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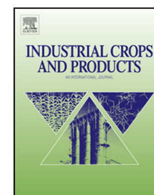

Production of an enzymatic protein hydrolyzate from defatted sunflower seed meal for potential application as a plant biostimulant

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ABSTRACT

An extensive protein hydrolyzate was produced starting from sunflower defatted seed meal, an abundant by-product of biodiesel chain oil extraction. Two-step enzymatic hydrolysis was carried out using sequentially Alcalase-Flavourzyme enzymes and a high quality hydrolyzate was produced with an interesting composition in terms of nutrient, amino acid, and peptide content for application in conventional and organic agriculture. The biostimulant properties of the hydrolyzate were investigated by carrying out auxin and gibberellin-like activity assays in Petri dishes on garden cress (*Lepidium sativum* L.) and lettuce (*Lactuca sativa* L.) seedlings and by conducting experiments in pot on maize plants. The sunflower hydrolyzate showed auxin-like, but no gibberellin-like activity and interesting effects on plant root elongation suggesting a potential use of the product as an effective biostimulant in the agricultural field.

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1. Introduction

Oleaginous crops are widely used for non-food applications in green chemistry and for energy production, as in the biodiesel chain. Lately, the amount of sunflower and rapeseed defatted seed meals, the main by-products of seed defatting, and of crude glycerol, the main by-product of oil esterification, have steadily increased and the valorization of these products is becoming a fundamental opportunity from an economical and environmental perspective (Katryniok et al., 2009; Lomascolo et al., 2012). The transformation of by-products into co-products, meaning their use as starting materials in a biorefinery chain, indeed makes it possible to decrease the amount of waste and to increase the efficiency of the entire industrial process. Sunflower defatted meal contains about 30% protein with a high quality amino acid profile, even if industrial oil extraction processes often affect its protein biological value, decreasing the solubility and bioavailability (Lomascolo et al., 2012). To overcome this aspect, sunflower protein hydrolyzates with enhanced functional properties have been extensively studied and used for food and feed applications (Gassman, 1983). In addition, polypeptides, peptides and amino acids of animal and plant origin, obtained by protein hydrolysis, have been increasingly applied in agriculture, not only as a source of organic nitrogen, but also as a sustainable and renewable tool to improve soil quality and nitrogen acquisition efficiency of plants, contributing to reduce chemical fertilizer input (Maini, 2006; Schiavon et al., 2008). Amino acid containing products, together with microbial inoculants, humic substances, seaweed extracts, and other natural origin hormone-like compounds are in fact studied for their biostimulant properties (Calvo et al., 2014). Biostimulants were recently defined as substances and materials that, when applied to plants, seeds, or growing substrates in specific formulations, have the capacity to provide potential benefits to growth, development and/or stress response (Du Jardin, 2012). Different mechanisms of action were observed as improvement of soil

Abbreviations: DSM, defatted sunflower meal; VPH, vegetal protein hydrolyzate; APH, animal protein hydrolyzate; AU, Anson unit; LAPU, leucine aminopeptidase unit; TNBS, 2,4,6-trinitrobenzenesulphonic acid; SMH, sunflower meal hydrolyzate; DH, degree of hydrolysis; OPA, o-phtalaldehyde-3-mercaptopropionic acid; FMOC, 9-fluorenylmethylchloroformate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; FPLC, fast protein liquid chromatography; IAA, indole-3-acetic acid; GA, gibberellic acid; LSD, least significance difference; ANOVA, analysis of variance; HS, Hoagland solution.

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microbial activity and soil enzymes as well as modulation of numerous plant physiology aspects, acting as positive growth regulators or metabolic enhancers. Over the past years, biostimulants have been the subject of common interest as alternative ways of increasing plant growth, limiting the extensive use of mineral fertilizers and recognizing the public's growing concern for human health conditions and environmental pollution. The biostimulating effect of hydrolyzates, containing free amino acids and oligo-peptides but also other bioactive non-protein components, has been demonstrated in many application studies, and related products have been successfully commercialized, with the largest market for biostimulants in 2012 in Europe (Calvo et al., 2014; Ertani et al., 2009; Kauffman et al., 2005; Maini, 2006). Accordingly, sunflower defatted meal is an interesting starting material, and products obtained by its protein hydrolysis, besides being a supply of nitrogen and microelements with fertilizing properties, could also play a role as a biostimulant.

In order to obtain small peptides and free amino acids from an extensive hydrolysis of sunflower meal, conventional chemical processes involving strong acids or bases and high temperatures have several drawbacks, concerning both the sustainability of the process and the chemical composition of the final product. For these reasons, the use of enzymes from fungi and bacteria has become the most common choice, thus preserving the quality of free amino acids. In particular, the use of two-step enzymatic hydrolysis by Alcalase and Flavourzyme, as endo and exo-peptidases, has been widely studied and applied to protein concentrates or isolates obtained by preliminary processing of the defatted seed meal (Parrado et al., 1991; Villanueva Clemente et al., 1999). The aims of the present study were: (i) to explore the possibility of obtaining hydrolyzates by an optimized enzymatic process performed directly on sunflower defatted meal; (ii) to characterize the sunflower meal hydrolyzate (SMH) composition and its qualitative and quantitative amino acid content; (iii) to evaluate the auxin and gibberellin-like activity of the SMH in a bioassay performed on garden cress and lettuce seedlings respectively; and (iv) to asses biostimulant properties of the SMH on maize plant seedlings.

