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ORIGINAL PAPER

Enzymatic Preparation of Mushroom By-product Protein Hydrolysates (Mb-PPHs)

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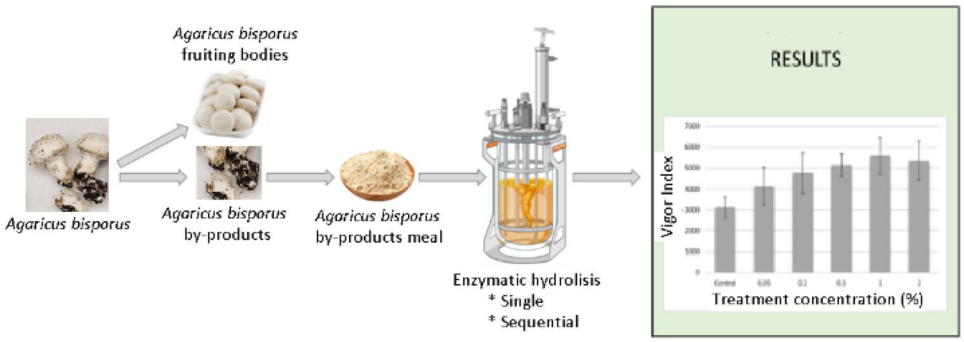
Abstract

The excessive use of chemical fertilizers can cause severe environmental damage. In recent decades, the application of biostimulants to improve soil composition and stimulate plant growth has contributed significantly to environmental preservation. In this paper, we studied the production and characterization of an amino acid/peptide-enriched biostimulant using edible mushroom (*Agaricus bisporus*) by-products (tails and nonmarketable mushrooms) as raw materials and commercial proteases as hydrolytic agents. A single hydrolytic process using four different endoproteases, Alcalase®, L-450, Flavourzyme® or papain, and a sequential hydrolytic process using two proteases, an endoprotease and an exoprotease, Alcalase® + Flavourzyme® or L-450 + Flavourzyme), were conducted. A preevaluation of potential plant biostimulants was also carried out, testing the biostimulant capacity of single and sequential Mb-PPHs to stimulate maize seed germination and root growth, as well as the evaluation of the vigor index (VI), with very promising results.

Graphical Abstract



Keywords *Agaricus bisporus* · Enzymatic hydrolysis · Biostimulants · Seed germination · Root growth · Vigor index

Renato Inca-Torres and José Raúl Aguilera-Velázquez have contributed equally to this work.

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Statement of Novelty

Currently, the overuse of chemical fertilizers has a huge environmental impact, hence, to the human and animal health. To improve the environmental health and replace the use of chemical fertilizers is necessary to look for sustainable biostimulants. The use of rich-protein by-products, such as tails and nonmarketable parts of *Agaricus bisporus* as a raw material to develop protein biostimulants by enzymatic hydrolysis, appears as an innovative, ecofriendly and viable alternative. Enzymatic hydrolysis is a clean and specific method to generate *taylor-made* mixers of peptides, oligopeptides and free amino acids according to the needs to the plant. In this work, we have evaluated the biostimulant capacity of single and sequential enzymatic hydrolysates to stimulate the maize growth with very promising results.

Introduction

The overuse of chemical fertilizers has contributed to increasing environmental pollution. In addition, this excessive use of chemical products has led to some negative effects, such as greenhouse gas production, acidification, and increases in soil and water pollution [1]. To promote the efficiency of plant nutrient uptake and reduce environmental pollution, new agricultural strategies based on the application of a wide spectrum of natural substances and/or beneficial microbes have been evaluated to reduce negative environmental impacts and improve crop performance and sustainability under adverse ecological conditions [2, 3]. This type of compound is known as a positive plant growth regulator or metabolic enhancer, as well as a biostimulant [4–6], and it is considered capable of enhancing plant growth and development when applied in small amounts to the soil or directly to the foliar surface. Biostimulants can be grouped into four main groups: humic substances, products containing amino acids and peptides, microbial inoculants (mycorrhizal fungi and rhizobacteria), and seaweed extracts [7, 8]. Specifically, biostimulants belonging to the amino acid- and peptide-containing group mainly consist of free amino acids, oligos and polypeptides [8] obtained by enzyme and/or chemical hydrolysis of proteins from animal or vegetal sources [6, 9–11].

Currently, most of the biostimulants based on protein hydrolysate on the market are obtained by chemical hydrolysis of organic materials derived from animal waste (epithelial or connective tissue, hen feathers and bone meal) [12] or from plant-derived material (carob germ protein, alfalfa residues, wheat-condensed distiller soluble, and seaweed proteins) [13], and only a small part (< 10%) is derived from enzymatic hydrolysis of plant biomass, especially Leguminosae crops, or animal-origin biomass [14]. Traditionally, chemical hydrolysis is achieved with chloride acid at high temperatures (> 110 °C), and generally, it produces products of low agronomic quality since some important amino acids (e.g., tryptophan, tyrosine, serine, asparagine, and glutamine) and peptides are destroyed during the production process [15]. One way to reduce the loss of amino acids and peptides during the process is through the digestion of proteins with enzymes [7]. The enzymatic hydrolysis system obtains high-quality protein hydrolysates using proteases and low temperatures (< 60 °C) [16]. Enzymatically produced protein hydrolysates contain not only free amino acids but also soluble peptides acting as signal molecules that regulate a spectrum of physiological processes [17, 18].

