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# Effect of the fermentation of whole soybean flour on the conversion of isoflavones from glycosides to aglycones

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ABSTRACT

The effect of fermentation of whole soybean flour (WSF) on the conversion of isoflavone glycosides to the aglycone form was analysed by liquid chromatography. WSF (200 g) with 35% moisture, was autoclaved at 121 °C for 20 min (AWSF), cooled and inoculated with 2 mL of spores of the fungus *Aspergillus oryzae* CCT 4359, and then incubated for 24 h and 48 h. The fermented flour was dried in a vacuum oven and 10 g of each flour, non-fermented and fermented, sieved and defatted. One gram of each flour was used for extraction of the isoflavones with 10 mL of an 80% methanol solution. Aliquots were injected into the HPLC under the following conditions: C18 column, 30 °C, gradient elution, mobile phase of (A) water: 5% acetic acid (v/v) and (B) methanol. The results showed that the FAWSF-48 h contained predominantly isoflavone aglycones (75.51%) when compared to the AWSF (6.94%) and WSF (2.67%).



## 1. Introduction

Soybean is highlighted as an important source of vegetable protein, whose main products are defatted flour, whole soybean flour and soy protein isolate and concentrate, all used primarily in processed foods and as animal protein replacers (Liu, 2000).

Whole soybean flour is obtained from the milling of the grain. It usually has a protein content between 35% and 40% and lipid content from 15 to 20%. This product is extremely nutritious, showing high levels of fibre, vitamins, minerals and phytochemicals. It is classified as a raw flour, enzymatically active and used for bread whitening or as a toasted flour for general use (Riaz, 2006).

In addition to its nutritional potential, there is a growing interest in the benefits of isoflavones in various aspects of human health, particularly in western and chronic diseases such as various types of hormone-dependent cancers, cardiovascular disease, osteoporosis and relief from symptoms related to the menopause (Delmonte, Perry, & Rader, 2006).

Soy isoflavones are found predominantly in the glycoside form and in low concentrations as aglycones (Fig. 1) (Izumi et al., 2000). The content and composition of isoflavones in soy-based products depend on the techniques used in processing, such as heat treatment, cooking, enzymatic hydrolysis and fermentation (Wang & Murphy, 1994).

Isoflavone conjugated glycoside are converted to aglycones under acidic or alkaline conditions or by the action of β-glycosidase (Wei, Wang, & Fang, 2004). The aglycone forms show greater potential for absorption in the intestine than the glycoside forms (Izumi et al., 2000).

Studies have shown that the fermentation process of soybean promotes changes in the phytochemical compounds, causing changes in the isoflavone forms, hydrolysing the proteins and reducing the antinutritional factors, by reducing trypsin inhibitor content (Kim, Peterson, & Barnes, 1998; Molteni, Brizio-Molteni, & Persky, 1995; Zhu, Hettiarachchy, Horax, & Chen, 2005). The development of products with fermented soy may increase the functional components, such as increased isoflavone aglycones and active peptides that have greater potential health benefits (Hong, Lee, & Kim, 2004; Mejia & Lumen, 2006).

Fermented soy is largely consumed in Asia, in countries such as Japan and China, and currently an increasing consumption of this legume in Western countries has been noticed, due to the presence of functional compounds such as isoflavone aglycones, which have health benefits (Mejia & Lumen, 2006).

This work aims to quantify by liquid chromatography the effect of the fermentation process of whole soybean flour (WSF) on the conversion of isoflavone glycoside into the aglycone form.

## 2. Materials and methods

### 2.1. Materials

The whole soybean flour (WSF) was obtained by grinding BRS 232 cultivar soybeans, acquired from Brejeiro S/A (Orlando, SP, Brazil), in a knifeblade mill (Marconi, Piracicaba, SP, Brazil). The

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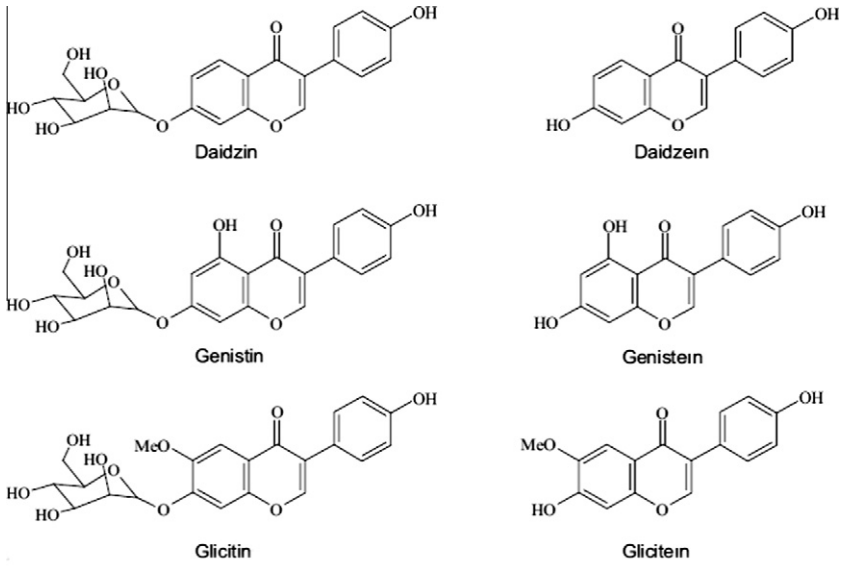


**Fig. 1.** Chemical structures of the isoflavones considered in this study.

strains of the filamentous fungus, *Aspergillus oryzae* (Ahlburg) Cohn, anamorph CCT 4359, were acquired from the Collection of Tropical Crops - Foundation André Tosello (São Paulo, SP, Brazil).

