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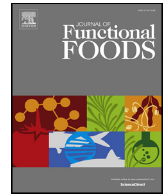

Enhanced estrogenic effects of biotransformed soy extracts

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ABSTRACT

Soy isoflavones have been associated with several beneficial effects to human health including estrogenic action. Due to the risks associated with pharmaceutical hormone replacement therapy, soy extracts rich in isoflavones and metabolites appear as a safe alternative for menopausal women. This study aimed to improve the process conditions for biotransformation of soymilk polyphenols by tannase to obtain higher amounts of isoflavone aglycones and metabolites such as equol. The estrogenic potential of the extracts was evaluated in *in vitro* assays. The results showed that the aglycones increased 36–46 times after reaction with tannase and proved that it is possible to produce equol in soymilk using an enzymatic bioprocess, without gut microbial intervention. Moreover, the soymilk biotransformed by tannase presented higher estrogenic action in the MCF-7 BUS cell line assay, making it a promising nutraceutical with possible effects in the attenuation or treatment of menopause symptoms.

1. Introduction

Soy-based products, an important source of nutrients and phytochemicals, have been appreciated worldwide as health promoting foods. Isoflavones, a class of phytoestrogens present in high concentration in soybeans, have been widely investigated because of their potential health-enhancing properties, including prevention and therapy of some chronic diseases such as cancers and cardiovascular diseases as well as their ability to relieve the symptoms of menopause in women (Rimbach et al., 2008; Sunita & Pattanayak, 2011; Uifălean, Schneider, Ionescu, Lalk, & Iuga, 2016).

Soybeans contain four distinct chemical structures of isoflavones known as aglycone (genistein, daidzein, and glycitein), glycoside (genistin, daidzin, and glycitin), acetyl glycoside (acetyl genistin, acetyl daidzin, and acetyl glycitin), and malonyl glycoside (malonyl genistin, malonyl daidzin, and malonyl glycitin) (Shao et al., 2011). The biological activity of these compounds in the human body depends on their chemical forms. Among them, isoflavone aglycones seem to have higher beneficial effects on human health than other forms (Izumi et al., 2000). Fortunately, isoflavone glycosides can be enzymatically converted to their respective aglycones, which has a significant effect on their bioavailability (Ismail & Hayes, 2005). Moreover, the isoflavone aglycones can be metabolized to many specific compounds in the human gut, with emphasis to equol, a most bioavailable metabolite and directly associated with the clinical efficacy of isoflavones (Rowland et al., 2003). Equol [7-hydroxy-3-(4-hydroxyphenyl)-chroman] is a nonsteroidal estrogen produced by daidzein metabolism and, according to the present literature, is formed exclusively by intestinal bacteria (Setchell, Brown, & Lydeking-Olsen, 2002; Yuan, Wang, & Liu, 2007). Therefore, there is growing interest in soy-based products with high bioactivity, being important to generate a commercial source of isoflavone aglycones and bioactive metabolite, such as equol.

A common method to convert glycosides to bioactive aglycones is enzymatic hydrolysis, which is considered more specific than chemical hydrolysis. The β-glycosidase, an enzyme naturally presents in soybeans and produced by various microorganisms, is able to catalyze the hydrolysis of isoflavone glycosides (Chen et al., 2013; Park, Alencar, Nery, Aguiar, & Pacheco, 2001). The use of β-glycosidase to produce food supplements rich in more bioavailable soy isoflavones is the subject of a patent with commercial exploitation (Park, Scamparini, Sato, & Alencar, 2004).

Our research group proposes the application of a different enzyme, named tannase (E.C. 3.1.1.20) or tannin acyl hydrolase, which has great potential to bioconvert the soy isoflavones into aglycones and further.

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De Queirós, Macedo, and Macedo (2016) demonstrated the potential of aglycones production and equol generation by this enzyme in soymilk bioprocessing. This was the first study that was able to produce equol through an enzymatic process of biotransformation of soy isoflavones.

Tannase is an esterase enzyme largely characterized by its activity on phenolic complex and its ability to perform hydrolysis reactions on ester bond and depside linkages of hydrolysable and condensed tannins (García-Conesa, Østergaard, Kauppinen, & Williamson, 2001). Furthermore, this extracellular enzyme also exhibits deglycosylation activity, demonstrating great potential (Ferreira, Macedo, Ribeiro, & Macedo, 2013).

In view of the knowledge accumulated by our research group and the broad range of action of tannase on phenolics in foods, it was important to further evaluate the capacity of the enzyme to biotransform soy isoflavones, since tannase has presented a more extensive action on phenolics than β-glycosidase, as well as higher bioactive potential on the generated extracts.

According to some authors, isoflavones present structural and functional similarities to the 17β-estradiol hormone, demonstrating affinity for estrogen receptors. The isoflavones can bind to both estrogen receptor subtypes (ERα and ERβ), which exert distinct biological functions, with a slightly greater affinity to bind to ERβ (Wuttke, Jarry, & Seidlová-Wuttke, 2007; Zhao & Mu, 2011). ERα is present mainly in mammary gland, uterus, ovary (thecal cells), and hypothalamic tissue, whereas ERβ is found in kidney, brain, bone, ovary (granulosa cells), heart, lungs, intestinal mucosa, prostate, and endothelial cells (Bai & Gust, 2009; Taylor, Levy, Elliott, & Burnett, 2009).

In hormone dependent tissues, estrogens play an essential role in several physiological processes, including cell proliferation, differentiation, or apoptosis. However, elevated levels of estrogens are a main risk factor for the progress of hormone dependent diseases, increasing the risk of developing breast, endometrial, and ovary cancers (Kumar, Lata, Mukhopadhyay, & Mukherjee, 2010; Schairer et al., 2000; Uifălean et al., 2016).