However, to the best of our knowledge, no report on mushroom protein hydrolysates for biostimulant uses has been published. The edible mushroom *A. bisporus* (white button mushroom) is the world's leading cultivated mushroom, representing more than 70% of the world's production of edible mushrooms [19, 20]. Its consumption is mainly attributed to its taste and flavor, but in addition, *A. bisporus* is a rich source of nutrients that includes proteins, amino acids, minerals, and vitamins [20], potentially useable in fields other than nutrition, for example, agriculture and/or cosmetics. The industrial processing of this mushroom generates a large amount of waste (25–30% of the global production), which is not generally used [21]. However, this byproduct has a high protein content (25–33%, d.w.) which can be converted by enzymatic hydrolysis into free amino acids, oligopeptides, and peptides that are potentially easily assimilated by plants, either through the roots or leaves. However, no reports have been conducted on the potential use of *A. bisporus* by-product hydrolysates as biostimulants.

In this study, the proteins from *A. bisporus* (tails and nonmarketable mushrooms) were hydrolyzed using single and sequential enzymatic processes. The aim was to generate mushroom by-product protein hydrolysates (Mb-PPHs), their characterization and their preevaluation as potential plant biostimulants.

Materials and Methods

Materials

By-products of white button mushrooms (*A. bisporus*) (tails and nonmarketable mushrooms), provided by Grupo Riberebro Integral S.A. (Haro, Logroño, Spain), were used as raw materials.

Enzymes

Alcalase® (endopeptidase from *Bacillus licheniformis*, 2.4 L) and Flavourzyme® (exopeptidase and endoprotease complex from *Aspergillus oryzae*, 500 L) were provided on loan by Novozyme® Spain (Madrid, Spain). Papain (endopeptidase from *Carica papaya*, (P3375) and Bioprotease-L-450 (endopeptidase from *Bacillus licheniformis*) were purchased from Sigma–Aldrich (Madrid, Spain) and Biocon® (Barcelona, Spain), respectively.

Chemicals

Free amino acid standards and reagents for amino acid analysis were obtained from Agilent Technologies. 2,4,6-Trinitrobenzenesulfonic acid (TNBS), L-leucine, sodium dodecyl sulfate (SDS), NaOH, KOH, citric acid, and all other chemicals used were purchased from Sigma–Aldrich and were of analytical reagent grade.

Preparation of Mushroom Tail Meal (MTM) and Enzymatic Hydrolysis

Agaricus bisporus was air-dried at 50 °C and ground into a fine powder with a Retsch® SM100 mill equipped with a 0.35 mm sieve (42 mesh). The powder was called mushroom tail meal (MTM). MTM samples were hydrolyzed by single hydrolysis using four proteases, Alcalase®, Flavourzyme®, papain and L-450, and by stepwise dual-enzymatic hydrolysis using the combinations of Alcalase® + Flavourzyme® and L-450 + Flavourzyme®, obtaining mushroom by-product protein hydrolysates (Mb-PPHs).

(i) Single hydrolysis

Enzymatic hydrolysis was performed on MTM with 28.6 ± 1.6% (d.w. basis) of protein concentration using the pH–stat technique [22] as described by Parrado et al. [23]. Briefly, MTMs were suspended in distilled water (10%, w/v) in the reactor, which was equipped with pH, temperature and agitation controls. The solution was adjusted to pH 8.5, 50 °C, and 100 rpm; once it reached 50 °C, the solution remained under these conditions for 15 min to maximize protein solubilization. After thermal pretreatment, hydrolysis was carried out with four different proteases (Alcalase®, Flavourzyme®, papain and L-450) using an E/S ratio of 0.3 for Alcalase®, Flavourzyme® and L-450 and 0.4 for papain. Hydrolytic processes were maintained for 2 h at pH 7.5 and 50 °C under constant agitation (100 rpm). The pH was maintained by the controlled addition of 1.0 M NaOH. The hydrolytic processes were stopped by raising the temperature to 90 °C and maintaining it for 90 min, followed by cooling to room temperature and adjusting the pH to 5.0 with 3.0 M HCl to minimize contamination. The hydrolysates were then recovered by centrifugation and concentrated by vacuum approximately 15 times, obtaining a syrup-like product, and stored at 4 °C until use. The obtained products were designated Mb-PPHs.

(ii) Stepwise dual-enzymatic hydrolysis or sequential hydrolysis

Sequential hydrolysis was performed with Alcalase® + Flavourzyme® or L-450 + Flavourzyme®. The Alcalase® and L-450 steps were carried out as described above, maintaining the hydrolytic process for 60 min, after which Flavourzyme® treatment was carried out for another 60 min, maintaining the same conditions. The recovery of the hydrolysate was carried out as described above (see Fig. 1B).