The solvents used for the extraction and chromatographic analyses were HPLC grade solvents (Mallinckrodt, Lexington, KY, USA). Isoflavone standards of 99% purity (daidzin, glycitin, genistin, daidzein, glycitein, genistein) were obtained from the company LC Laboratories (Woburn, MA, USA).

## 2.2. Methods

### 2.2.1. Preparation of the inoculum

A pure culture of the filamentous fungus, *A. oryzae* (Ahlburg) Cohn, anamorph CCT 4359 was initially reactivated with successive inoculations for test tubes with PDA (Potato Dextrose Agar), incubated at 30 °C for 4 days, transferred to 250 mL conical flasks containing 100 mL PDA (Potato Dextrose Agar) medium and incubated at 30 °C for 6 more days for sporulation of the microorganism on the surface of the culture medium. After this period, 50 mL of a Tween 40 solution (15 $mgL^{-1}$) (Atlas Chemical Industries, Inc.) were added to each flask plus a set of glass beads, stirred for 1 min and then transferring to an empty, sterilised flask, producing, after homogenisation, a suspension of spores containing $1.85 \times 10^7$ spores $mL^{-1}$, which was stored at 4 °C for subsequent inoculation into the autoclaved whole soybean flour (AWSF) (Pamboukian, 1997).

### 2.2.2. Fermentation process

For the semi-solid fermentation process, 200 g of BRS 232 cultivar WSF, with a moisture content of 35%, were sterilised at 121 °C for 20 min, cooled, inoculated with 2 mL of a spore suspension ($1.85 \times 10^7$ spores $mL^{-1}$) of the fungus *A. oryzae* CCT 4359, and incubated in a BOD oven at 30 °C for 24 and 48 h. The fermented product was then dried in a vacuum oven at 60 °C to a final moisture content of 10% and finally crushed in a small cyclone mill (Marconi, Piracicaba, SP, Brazil) to obtain the fermented autoclaved whole soybean flour (FAWSF) (Aguiar & Park, 2004; Hong et al. 2004).

### 2.2.3. Sample preparation and extraction of the isoflavones

The isoflavones were extracted using the methodology described by several authors (Coward, Barnes, Stcbell, & Barnes, 1993; Fukutake et al., 1996; Lui, 2004), with adjustments for the use of whole soybean flour. Ten grams samples of the fermented autoclaved whole soybean flour (FAWSF), autoclaved whole soybean flour (AWSF) and control – whole soybean flour (WSF) were sieved through a 35 mesh sieve, defatted with petroleum ether in a Soxhlet type extractor for 4 h, and 1 g of each flour submitted to extraction with 10 mL of an 80% methanol solution for 2 h at 25 °C, centrifuged at 5000*g* for 10 min and the supernatant filtered through a PTFE micro filter with 0.22 μm porosity (Millipore, São Paulo, Brazil). The filtrates were stored at 4 °C for a maximum of 6 days and analysed by HPLC (High Performance Liquid Chromatography) with a photodiode detector.

*2.2.3.1. HPLC analysis of the isoflavones.* The HPLC method followed the procedure developed by Aguiar and Park (2004). The analysis was carried out using a modular Shimadzu LC-10 system (Columbia, MD) comprised of a LC-10AT VP pump, a CTO-10AS VP column oven, a SPD-M20A VP diode array detector (DAD), a SCL-10A interface and a Class VP Workstation. A Gemini C18 column (250 × 4.6 mm × 5 μm) (Phenomenex, Torrance, CA, USA) was used with an oven temperature of 30 °C. The DAD was operated between 200 and 800 nm. Chromatograms for quantitative analysis were extracted at 254 nm. The samples were eluted at 0.5 mL $min^{-1}$ with a linear gradient of 5.0% (v/v) acetic acid in water (A) and methanol (B). The gradient was started immediately after injection followed by a four step linear gradient. The elution gradient was as follows: from 20% B to 40% B over 15 min, then to 50% B over 40 min, then to 70% B over 27 min and finally to 20% B over 5 min. At the end of the run, the column was re-equilibrated with 20% B for 13 min before the next run. The injection volume was fixed at 20 μL for all the analyses.

*2.2.3.2. Quantitative analysis of the isoflavones.* The isoflavones were quantified by analysing a methanolic extract of the soybeans by high performance liquid chromatography using a diode array detector (HPLC-DAD), following the procedure described by Aguiar and Park (2004).

The isoflavones were quantified by an external standard method. Standard solutions of known concentrations of daidzin and genistin (200, 80, 40, 20 and 10 μg $mL^{-1}$), glycitin (200, 100, 40, 20 and 10 μg $mL^{-1}$) and daidzein, glycitein and genistein (100, 50, 20, 10 and 5 μg $mL^{-1}$), were used. The standards were injected