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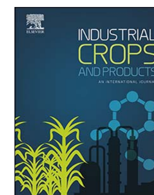

Soybeans isoflavone aglycone-rich extracts: Optimization by different bioprocesses and production of a purified fraction with promising wound healing property

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ABSTRACT

Positive skin effects have been reported for isoflavones, mainly for their aglycone forms. In this way, the present study aimed to produce a fraction enriched in isoflavone aglycones (IA) from soybeans and to evaluate its potential for skin cells proliferation. For that, it was firstly obtained hydrolyzed extracts from defatted soybeans by different hydrolytic mechanisms, including acid, enzymatic and fermentation processes. The analysis of IA, sugars, furanic compounds, saponins and triterpenes were performed and compared for each hydrolyzed extract. The acid hydrolysis was accomplished with a classical process; however, the conditions used during enzymatic hydrolysis (EH) and fermentative process (FP) were screened by Plackett-Burman design, and subsequently optimized by Box Behnken design. The procedures were carried out using a sample corresponding to an extract obtained from 1.0 g of defatted soybeans. The optimum conditions to enhance IA content were obtained by EH using 838 units of β-glucosidase during 4.5 h at pH 6.0 and 37 °C, and by FP using 1500 mg of commercial bakery yeast (*Saccharomyces cerevisiae)* during 24 h at pH 7.6 and 33 °C. All hydrolyzed extracts were partitioned with ethyl acetate to obtain IA-rich fractions. The most pure and easily obtained fraction was the one from enzymatic hydrolyzed extract. This fraction was considered non-cytotoxic for keratinocytes after 24 and 48 h of treatment with concentrations between 0.1–1.0 μg of total IA/mL. Moreover, this fraction showed a proliferative effect at 0.1 μg of total IA/mL, suggesting its potential as an ingredient for skin regeneration during wound healing.

1. Introduction

Soybeans (*Glycine max* (L.) Merrill) are a rich source of proteins, carbohydrates, lipids, and other phytochemicals, such as sterols, isoflavones and saponins (Cederroth et al., 2012). The consumption of this legume, or derived products, is associated with many health benefits, mostly linked to the presence of isoflavones (Barnes, 2010). These compounds play a key role in several health effects due to their estrogen receptors binding ability (Barnes, 2010; Morito et al., 2001). Some estrogen-like properties exhibited by isoflavones are the stimulation of hyaluronic acid and collagen synthesis by keratinocytes and fibroblasts, respectively, resulting in skin repair processes that include reduction of wrinkles and wound healing (Miyazaki et al., 2002; Sudel et al., 2005).

Isoflavones naturally occur in soybeans as glucosidic conjugates (Albulescu and Popovici, 2007). Because of this, normally the acidic, basic, and enzymatic hydrolysis processes are applied to obtain their bioactive forms from soy products. These processes are responsible for the breakdown of the conjugated isoflavones, resulting in the aglycone units of genistein, daidzein and glycitein (Schwartz and Sontag, 2009). The acid hydrolysis has advantages regarding coast and speed of process. However, the greatest disadvantage is the lack of specificity for a specific target, resulting in undesired degradation products (Chen et al., 2014; Schwartz and Sontag, 2009). Recently, it was demonstrated that acid hydrolysis of soybeans not only transform the conjugated isoflavones into their aglycones, but also degraded the soybean oligosaccharides until furanic compounds (Nemitz et al., 2015a). The mainly sugar degradation products formed are hydroxymethylfurfural (HMF) and ethoxymethylfurfural (EMF), both considered genotoxic compounds when in high amounts (Nemitz et al., 2016).

In order to obtain an isoflavone aglycones-rich fraction (IAF) intended to healthcare products development, recently Nemitz et al.

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(2015a) developed a purification method capable to remove furanic impurities from soybean acid hydrolyzed extracts. However,although the method has allowed obtaining an IAF with high purity, it cannot be considered a good candidate for scaling up during industrial routine, since it requires several steps making the process slow and unfavorable. Taking this into account, when the intention is the large production of IA from soybeans, it is recommended to seek alternatives using more selective methods of hydrolysis. Therewith, fewer impurities probably would be formed, and the purification process would be simpler and more appropriate for industrial application.

In this context, the use of enzymes can be a very interesting hydrolysis alternative (Singh et al., 2016). In the case of isoflavone biotransformations, the use of β-glucosidases is largely reported in literature (Ismail and Hayes, 2005; Lee et al., 2008). Besides, this kind of process has been performed during procedures in some industrial companies (Shen and Bryan, 1997, 1998). The enzymatic hydrolysis can be carried out using purified β-glucosidases isolated from natural sources, or using microorganisms that expressed this kind of enzyme (Singh et al., 2016). Considering the last option, several fungal and yeasts containing β-glucosidases are applied during food industrial processes to obtain fermented soy derivatives rich in IA for dietary products. Some microorganisms used for this purpose are the strains of species from the genera *Aspergillus*, *Rhizopus*, *Mucor*, *Actinomucor*, *Monascus, Saccharomyces, Neurospora, Acetobacter, Bacillus* and *Lactobacillus* (Chen et al., 2012; Rosa et al., 2009).

Several yeasts have high β-glucosidase activity, resulting in the breakdown of all sugar β-conjugated compounds present in soybeans during fermentation process. However, yeasts that have low enzymatic activity cannot carry out the hydrolysis process with high performance, producing an incomplete biotransformation of isoflavones (Dueñas et al., 2012). In these cases, it is important to highlight that yeast fermentation process could be performed not only intended to IA production, but also to promote complementary steps for further processes (Rekha and Vijayalakshmi, 2010). These practices are mainly reported by food engineering.