Degree of Hydrolysis

The degree of hydrolysis (DH) was calculated by the following equation:

$$DH(\%) = (h/h_t) \cdot 100\% = [(B \cdot N_b)/\left(Mp \cdot \alpha \cdot h_t\right) \cdot 100\%$$

where h is the number of broken peptide bonds, h_{tot} is the total number of peptide bonds in the studied substrate, B is the amount of base consumed (ml) during the reaction, N_b is the normality of the base, M_P is the mass (g) of protein ($N \times 5.5$), α is the average degree of dissociation of the α-NH_2 released during hydrolysis and h_t is defined as 7.9 meq/g [24].

Chemical Characterization of MTM and Mb-PPHs

The moisture, dry matter, ash, organic matter, crude fat, total carbohydrates, total nitrogen, and protein content were characterized for the MTM and Mb-PPHs following standard methodologies [25].

Total glucan and α-glucan contents were determined using a Mushroom and Yeast Assay Kit (Megazyme International, Ireland) based on the McCleary and Codd enzymatic method [26] according to the manufacturer's protocol. The β-glucan content was calculated by subtracting the α-glucan content from the total glucan content (β-glucans = total glucans − α-glucans).

Free Amino Acid and Total Amino Acid Analysis

Lyophilized Mb-PPHs were used to determine FAA. FAA extraction was carried out using 0.1 M HCl as solvent, using a sample:solvent ratio of 1:10. After the extraction, 6% trichloroacetic acid was added to precipitate proteins and centrifuged at 6000 × g for 10 min at room temperature. The supernatant, filtered through a 0.2 µm filter, was used

