cleave the N-terminal region of angiotensinogen and produces a decapeptide, angiotensin-I, which circulates in the blood until its C-terminal residue is also cleaved by Angiotensin-I Converting Enzyme (ACE), with the formation of an 8 amino acid peptide, called Angiotensin-II, which is a powerful vasoconstrictor. Current hypertension medications (Captopril, Enalapril and Lisinopril) are based on ACE inhibition, but the need to reduce the side effects of nausea, vomiting and dry cough is pushing researchers to find natural alternatives, that is to say peptides deriving from the hydrolysis of food proteins, such as hemp seed or wheat bran.

The Hemp Seed Proteins (HSPs) can be obtained during the production of an edible oil: starting from the Hemp seed Protein Meal (HPM), which has a protein content of 37%, HSPs can be isolated for the preparation of a PH through the use of different proteolytic enzymes. By testing the *in vitro* effects of HSP hydrolysates, it was noted that these act by inhibiting the renin and ACE activity, thus preventing blood pressure from rising (Malomo et al., 2015).

Another example of antihypertensive bioactive peptide is represented by the Wheat bran PH (WPH). Wheat bran, generated as a by-product of the wheat industry, mainly consists of the seed coat resulting from the transformation of wheat into flour (Coda et al., 2014). As of today, most wheat bran is used as a low-value component for animal feed and human consumption. The WPH can instead be seen as a high-value added product with significant biological activity. In particular, highly hydrolysed WP (peptide fraction < 1 kDa) were found to have high antihypertensive efficiency (Zou et al., 2019), supposedly because these small peptides can either be better absorbed in the intestine or show increased ability to interact and thus inhibit the involved enzymes (ACE and renin). Moreover, this WPH fraction also exhibits high antioxidant activity against oxygen radicals, which is another relevant feature to be pursued.

This body of evidence gives information on how the PHs obtained from both hemp seeds and wheat bran can be used effectively for the formulation of functional foods and nutraceuticals with antihypertensive activities.

Lastly, PHs have recently been tested as promoters of non-haem iron absorption. Iron is one of the trace elements most present in the human body and its deficiency, in addition to leading to anaemia, can also decrease productivity at work, impair physical capacity and reduce endurance in sports. In this view, the ability of PHs to form ferrous chelates, *i.e.* molecules linked to ferrous ($Fe^{2+}$) ions, is a desirable quality to promote iron absorption, thus preventing and reducing fatigue (Li et al., 2017). Fatigue is a complex physiological and biochemical process, which represents a widespread social problem common to those people who, due to strong competition in the workplace or irregular lifestyle, are subject to enormous pressure. Mineral-based products against fatigue are therefore a developing field (Huang et al., 2015).

For these reasons, PHs able to chelate iron and other metals, thus enhancing their absorption, have been arousing increasing interest. PHs can indeed be exploited to keep iron soluble, reduce ferric iron to ferrous iron and promote transport across cell membrane, especially into the gut. In this respect, barley, chickpea, rice and soybean PHs can all be listed among mineral chelating peptides (Cao et al., 2007; Eckert et al., 2016; Li et al., 2017; Lv et al., 2009; Lv et al., 2013; Torres-Fuentes et al., 2012; Wakabayashi et al., 1989; Zhang et al., 2014).

## CHEMICAL HYDROLYSIS

Until recently, the production of PHs was mainly carried out chemically, employing acid or basic hydrolysis at high temperatures. However, these types of processes are difficult to control and above all lead to the formation of poor quality products due to the loss of assimilable amino acids and the production of modified amino acids (Tavano, 2013). Acid hydrolysis is usually carried out by using 6 M HCl at 110°C for more than 24 hours leading to the degradation of some amino acids, namely arginine and tryptophan, and causes the formation of unwanted secondary compounds such as chlorides (Corte et al., 2014; Tsugita & Scheffler, 1982).

Similarly, alkaline hydrolysis, conducted with strong bases, namely NaOH or KOH, causes the degradation of amino acids such as cysteine, arginine, threonine, serine, and isoleucine and also leads to the formation of modified amino acids such as lysinoalanine and lanthionine (Fountoulakis & Lahm, 1998; Tavano, 2013). Furthermore, the high content of acid or basic residues in the final hydrolysates limits their applicability especially in the agricultural sector and in the food industry (Chervan & Deeslie, 1984).

