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Bitter vetch (*Vicia ervilia*) seed protein concentrate as possible source for production of bilayered films and biodegradable containers

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ABSTRACT

The manufacture of biodegradable and renewable containers from bitter vetch seed protein-based "bioplastics" was investigated. A bitter vetch seed protein concentrate was prepared and analyzed for proteins, carbohydrates, phenols, other several organic compounds and multi-elements. Protein film forming solutions were cast in the presence of two different glycerol concentrations and the film containing higher plasticizer amount was laminated with an additional corn zein layer. Both lamination process and lower glycerol concentration led to reduce film moisture content, total soluble matter and elongation at break, while both film tensile strength and water vapour barrier properties resulted enhanced. The obtained bioplastics were then processed by a new laboratory plastic moulding equipment specifically designed and fabricated to convert protein-based films to shaped containers. The use of either lower plasticizer concentration or corn zein lamination gave rise to potentially satisfactory vacuum thermoformed containers with acceptable resistance and stability.

1. Introduction

The dramatic increase in production and lack of biodegradability of commercial polymers, particularly commodity plastics used in packaging industry and agriculture, focused public attention on a potentially huge environmental accumulation and pollution problem that could persist for centuries. In fact, petroleum-based synthetic polymers occur in the ecosystem as industrial waste products generating multiple serious problems, e.g. visual pollution, blockage of drains, livestock deaths and threat to aquatic life (Shimao, 2001). There is, thus, an increasing interest toward bio-based polymers to produce eco-friendly materials. In fact, a future extensive use of renewable and biodegradable plastics would allow to avoid the petroleum dependence, as well as the incineration and landfill pitfalls, making these "bioplastics" strategic for a sustainable development. There are three main ways to produce bioplastics: i) by starting from bio-based monomers obtained by fermentation or conventional chemistry and polymerizing them in a second step (e.g. polylactic acid), ii) by producing bio-based polymers directly in microorganisms or in genetically modified crops (e.g. polyhydroxyalkanoates), iii) by using different natural polymers (e.g. polysaccharides and proteins). Natural polymers are often modified mechanically, physically or chemically, as well as combined with plasticizers or further polymeric additives, to overcome several problems of different origin. Therefore, most of the raw material converted to bioplastics may come from the nature, green plants and microorganisms, or even from eatable vegetables. Two examples are sugarcane and wheat straw, annually renewable resources, the by-products of which (bagasse and straw waste) can be turned into several articles normally made from plastic or paper. Further widely used biomaterials are polylactic acid, polybutylenes succinate, poly3-hydroxybutyrate, polycaprolactone, starch- and cellulose-based biopolymers (Smith, 2005).

Among the possible natural biomacromolecules, proteins offer a valid alternative to the mentioned biopolymers (Arcan & Yemenicioglu, 2011; Bambdad, Goli & Kadivar, 2006; Borreani & Tabacco, 2015; Giosafatto et al., 2014; Peelman et al., 2013; Razavi, Amini, & Zahedi, 2015; Tang, Xiao, Chen, & Yang, 2011). Numerous potential applications for protein-based bioplastics are imaginable, from biodegradable containers to packaging materials, from agricultural mulching films to food service utensils. However,

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to date only few commercial products have been made by using proteins as raw material, even though different vegetable and animal proteins have been suggested for obtaining edible coatings and biodegradable packages (Anderson & Lamsal, 2011; Cho, Lee, & Rhee, 2010; Cuq, Gontard & Guilbert, 1998; Peelman et al., 2013). Among these, whey and soy proteins represent a biodegradable and reproducible resource and, exhibiting an acceptable biocompatibility and processability, show an interesting potential mostly in both food and agricultural biotechnology (Di Pierro et al., 2013 and Mariniello et al., 2003). Some applications of whey and soy protein-based materials, especially for food preservation and packaging technology, have been reported (Di Pierro, Sorrentino, Mariniello, Giosafatto, & Porta, 2011; Grewell, Schrader, & Srinivasan, 2014; Rossi Marquez, Di Pierro, Esposito, Mariniello & Porta, 2014; Song, Tang, Wang & Wang, 2011). Also zein, a protein extracted from corn (*Zeamays* L.) during the wet-milling process (Shukla & Cheryan, 2001) has many properties that make it an appealing bioplastic feedstock, being an ethanol hydrophobic by-product and, thus, quite water resistant (Anderson & Lamsal, 2011; Arcan & Yemenicioglu, 2011; Ghanbarzadeh et al., 2006; Helgeson, Graves, Grewell, & Srinivasan, 2009). Some species of leguminous family are cheap protein sources, generally used only for animal feeding (Saki et al., 2008). One of these, bitter vetch (*Vicia ervilia*; BV), is widely cultivated throughout the temperate areas of Europe, western and central Asia, north Africa and Americas for the utilization of its seeds and hay. BV seeds contain up to 25% of protein (Larbi, El-Moneim, Nakkoul, Jammal, & Hassan, 2011; Pastor-Cavada, Juan, Pastor, Alaiz, & Vioque, 2011; Reisi, Zamani, Vatankhah, & Rahimiyan, 2011; Sadeghi, 2011) and, therefore, were recently analyzed as sustainable alternative source to produce biodegradable films, edible coatings and, potentially, properly shaped biodegradable containers (Arabestani, Kadivar, Shahedi, Goli, & Porta, 2013; Porta et al., 2015). Therefore, the specific objective of the present work was to develop biodegradable containers made of BV seed proteins using a simple laboratory equipment newly designed and fabricated. For this purpose a BV protein concentrate (BVPC) was prepared and its content in proteins, carbohydrates, as well as in multi-element, phenolic and other organic compounds, preliminarly analyzed.