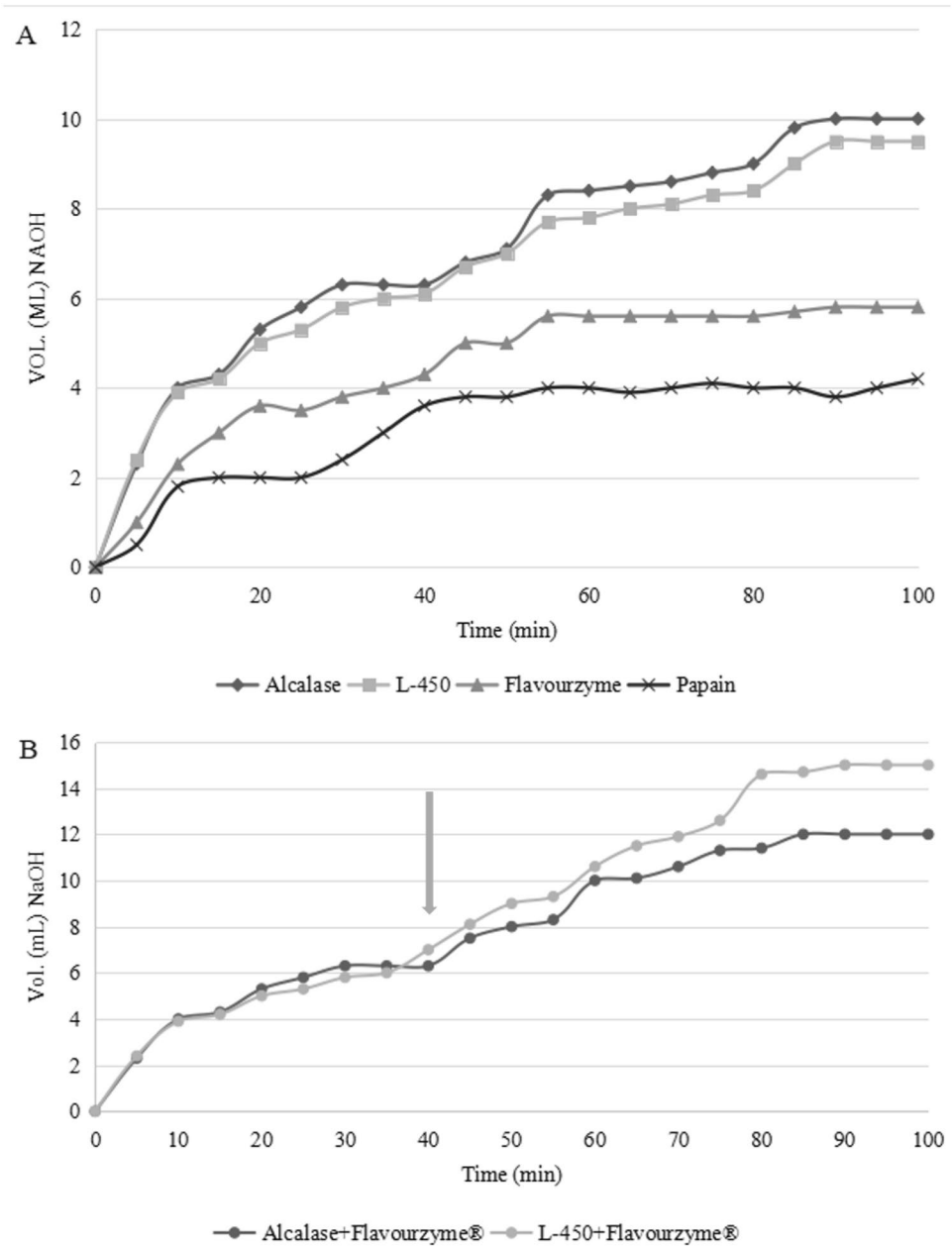

Fig. 1 Hydrolysis curve of MTM **A**: by single hydrolysis (Alcalase®, Bio-L-450, Flavourzyme® or papain) and **B** by sequential hydrolysis (Bio L450 + Flavourzyme® and Alcalase® + Flavourzyme®). The arrow indicates the change in the enzyme

to determine FAAs by reversed-phase HPLC analysis and automated precolumn derivatization with o-phthalaldehyde (OPA) for primary amino acids and 9-fluorenylmethyl-chloroformate (FMOC) for secondary amino acids [27] with slight modifications. Each sample was analyzed in triplicate.

Soluble proteins and peptides were hydrolyzed with 6 M HCl for 24 h at 110 °C according to standard methods, and the AA composition was determined as described above.

Gel Filtration Chromatography

The MTM and Mb-PPH peptide profiles were analyzed by size exclusion chromatography using an ÄKTA fast protein liquid chromatography (FPLC) system equipped with a Superdex™ 30 Increase 10/300 GL column (GE Healthcare Bio-Science AB, Uppsala, Sweden) with a resolution range of 7000–100. The Mb-PPHs were dissolved (10 mg ml^{-1}) in 50 mM phosphate buffer pH 7 and 0.15 mM NaCl eluent and centrifuged at 6000 × g, and the supernatant was filtered through a mesh membrane of 0.2 µm. Two hundred microliters was injected into the FPLC system, and the run was performed at a flow rate of 0.4 ml min^{-1}. Elution was monitored at 280 and 215 nm with a UV UCP-900 monitor (Amersham Biosciences). Molecular weights were determined by interpolation of a calibration curve obtained with the following calibration

standards: cytochrome C (M_r 12,400), aprotinin (M_r 65,000), vitamin B12 (M_r 1355), triglycine (M_r 189) and glycine (M_r 75).

Evaluation of Mb-PPH Biostimulant Activity

i. Seed priming

Maize seeds (Zea mays L.) were sterilized in a solution containing hypochlorite (0.25%) for 3 min. Then, the seeds were repeatedly rinsed with distilled water. The seeds were then left in contact with a 10 ml solution containing 0.01, 0.05, 0.10, 0.50, 1.00, 2.00, 5.0 and 10% Mb-PPH (single and sequential) overnight (approximately 12 h), using 10 ml of distilled H_20 as a control.

ii. Seed germination

After overnight priming, the seeds were placed on covered Petri dishes (15 cm diameter) with 25 seeds/plate (in triplicate for each treatment), lined with filter paper and moistened with 10 ml water. These were placed in a growth chamber in the dark at 24 ± 1 °C. Germination was recorded 4 days after priming, and radicle length was recorded 7 days after priming.

iii. Root growth evaluation

The primed maize seeds were transferred to hydroponic solutions (three replications per treatment and ten plants for each treatment) and grown according to a published procedure [28]. The nutrient solution contained 2 mM $Ca(NO_3)_2{\cdot}4H_2O$, 0.5 mM $MgSO_4{\cdot}7H_2O$, 0.7 mM K_2SO_4, 0.1 mM KCl, 0.1 mM KH_2PO_4, 1 µM H_3BO_3, 0.5 µM $MnSO_4{\cdot}H_2O$, 0.5 µM $CuSO_4$, 0.5 µM $ZnSO_4{\cdot}7H_2O$, 0.01 µM $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, and 100 µM Fe-EDTA. The samples were maintained at 24 ± 2 °C and a light intensity of 150 µmol m^{-2} s^{-1}, with a light/dark photoperiod of 12/12 h. Three weeks after sowing (21 days), plants were harvested and subjected to the following analysis: root and shoot length, fresh root weight, dry root and shoot weight, and plant vigor index.

iv. Calculation of vigor Index

The degree of deterioration, or severity of the genetic deficiency, was inversely proportional to the vigor of the seeds. The vigor index (VI) was calculated using the following equation:

$$VI = (L_{ROOT} + L_{shoot}) \cdot G$$

where L_{root} is the length of the root and L_{shoot} is the length of the aerial part. This index was calculated using the length of the root and aerial part of each seedling and the germination of seeds with equal treatment.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 5.03 software. Student's *t* test for unpaired data was applied when two independent variables were compared, and a p value < 0.05 was accepted as statistically significant. Data are presented as the mean ± standard error of the mean.