From the context here presented, this study was conducted in order to cover two main goals: (1) obtaining fraction with high IA purity through a simple purification process, and (2) obtaining IAF intended to be used as ingredient of dermal products, especially for wound treatments. To achieve these purposes, different hydrolysis mechanisms in soybean extracts, including acidic, enzymatic and fermentative processes were investigated. The hydrolysis methods mediated by biocatalysts were optimized by factorial experimental designs to maximize the IA content. The chemical composition of hydrolyzed extracts was compared not only for IA, but also for sugars, furanic compounds, saponins and triterpens. Purification process with ethyl acetate partition was performed in extracts, and the chemical compositions of IA-rich fractions were analyzed and compared. Finally, to suggest the application of an IAF for wound treatment, keratinocyte viability after IAF treatment was evaluated by the MTT assay, and the proliferation effect by Ki-67 assay.

2. Materials and methods

2.1. Chemicals

Soybeans from EMBRAPA BRS 262 cultivar were obtained by donation of SEMEL seeds (São Paulo, Brazil). Isoflavone standards, daidzein, glycitein and genistein, as well as the β-glucosidase enzyme were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Glucose, furanic standards HMF and EMF were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fresh baker's yeast of the species *Saccharomyces cerevisiae* (Fleischmann®) was acquired from a local supermarket. The UFLC solvents were purchased from Merck (Darmstadt, Germany). Celite® resin was supplied by Merck Millipore (Darmstadt, Germany). Other reagents, e.g., hydrochloric acid (HCl), ethylenediaminetetraacetic acid (EDTA), dimethyl sulfoxide (DMSO) were acquired from Nuclear (Diadema, SP, Brazil). For cells culture, Dulbecco's modified Eagle's medium (DMEM), trypsin/EDTA solution and 3-(4,5-dimethylthiazol- 2yl) 2,5-diphenyl-tetrazolium bromide salt (MTT) were supplied by Sigma-Aldrich (St Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Gibco (Grand Island, NY, USA). Ki-67 antibody (sc-23900 PE) was supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Hydroethanolic soybean extraction

Soybeans seeds were grinded with an analytical mill. Then, they were defatted by Soxhlet extraction with *n*-hexane. The defatted soybean (DS) powder was extracted in a Soxhlet using a protocol of 10.0 g of DS and 200 mL of ethanol 80% (v/v), during 8 h at 70–80 °C. Several extractions were performed, pooled, filtered and stored at −4 °C.

2.3. Hydrolytic procedures

Before performing the hydrolysis processes, the ethanol present in the hydroethanolic soybean extract (HSE) was removed by evaporation under reduced pressure. Each 200 mL of HSE was reduced to approximately 20 mL, and then, the volume was adjusted to 40 mL with purified water. The process was repeated several times, pooled and stored at −20 °C. Each 4 mL of aqueous extract corresponds to the content that has previously extracted from 1.0 g of DS.

2.3.1. Controls – extracts without hydrolysis and with acid hydrolysis

For negative control it was used a non-hydrolyzed extract, in which 4 mL of aqueous extract were diluted with ethanol up to a final volume of 50 mL. For positive control it was used an acid hydrolysis protocol, in which 4 mL of aqueous extract were diluted with ethanol and HCl was added up to a final acid concentration of 1.3 M. The mixture was adjusted to a volume of 40 mL with ethanol and refluxed at 80 °C for 2 h. At the final of the process, the acid hydrolyzed extract was diluted up to a final volume of 50 mL with ethanol.

2.3.2. Screening protocols of hydrolysis

Hydrolysis conditions were screened by Plackett-Burman design to visualize the significant factors during enzymatic hydrolysis (EH) and fermentation process (FP). The Minitab 17® software was used to generate and analyze the experimental designs. The twelve-run designs are presented in Table 1, and the assays were accomplished to select the most critical factors during conversion of isoflavone glucosides to aglycones. The EH independent variables (−1; +1) were: x_1: enzyme concentration (200 U; 800 U), x_2: pH (5.8; 7.8); x_3: time (2 h; 8 h), x_4: temperature (25 °C; 50 °C). Whereas, the FP independent variables (−1; +1) were: x_1: yeast concentration (300 mg; 900 mg), x_2: pH (5.8; 7.8), x_3: time (2 h; 8 h), x_4: temperature (25 °C; 50 °C). To execute each hydrolysis process, an aliquot of 4 mL of aqueous extract was diluted with an adequate buffer solution, and an amount of enzyme or yeast was added up to the desired concentration. The mixture was maintained during pre-established times. At the end of the process, ethanol was added to stop the reactions, the solution was filtered, and then, the extract was diluted to 50 mL with this same solvent.

2.3.3. BBD optimizations

For optimization of EH it was used a design with three factors in three different levels, as show in Table 2. A total of 15 experiment trials were conducted in randomized runs and with three center points to estimate the pure error. The protocol was carried out at 37 °C, and with 4 mL of aqueous extract. The BBD independent variables (−1; 0; +1) were: x_1: enzyme concentration (200 U; 600 U; 1000 U), x_2: pH (5.8; 6.8; 7.8), x_3: time (2 h; 4 h; 6 h).

For optimization of FP it was used a design with four factors in three

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