Table 1. Functions of agri-food waste biomass hydrolysates listed by area of application

| **Area of application** | **Biomass** | **Function** | **Ref.** |
|---|---|---|---|
| Nutrition | Rice bran | Flavour enhancers in food<br>Whiteners for coffee | Fabian & Ju, 2011;<br>Phongthai et al., 2017 |
| | Soybean | Diet supplements to improve growth-performances | Hou et al., 2017 |
| | Wheat Gluten | Palatability enhancers | Nagodawithana et al., 2008 |
| Farming | Vegetables | Biostimulant effect on yield performance and nutritional quality of greenhouse tomato | Colla et al., 2017 |
| | Soybean | Increasing crop yields and enhancing the activity of microorganisms in the soil | Liu et al. 2019 |
| Medicine | Soybean and Wheat | Increasing lymphocytic proliferation and phagocytic capacity of macrophages | Kiewiet et al., 2018 |
| | Rice bran | Anti-inflammatory properties by decreasing pro-inflammatory cytokines | Boonloh et al., 2017 |
| | Hemp seed | Antihypertensive power by inhibiting the renin and ACE activity | Malomo et al., 2015 |
| | Wheat bran | Antihypertensive power by inhibiting the renin and ACE activity | Zou et al., 2019 |
| | | Antioxidant activity against oxygen radicals | |
| | Barley | Promoters of non-haem iron absorption | Eckert et al., 2016 |
| | Chickpea | Promoters of non-haem iron absorption | Torres-Fuentes et al., 2012 |
| | Rice | Promoters of non-haem iron absorption | Cao et al., 2007 |
| | Soybean | Promoters of non-haem iron absorption | Lv et al., 2009;<br>Lv et al., 2013;<br>Wakabayashi et al., 1989;<br>Zhang et al., 2014 |

The chemical hydrolysis obtained with both acid and basic agents is therefore an extremely aggressive process, which, on the one hand, allows a high percentage of free amino acids to be obtained, but on the other hand, puts them through structural changes making them no longer assimilable.

One of the methods for determining the quality of the hydrolysate is to evaluate the degree of racemization, which is the phenomenon that causes the passage of amino acids from their levogyrous form, the biologically active form, to a dextrogyrous one. The latter represents the most abundant form in the PHs obtained chemically as the aggressiveness of the process modifies the natural form of the amino acids. A high degree of racemization therefore represents a negative quality index, since the right-handed amino acids cannot be assimilated by either animal or vegetable living organisms (Rikken & Raupach, 2000).

## MICROBIAL HYDROLYSIS

Another viable route to the preparation of PHs is based on the use microorganisms, which, through the action of specific enzymes such as proteases, lead to the formation of small peptides and free amino acids. Microorganisms are endowed with a whole series of enzymatic kits that enable the degradation of organic matter, not only of protein origin but also polysaccharides and lipids (Hou et al., 2017; Smid & Lacroix, 2013).

The microbiological hydrolysis of the biomass protein component is divided into liquid or solid depending on the percentage of humidity of the substrate used; as a rule, what varies is the microbial flora involved.

One of the first products obtained through microorganism-mediated hydrolysis was soy sauce (Hou et al., 2017; Pasupuleti, et al., 2008). Microbiological hydrolysis is now widely used to obtain hydrolysates starting from plant biomasses (Bah et al., 2016; Li-Chan, 2015; Lòpez-Barrios et al., 2014) and in the dairy industry (Hou et al., 2017).

A series of clear advantages comes with the employment of microorganisms to produce PHs. This is mainly linked to the fact that by not resorting to aggressive chemical agents and high temperatures, the formation of biologically active small peptides and free amino acids can be secured; moreover, it has been shown how microorganisms can also remove hypoallergenic or anti-nutritional substances from the final products.

However, even microbiological hydrolysis suffers some disadvantages, expressly due to the high production costs, as well as to the susceptibility of microorganism activity to changes in the environmental conditions (Hou et al., 2017).

## ENZYMATIC HYDROLYSIS

PHs can also be obtained enzymatically by resorting to purified enzymes in their free or immobilized form. In this case, the hydrolysis process is again more advantageous than the chemical one, since it is carried out under mild conditions of temperature (40-60°C) and pH (6-8), resulting extremely favourable from an economic and environmental point of view.

Enzymatic hydrolysis does not lead to the formation of unwanted and often toxic secondary products and, just like microbial hydrolysis, preserves the structure of the amino acids, which maintain their biologically active levogyrous form (Clemente et al., 1999; Clemente et al., 2000). The hydrolysates obtained through the enzymatic approach are also soluble, more resistant to heat and more resistant to precipitation (Clemente et al., 2000; Fox et al., 1982).