Results and Discussion

Mushrooms (tails and nonmarketable mushrooms) were dried by hot air (50 °C) and ground to for a powder (42 mesh) that we called MTM. As shown in Table 1, the basic composition of MTM is characterized by an important carbohydrate and protein content: 53.62 ± 3.9% and 28.76 ± 1.63%, d.w., respectively, and a low fat content (3.21 ± 0.22%), which makes it a good substrate for the preparation of protein hydrolysates that are potentially usable in the food and/or agronomic industry.

Enzymatic Hydrolysis of MTM

The proteins present in MTM were hydrolyzed by two procedures: single hydrolysis with one protease (Alcalase®, Flavourzyme®, L-450 or papain) and sequential hydrolysis with two proteases ("Alcalase® + Flavourzyme®" or "L-450 + Flavourzyme®"). Figure 1A and B show the hydrolysis curves as a function of base consumption (ml of 1 M NaOH) vs. hydrolysis time (min) and DH vs. hydrolysis time (min) obtained by single and sequential hydrolysis, respectively.

As Fig. 1A shows, the highest DH was obtained with Alcalase® and L-450, and both were similar at 23.7 ± 0.5 and 23.0 ± 0.7, respectively, which was not surprising because both enzymes are highly active endopeptidase from *Bacillus licheniformis*, one from Novozyme® and the other from Biocon®, with serine-type activity. Lower DHs were obtained with Flavourzyme® and papain, 13.9 ± 0.5 and 9.6 ± 0.4, respectively, where the first was a mixture of exo- and endoproteases with a predominance of exoprotease: activity, and the second was an endopeptidase with mainly cysteine-type activity.

Figure 1B shows that a significantly higher HD was observed in the sequential process with two enzymes than in the single hydrolysis, increasing DH from 23.7 ± 0.5 to 28.7 ± 0.9 ($p < 0.01$) and from 23.0 ± 1.2 to 27.3 ± 1.6% ($p < 0.01$) for "Alcalase® + Flavourzyme®" and "L-450 + Flavourzyme®", respectively. This result can be attributed to the action of the exoprotease, which showed that a greater number of attack points led to greater activity and, therefore, broke a greater number of peptide bonds. Consequently, both combinations of enzymes were

Table 1 Basic compositions of MTM and dried Mb-PPHs obtained by single (Alcalase®) and sequential ("Alcalase® + Flavourzyme®") enzymatic hydrolytic processes

	MTM	Mb-PPH$_{(Alcalase®)}$	Mb-PPH$_{(Alcalase®+Flavourzyme®)}$
Moisture (%)	7.32 ± 1.27	9.30 ± 0.63	9.44 ± 0.55
Dry matter (%)	92.75 ± 1.23	90.70 ± 0,63	90.56 ± 0.55
Ash (% d.w.)	8.81 ± 2.32	15.31 ± 0.97	16.03 ± 0.84
Organic matter (% d.w.)	91.19 ± 2.32	84.69 ± 0.97	83.97 ± 0.84
N_t (g/100 g d.w.)	5.23 ± 0.39	10.11 ± 0.41	10.24 ± 0.28
Protein (% d.w.)	28.76 ± 1.63*	63.19 ± 1.36**	64.01 ± 1.48**
FAAs[#] (% d.w.)	1.04 ± 0.08 (3.62%)	3.85 ± 0.22 (6.09%)	7.28 ± 0.63 (11.37%)
Oligopeptides[#] (% d.w.)	9.49 ± 0.12 (33.00%)	18.12 ± 0.51 (66.64%)	18.93 ± 1.76 (67.15%)
Peptides + Proteins[#] (% d.w.)	18.23 ± 0.98 (63.38%)	5.20 ± 0.38 (19.12%)	4.13 ± 0.18 (14.65%)
Total Carbohydrates (% d.w.)	53.62 ± 3.91	11.63 ± 3.22	11.98 ± 4.12
β-Glucans (% d.w.)	14.14 ± 1.62 (26.37%)	7.74 ± 0.62 (66.55%)	8.02 ± 0.54 (66.94%)
Crude Fat (% d.w.)	3.21 ± 0.22	n.d	n.d
Others (% d.w.)***	5.8	9.89	8.78

Data are expressed as the mean ± standard deviation

*$N_t \times 5.5$

**$N_t \times 6.25$

***Determined by difference in the means

#FAAs: < 0.2 kDa; Oligopeptides: > 0.2 kDa and < 5 kDa; Peptides + Proteins: > 5 kDa

FAAs free amino acids, *n.d.* not detectable

applicable. Although the sequential process is more expensive than single hydrolysis, due to the use of two proteases, the content of FAAs and oligopeptides (> 5 kDa) is much higher than that in single hydrolysis, as we will discuss below. This scenario can be explained by a synergistic effect of endopeptidase and exopeptidase activities.

Characterization of Mb-PPHs

Table 1 also shows the basic composition of the dry Mb-PPHs obtained by single (Alcalase®) and sequential ("Alcalase® + Flavourzyme®") hydrolytic processes. These results showed that no significant differences were observed in the basic compositions, although significant differences were observed for FAAs, oligopeptides (> 0.2 and < 5 kDa) and peptides + proteins (> 5 kDa), as well as for β-glucans, between the hydrolysates obtained by single and sequential processes and with respect to MTM. This can be explained by the enzymatic activity of the endo- and exoenzymes used in the study, as we will discuss below.