2. Materials and methods

2.1. Materials

Defatted sunflower meal (DSM), produced by an industrial mechanical-hexane oil extraction process, was provided by Italcol S.p.A Castelfiorentino (Florence, Italy).

Protein hydrolyzates obtained by controlled hydrolysis of organic substances from plant origin (vegetal protein hydrolyzate, VPH) and from animal origin (animal protein hydrolyzate, APH) were purchased from the market and used for comparison with SMH.

2.1.1. Enzymes

Alcalase® (endopeptidase from Bacillus licheniformis, 2.4 L) and Flavourzyme® (exopeptidase and endoprotease complex from Aspergillus oryzae, 500 L) were purchased from Sigma–Aldrich. The activity of Alcalase was 2.4 Anson units (AU) g^{-1}, where one AU is the amount of enzyme which digests hemoglobin, at an initial rate that produces an amount of trichloro acetic acid-soluble product which gives the same color with the Folin–Ciocalteu reagent as one milliequivalent of tyrosine per minute at 25 °C at pH 7.5. The activity of Flavourzyme was 500 leucine aminopeptidase units (LAPU) g^{-1}. One LAPU is the amount of enzyme that hydrolyzes 1 μmol of L-leucine-p-nitroanilide per minute.

2.1.2. Chemicals

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

2.2. Enzymatic hydrolysis

Enzymatic hydrolysis was first performed on three starting materials: (i) DSM as such, (ii) enriched DSM, (iii) protein isolate DSM. Enriched DSM was prepared by following the sedimentation/flotation fractionation described by Parrado et al. (1991). The protein isolate DSM was prepared as described by Villanueva Clemente et al. (1999), with the difference that alkaline extraction and protein precipitation at isoelectric point (pH 4.3) were carried out on DSM instead of on a protein concentrate. The protein content of the two different DSM-derived products was compared to the initial content in DSM. The three starting materials were then subjected to hydrolysis with Alcalase enzyme and the degree of hydrolysis (DH) was evaluated (Section 2.3). Hydrolysis was performed with the pH-stat technique as follows: products were suspended in distilled water (10%, w/v) in the reactor of an automatic titrator (Mettler Toledo DL 50 Graphix); the solution was adjusted to pH 8 and then incubated for 15 min at 50 °C for maximum protein extraction/solubilization; after thermal pre-treatment, 0.2 AU g^{-1} (Alcalase-meal protein ratio) were added and the hydrolysis was carried on for 60 min, at pH 8 and 50 °C. Subsequent hydrolysis protocols were performed on DSM as such and a two-step hydrolyzing process was applied: the Alcalase step, as described before, followed, after 60 min, by the Flavourzyme hydrolysis with 50 LAPU g^{-1} (enzyme-meal protein ratio), for 120 min at 50 °C and pH 7. During each reaction the pH was adjusted with 2.0 M KOH. The hydrolysis processes were stopped by adjusting the pH to 6.0 with citric acid; hydrolyzates were then recovered by centrifugation, freeze-dried, and stored at room temperature. This process made it possible to obtain the final SMH, subsequently characterized and used for the experiments described below.

2.3. Degree of hydrolysis

The DH is defined as a percentage of peptide bonds cleaved during enzymatic hydrolysis. The hydrolysis progress with Alcalase was evaluated with the pH-stat method (Adler-Nissen, 1986), based on the titration of protons released during hydrolysis. The volume of the appropriate base titrant (2 M KOH) was automatically recorded and used to calculate the DH. The Flavourzyme DH was determined by a spectrophotometric method measuring the chromogenic product formed by the reaction between free primary amino acids and 2,4,6-Trinitrobenzene Sulfonic Acid (TNBS), according to the Adler-Nissen method (1979). A UV–VIS spectrophotometer (Varian Cary-300 BIO) was employed for absorbance measurement (340 nm) and L-leucine (0.5, 1.0, 1.5, 2.0 mM) was used to generate a standard curve.

2.4. Chemical characterization of DSM and SMH

DSM and SMH were characterized for moisture, residual oil, protein, ash, organic matter and carbon content following methodologies described in De Nicola et al. (2013). Fiber content was determined with an FIWE fiber analyzer (Velp Scientifica) following the Van Soest method (Van Soest and McQueen, 1973). Micro and macroelement, sugar and indol-3-acetic acid (IAA) content analyses were performed by the Research Institute for Agroindustry s.r.l. (Modena, Italy): P, Ca, K, B, Cu, Mg, Zn

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