Therefore, due to the risks mentioned above, pharmaceutical hormone replacement therapy, although effective in most cases, does not seem to be the best treatment option in menopause. So, soy extracts rich in isoflavones and metabolites appear as an alternative to relieve and improve the symptoms in menopausal women.

Continuing the endeavors of our research group, this study aimed to improve the process conditions for biotransformation of soymilk polyphenols by tannase to obtain higher amounts of isoflavone aglycones and metabolites, such as equol. The estrogenic potential of the obtained extracts in *in vitro* assays were also verified. The traditional enzymatic biotransformation of soy isoflavones with β-glycosidase was performed altogether to compare the results with an established process.

2. Materials and methods

2.1. Materials

Soybeans (IAC Foscarin 31 variety) were obtained at the Agronomic Institute of Campinas, Campinas, SP, Brazil. Analytical standards daidzin, genistin, daidzein, genistein, and equol, gallic and tannic acids, β-glycosidase enzyme (naringinase from *Penicillium decumbens*), sodium alginate, Folin-Ciocalteu reagent, sulforhodamine B sodium salt and Trizma® base were purchased from Sigma-Aldrich (Steinheim, Germany). All other chemicals were acquired in the grade commercially available. Cell culture reagents were purchased from Invitrogen®.

2.2. Soymilk preparation

Soymilk was prepared following the methodology developed by Embrapa Soja, Brazil, with modifications (Mandarino & Carrão-Panizzi, 1999). Whole soybeans were macerated in distilled water (1:3 w/v) for 6 h at room temperature. After that, the beans were boiled (1:6 w/v) for 5 min and then blended in distilled water (1:6 w/v) for 2 min. The resultant slurry was centrifuged at 5000 rotations per minute (rpm) for 15 min at room temperature (Megafuge 16R, Thermo Electron Led GmbH, Langenselbold, Germany). The supernatant, designated soymilk, was pasteurized at 85 °C for 15 s, freeze-dried, and stored at −20 °C.

2.3. Enzymes

Tannase from *Paecilomyces variotii* was obtained according to a previously published procedure by Battestin and Macedo (2007). For comparison, commercial β-glycosidase enzyme (naringinase from *Penicillium decumbens*) was also used in the present study.

For tannase and β-glycosidase immobilization, sodium alginate (viscosity 20,000–40,000 cps) was used as carrier for ionic gelation. Fifteen milliliters of the 5% sodium alginate media were mixed with 200 mg of tannase or 1050 mg of β-glycosidase powder, based on enzymatic activity (β-glycosidase activity of 4.2 U/mg was fixed to both enzymes). The mixture was dropped into 90 mL of 0.1 M calcium chloride using a peristaltic pump to obtain equally sized polymeric beads (20 mm) composed of calcium alginate. After repose for 6 h at 7 °C, the beads were washed with 200 mL of distilled water to remove the excess calcium ions and the unbound enzyme. The beads were dried for 16 h in refrigerated environment and stored in the same conditions until analysis (Mohapatra, Mondal, & Pati, 2007).

Both enzymes (from *P. variotii* and *P. decumbens*) were assayed for β-glycosidase activity. Free and immobilized enzymes were tested according to Matsuura, Sasaki, and Murao (1995), with modifications, using the chromogenic substrate ρ-nitrophenyl-β-D-glucopyranoside (ρ-NPG). The enzymatic activity was evaluated by monitoring the release of ρ-nitrophenol at 410 nm, using a standard curve of ρ-nitrophenol, ranging from 0.036–0.576 μmol/mL. One unit of β-glycosidase activity (U) was defined as the amount of enzyme required to release 1 μmol of ρ-nitrophenol per minute of reaction.

Tannase activity of enzyme from *P. variotii*, in free and immobilized forms, was also evaluated according to Sharma, Bhat, and Dawra (2000), adapted using tannic acid as substrate. The enzymatic activity was measured by monitoring the release of gallic acid at 520 nm, using a calibration curve of gallic acid ranging from 0.013–0.321 μmol/mL. One unit of tannase activity (U) was defined as the amount of enzyme that released 1 μmol/min of gallic acid.

For specific enzymatic activity calculation, the protein content was determined according to Bradford (1976).

2.4. Optimization of biotransformation process parameters

The first set of conditions were fixed as soymilk (lyophilized powder) at the concentration of 200 mg/mL of distilled water, pH 6.5, temperature of 40 °C and reaction time of 30 min, based on previous work by De Queirós et al. (2016). In this condition, the concentrations of immobilized tannase tested were 1, 5, 10, and 15% (w/v). After the concentration of enzyme was fixed, the following variables were tested one at a time: hydrogen potential – pH (5, 6, 6.5, and 7), temperature (30, 40, 50, and 60 °C), and reaction time (15, 30, 45, and 60 min). The samples were incubated in thermostated bath (model B12D, Micronal, São Paulo, Brazil) with stirring at 100 rpm and the reaction was stopped collecting the immobilized enzyme by filtration.

After selecting the best parameters of reaction with immobilized tannase, the soymilk biotransformed with commercial β-glycosidase in immobilized form was tested in the same conditions in order to compare with the established process.

For free tannase, according to De Queirós et al. (2016), the soymilk was incubated with 20 mg of tannase powder (β-glycosidase activity of 4.2 U/mg), also with the best conditions fixed above (pH, temperature, and reaction time), and the hydrolysis process was stopped by placing the reaction in an ice bath for 15 min.

As control, soymilk (lyophilized powder) at the concentration of

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