These results also showed that almost half of the dry matter contained carbohydrates (mainly single sugars,

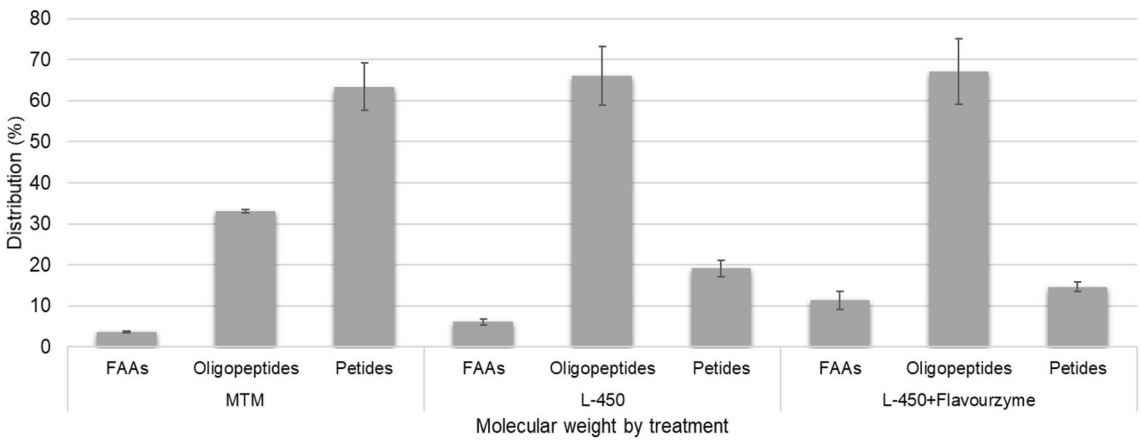

Fig. 2 Molecular weight distribution (%) of FAAs, Oligopeptides and Peptides + Proteins in MTM, Mb-PPH$_{Alcalase®}$ and Mb. PPH$_{Alcalase®,+ Flavourzyme®}$

Table 2 Amino acid composition of proteins found in MTM and Mb-PPHs obtained by single (Alcalase®) and sequential hydrolysis (Alcalase® + Flavourzyme®)

	MTM	$MbPPH_{L\text{-}450}$	$MbPPH_{LA450+Flavourzyme®}$
Ala	28.96 ± 0.83	54.97 ± 2.12	59.42 ± 1.17
Arg	14.66 ± 0.25	34.39 ± 1.48	38.48 ± 2.06
Asx	27.94 ± 1.02	52.50 ± 1.98	58.61 ± 2.01
Cys	0.95 ± 0.62	1.43 ± 0.71	1.02 ± 0.32
Glx	53.75 ± 1.04	125.19 ± 4.34	130.32 ± 5.21
Gly	15.12 ± 0.42	32.12 ± 1.89	30.18 ± 1.45
His	8.08 ± 0.30	15.91 ± 0.87	14.58 ± 0.56
Ile	11.90 ± 0.47	26.33 ± 1.43	23.78 ± 1.64
Leu	19.66 ± 0.54	74.87 ± 2.21	73.85 ± 1.98
Lys	20.81 ± 0.78	21.15 ± 1.54	24.85 ± 1.09
Met	5.89 ± 0.23	3.08 ± 0.26	2.96 ± 0.31
Phe	10.63 ± 0.58	25.61 ± 1.93	22.82 ± 2.01
Pro	12.10 ± 0.43	30.56 ± 2.11	24.74 ± 1.84
Ser	15.83 ± 0.52	29.41 ± 1.75	31.12 ± 1.16
Thr	16.58 ± 0.72	31.53 ± 2.23	28.82 ± 1.43
Trp*	5.58 ± 0.32	5.33 ± 0.44	5.39 ± 0.61
Tyr	4.26 ± 0.29	16.10 ± 1.21	15.76 ± 2.02
Val	17.42 ± 0.51	51.37 ± 4.32	53.28 ± 3,85
PEAAs	116.55	255.18	250.33

The results are expressed as the mean ± standard deviation of mg/g of product

*Determined by basic hydrolysis

PEAAs Plant essential amino acids (Trp, Thr, Val, Lys, Leu, Met, His, Phe, and Ile)

oligosaccharides and soluble polysaccharides). It should be noted that soluble β-glucans represented 7.74 ± 0.62 and 8.02 ± 0.54% of the product obtained by single and sequential hydrolysis, respectively, representing 16.25 and 17.07% of the total soluble carbohydrate content. Although the content of β-glucans in the MTM was significantly higher (14.14 ± 1.62%) than that found in Mb-PPH, the β-glucans that occurred in the MTM included both soluble and insoluble β-glucans, while in the Mb-PPH, there were only soluble β-glucans. The presence of β-glucans and β-oligoglucans in Mb-PPHs is of great importance since they can act as elicitors against certain pests [29, 30].