2. Materials and methods

2.1. Materials

BV seeds were obtained from a local market in Isfahan, Iran. Zein powder (containing 90% protein dry weight) was purchased from Suvchem (India). Ethanol (96%) was supplied from Sepahan Teb Company (Isfahan, Iran) and polyethylene glycol 400 from Serva Electrophoresis GmbH (Heidelberg, Germany). Sodium hydroxide, hydrochloric acid (37%) and glycerol (about 87%) were purchased from the Merck Chemical Company (Darmstadt, Germany). All reagents for multi-element, organic compound, carbohydrate and protein analyses were from Sigma Chemical Company (St. Louis, MO).

2.2. Preparation of BV seed proteins and BVPC

Proteins were extracted from BV seeds according to Monsoor and Yusuf (2002) by milling the seeds to a fine powder (40 mesh) and soaking the latter in distilled water (1:10, w/v) brought at pH 10 by 0.1 N NaOH. After stirring (IKA® RH basic 2, Germany) at medium speed for 1 h at 25 °C, the mixture was centrifuged at 3200 g for 10 min and the precipitate discarded. The pH of the supernatant was then adjusted to pH 5.4 by 0.1 N HCl addition and the resulting precipitate, collected after centrifugation at 3200 g for 10 min, was finally dissolved by adding 0.1 N NaOH until pH 7.0 was reached and the obtained solution dried in a vacuum oven at 40 °C. The obtained dry concentrate of BV proteins was finally minced in a coffee grinder.

Protein content of both BV seeds and of the derived final BVPC was determined by the Kjeldahl's method (AACC, 2003), using a nitrogen conversion factor of 6.25.

2.3. BV protein identification

In gel trypsin digestion and protein identification. Proteins contained in BVPC sample were analyzed by 12.5% SDS-PAGE. The gel (1.5 mm) was run at constant 25 mA for 1 h, then stained with colloidal Coomassie, and the blue-stained protein bands excised from the gels and analyzed as follows. Gel particles were first separately washed with acetonitrile and with 0.1 M ammonium bicarbonate, then incubated with 10 mM DTT for 45 min at 56 °C and, finally, the cysteine residues alkylated by 5 mM iodoacetamide treatment for 15 min at room temperature in the dark. After washing with ammonium bicarbonate and acetonitrile, an enzymatic digestion was carried out by incubating the sample in the presence of trypsin (10 ng/μL) in 50 mM ammonium bicarbonate buffer, pH 8.5, at 4 °C for 2 h to allow the enzyme to enter the gel. The solution was then removed and a new aliquot of buffer was added and the incubation continued for 18 h at 37 °C. A minimum reaction volume, sufficient for a complete gel rehydration, was used. At the end of the incubation the peptides were extracted by washing the gel particles with 0.1% formic acid in 50% acetonitrile at room temperature and the sample freeze-dried.

Mascot identification. The sample was analyzed by LCMSMS on a 6520 Accurate-Mass Q-TOF LC/MS System (Agilent Technologies, Palo Alto, CA) equipped with a 1200 HPLC system and a chip cube (Agilent Technologies). After loading, the peptide mixture was first concentrated and washed in 40 nL enrichment column (Agilent Technologies chip), with 0.1% formic acid in 2% acetonitrile as eluent. The sample was then fractionated on a C18 reverse-phase capillary column (Agilent Technologies chip) at a flow rate of 400 nL min^{-1}, with a linear gradient of eluent B (0.1% formic acid in 95% acetonitrile) in eluent A (0.1% formic acid in 2% acetonitrile) from 5 to 80% in 50 min. Peptide analysis was performed using data-dependent acquisition of one MS scan (mass range from 300 to 1800 m/z) followed by MS/MS scans of the five most abundant ions in each MS scan. MS/MS spectra were measured automatically when the MS signal surpassed the threshold of 50,000 counts. Double and triple charged ions were preferably isolated and fragmented. The acquired MS/MS spectra were transformed in Mascot generic format (.mgf) and used for protein identification in the unreviewed set of protein entries that are present in the NCBInr database for all bacteria, with a licenced version of Mascot software (http://www.matrixscience.com) version 2.4.0. Additional Mascot search parameters were: peptide mass tolerance 10 ppm, fragment mass tolerance 0.6 Da, taxonomy Viridiplantae (Green Plants, 2,907,072 sequences) allowed trypsin missed cleavages up to 3, carbamidomethylation of cysteines as fixed modification, oxidation of methionine, pyro-Glu N-term Q, as variable modifications. Only doubly and triply charge ions were considered. Ions score was −10 log(P), where P is the probability that the observed match is a random event. Individual ion scores > indicated identity or extensive homology ($P < 0.05$).

2.4. Analysis of low MW carbohydrates and other organic compounds

1 mg of BVPC was dissolved in 500 μL of 1 M methanolic-HCl at 80 °C for 16 h. The re-N-acetylation of the monosaccharide mixture