The most preferred enzymes for the preparation of PHs are proteases of microbial origin, composed of exopeptidases and endopeptidases according to the type of catalytic reaction performed; in fact, the exopeptidases make cuts at the level of the terminal region of the protein, while endopeptidases give internal cuts (Hou et al., 2017; Wu et al., 2013).

The enzymes involved in the production of PHs can derive from animal, vegetable or microbial sources. Pancreatin, trypsin, and pepsin are the main proteolytic enzymes obtained from animal sources, while papain and bromelain are extracted from plant organisms. Bacteria and fungi, however, represent the largest source of proteolytic enzymes, as they release enzymes directly into the extracellular environment, making the latter easier to extract. Moreover, proteases of bacterial and fungal origin exhibit a large range of optimal temperatures and pH (Dixon, 1979; Hou et al., 2017; Kunst, 2003).

The properties and composition of PHs obtained through enzymatic hydrolysis are crucial for their positioning in the market as commercial products. For example, the PHs produced enzymatically from perilla seed meal (PSM), a by-product of the production of perilla seed oil, were found to have a very high content of essential amino acids (*e.g.* lysine). Additionally, PSM hydrolysates feature better functionality relative to PSM protein isolates, as the former exhibit higher solubility, greater oil absorption capacity, and superior emulsifying and foaming properties (Park & Yoon, 2019). Another example is the partial hydrolysis of quinoa protein (QP) isolates, by means of a fungal serin peptidase, for the production of QP hydrolysates to be used as food additives in semi-solid healthy foods with both antioxidant activity and gelling capability (Galante et al., 2020). Again, Maqsoudlou et al., 2019 proposed the hydrolysis of pollen using pepsin and trypsin under controlled conditions for the generation of peptides possessing different sizes. A heterogeneous composition was revealed in the peptide sequences, with glycine and alanine being the two most abundant hydrophobic amino acids. Pollen PHs also possess significant bioactivities, namely antioxidant and ACE inhibitory activities, which make them promising functional ingredients in food formulations.

However, although enzymatic hydrolysis is extremely advantageous for the low environmental impact, thanks to the mild reaction conditions required, on the other hand, it is very expensive due to the need for the enzymes to be purified. Once the final hydrolysate is obtained, further purification steps are also required when free enzymes are employed, as well as deactivation processes so that the enzyme does not alter the desired products. Indeed, it is necessary to use high temperatures to inactivate proteolytic enzymes, but at the same time, these stringent conditions cause the irreversible denaturation of the enzymes, making their recycling impossible (Rocha et al., 2011; Huang et al., 1999).

In order to overcome these drawbacks, enzymes immobilized on inert substrates (*e.g.*

films, column reactors, nanoparticles) have recently been developed. By doing so, enzymes maintain their catalytic capacity and their stability under wide reaction conditions, but at the same time they become easily separable from the reaction product through sedimentation, filtering or centrifugation.

There are many examples in which PHs have been prepared through the use of immobilized enzymes. For example, active corn peptides were obtained from zein by alcalase and trypsin co-immobilized on a calcium alginate-chitosan composite carrier (Wang et al., 2014). In a study by Wei et al., 2018, immobilized alcalase and flavourzyme were instead used to prepare flaxseed PHs capable of improving the flavour in Maillard reaction products. More recently, alkaline proteinase was covalently immobilized on amino-functionalized magnetic nanoparticles, resulting in better storage stability compared to free enzyme, for soy protein hydrolysis (Zhu et al., 2019).

## CONCLUSIONS

In a society where the search for new sources of energy is becoming more and more vital, waste biomasses cannot be seen as something to be disposed of, but instead as new resources to take advantage of.

Agriculture and food industry generate large amount of waste biomasses, rich in protein content, so that they can be exploited for the production of PHs to be reintroduced into the market, thus enjoying the ride of Circular Economy.

Further to this, in view of their many applications ranging from nutritional to biological and clinical, PHs have been awarded the deserved title of high added-value products. Here we have highlighted a long list of effective practical applications for PHs derived from agri-food waste biomasses, unravelling their endless potential.

In addition, we discussed the key strength of microbiological and enzymatic catalysis, which allow mild reaction conditions with respect to harsh chemical hydrolysis. Finally, we brought into clear focus the possibility to resort to immobilized enzymes in order to carry out protein hydrolysis: this is an ever-growing strategy which has been gaining a position of special prominence, as it combines the eco-friendliness of the enzymatic approach with the economic advantage of reusing immobilized enzymes.

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