Figure 2 shows the molecular distribution obtained by size-exclusion chromatography for MTM, $Mb\text{-}PPH_{Alcalase®}$ and $Mb\text{-}PPH_{Alcalase®+Flavourzyme®}$, grouped into three groups: FAA (< 0.2 kDa), oligopeptides (> 0.2 and < 5 kDa) and peptides + proteins (> 5 kDa). As these results show, the main protein components of MTM are molecules with Mw > 5 kDa (peptides + proteins; 63.38 ± 1.21%), while in $Mb\text{-}PPH_{Alcalase®}$ and $Mb\text{-}PPH_{Alcalase®+Flavourzyme®}$, the main components are molecules with Mw > 0.2 and < 5 kDa (oligopeptides, 66.64 and 67.15%, respectively), obtaining the highest

Table 3 Effect of seed priming with Mb-PPHs on germination and radicle length

[Mb-PPH]	% of seed germination	Radicle length (cm)
0.01%	81.3 ± 4.6	3.1 ± 0.2
0.05%	89.0 ± 2.3	3.4 ± 0.2
0.10%	93.3 ± 4.6	3.5 ± 0.1
0.50%	94.7 ± 2.3	4.1 ± 0.2
1.00%	98.3 ± 2.3	4.3 ± 0.2
2.00%	97.3 ± 2.3	4.1 ± 0.4
5.00%	94.7 ± 6.1	4.0 ± 0.3
10.00%	92.0 ± 4.0	4.0 ± 0.3
Control	81.3 ± 1.2	2.9 ± 0.2

Data are expressed as the mean ± standard deviation

FAA concentrations in $Mb\text{-}PPHs_{Alcalase®+Flavourzyme®}$, as expected.

This type of protein hydrolysate with high oligopeptide and/or FAA concentrations has different beneficial effects in modern agriculture. Protein hydrolysates can improve crop tolerance to abiotic stresses; therefore, root applications of plant-derived protein hydrolysate have been observed to improve salinity tolerance by improving nitrogen metabolism and a higher K/Na ratio and proline accumulation in leaves [31]. Biostimulants in the presence of oligopeptides could also act as plant regulators; in this respect, several bioactive oligopeptides produced from a variety of plants have been found to have phytohormone-like activities [32, 33].

Compositional analysis shows (see Table 1) that the FAA content of MTM (1.04 ± 0.08%, d.w.) was significantly lower ($p < 0.01$) than that of the Mb-PPHs at 3.85 ± 0.22%, d.w. for the hydrolysate obtained by simple hydrolysis and 7.28 ± 0.63%, d.w. for that obtained by sequential hydrolysis. As expected, the highest content of FAAs was found in

Table 4 Effects of seed priming treatment with Mb-PPHs on the root length and fresh and dry weight of maize seed growth by the hydroponic method over 21 days

[Mb-PPH]	Root length (cm)	Root fresh weight (g/plant)	Root dry weight (mg/plant)
0.05%	17.22 ± 4.12	0.29 ± 0.12	17.11 ± 2.55
0.10%	19.56 ± 4.15	0.33 ± 0.11	19.31 ± 3.58
0.50%	20.98 ± 3.13*	0.55 ± 0.16*	28.21 ± 2.23**
1.00%	22.48 ± 3.31**	0.67 ± 0.24**	33.22 ± 3.71**
2.00%	20.93 ± 3.08*	0.61 ± 0.21**	28.53 ± 3.11**
Control	14.65 ± 3.12	0.23 ± 0.08	13.74 ± 2.16

Data are expressed as the mean ± standard deviation

*$p < 0.05$

**$p < 0.01$

Table 5 Effects of seed priming treatment with Mb-PPHs on the shoot length and fresh and dry weight of maize seed growth by the hydroponic method over 21 days

[Mb-PPH]	Shoot length (cm)	Shoot fresh weight (g/plant)	Shoot dry weight (mg/plant)
0.05%	29.07 ± 4.92	1.33 ± 0.33	78.89 ± 5.77
0.10%	31.21 ± 3.98	1.34 ± 0.34	82.31 ± 4.34
0.50%	33.22 ± 4.01	1.45 ± 0.37	88.39 ± 7.86
1.00%	34.28 ± 4.23	1.65 ± 0,22	99.26 ± 6.86
2.00%	33.87 ± 5.12	1.59 ± 0.42	92.35 ± 8.34
Control	23.83 ± 2.44	0.92 ± 0.17	74.82 ± 3.86

Data are expressed as the mean ± standard deviation

the hydrolysate obtained by sequential hydrolysis due to the exoproteasic activity of Flavourzyme®.

Table 2 shows the amino acid composition, after acid hydrolysis, of the protein material present in MTM, Mb-PPH$_{Alcalase®}$ and Mb-PPH$_{Alcalase®+Flavourzyme®}$. As these results show, the content of essential amino acids for plants (Trp, Thr, Val, Lys, Leu, Met, His, Phe and Ile) increased significantly in the Mb-PPHs compared to content in MTM, showing that the use of these hydrolytic enzymes resulted in an increase in the content of plant essential amino acids.

Regarding FAAs, practically all (18 AAs) were detected in both hydrolysates, while only 10 AAs were detected in the MTM (Ala, Asx, Glx, Gly, Leu, Lys, Phe, Ser, Tyr and Val).

Due to the amino acid, oligopeptide, peptide and carbohydrate contents, these hydrolysates could be rooting and defense protein enhancers for plants [17, 18]. Therefore, these potential activities will be studied in detail in future works, anticipating preliminary results in relation to the effect of seed priming on maize seed germination and root growth.

Effect of Seed Priming with Mb-PPHs on Germination and Growth

Seed priming was assessed at 0.00% (control), 0.01, 0.05, 0.10, 0.50, 1, 2, 5 and 10% Mb-PPHs (single and sequential) overnight (approximately 12 h), which was found to be the best period for this study (data not shown). As shown in Table 3, all concentrations assayed, except 0.01%, showed a higher germination percentage than that of the control. Of the treatments, the 1% treatment had the highest percentage of germination (98.3 ± 2.3%). Seed priming with concentrations higher than 1% (2, 5 and 10%) did not show any improvement in maize germination and even had a negative effect. Seed priming with all concentrations of Mb-PPH other than 0.01% significantly increased the length of the maize seed radicle, obtaining the highest value (4.3 ± 0.2 cm) for the 1% treatment; no increase was observed at higher concentrations.

For the hydroponic study, the concentration of Mb-PPH was reduced to the following concentrations: 0.05, 0.10, 0.50, 1 and 2%. The results obtained for root/shoot length, fresh root/shoot weight and dry root/shoot weight are shown in Tables 4 and 5. These data show that in comparison to the control, seed priming with Mb-PPH had a positive effect on root/shoot length and this effect was statistically significant for concentrations > 0.05% for the three parameters measured (root/shoot length, fresh root/shoot weight and dry root/shoot weight).

Taking into account that the vigor index (VI) was a parameter that gave information about the effect of seed priming on the development of the plant, as it combined germination data with shoot and root length, its value was determined from the results shown in Tables 3, 4 and 5. The results are shown in Fig. 3, showing that seed priming with 1.00% Mb-PPH presented the highest increase in vigor index (VI); concentrations of 0.10, 0.50 and 2.00% also yielded

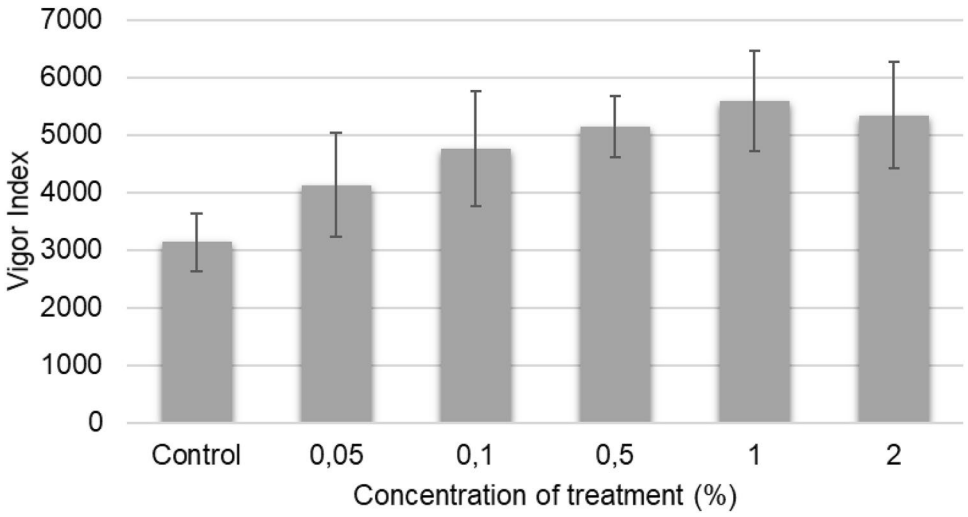

Fig. 3 Effect of seed priming with Mb-PPH on the plant vigor index (VI) of maize

a significantly different VI value from that in the control, while priming with 0.05% had no effect.

Conclusions

This work shows that using enzymatic hydrolysates from nonmarketable parts of the mushroom *Agaricus bisporus* induced the germination of seeds and stimulated root and shoot growth in maize. The combination of different proteases led to the formation of a mix of certain peptides, oligopeptides and amino acid patterns. Avoiding chemical treatments such as acid hydrolysis, we were able to conserve valuable agronomic amino acids such as tryptophan, tyrosine, serine, and glutamine. Similarly, other beneficial substances to plants, such as soluble β-glucans, were shown to prevent the need for aggressive treatment. These hydrolyzates will be tested in future studies to complete the data.

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Data Availability The datasets generated during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interest The authors have no relevant financial or non-financial interests to disclose.

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