A New Biotechnological Process to Enhance the Soymilk Bioactivity

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Abstract Equol, a daidzein metabolite produced exclusively by intestinal bacteria in some, but not all, humans, exhibits a wide range of beneficial health effects owing to its superior nutraceutical effect compared with isoflavones of soy. The aim of this work was to develop bioprocesses capable of increasing the bioactive properties of soymilk and, most importantly, increase the equol content by a biotechnological process *in vitro*. Biotransformation processes based on soymilk fermentation by probiotic lactic bacteria and application of the enzyme tannase caused an increase in the bioactive isoflavones and antioxidant capacity of soymilk. Furthermore, these processes approximately resulted in a 10-fold increase in the equol content of the soymilk. This is the first study to produce a significant equol concentration in soymilk using enzymatic processing only. The results suggest a new and effective biotechnological process, with major commercial potential, capable of producing a bioactive soy extract that intends to be “functional for everyone.”

Keywords: isoflavones, equol, tannase, biotransformation, antioxidant capacity

Introduction

Soybeans have attracted considerable worldwide attention for their health-promoting and disease-preventing effects. These effects are mainly attributed to isoflavones, a group of flavonoids present in relevant amounts in soybean and soybean-based products (1). Isoflavones are a class of natural heterocyclic phenols that act as phytoestrogen compounds because they are structurally and functionally similar to 17 β -estradiol. Soy isoflavones have been reported to bind to estrogen receptors ER α and ER β , playing an important role in estrogenic activity in various tissues (2).

The composition and distribution of different chemical forms of isoflavones in soy-based products can be affected during processing methods (3,4). In native soybeans, isoflavones exist mainly in glycoside forms. However, some food processing such as fermentation converts the glycosides into their respective aglycone forms (5,6). These changes to isoflavone composition may affect the bioavailability of soy isoflavones in soy products because the aglycones are known to be more bioactive than their respective glycosides (7,8).

Epidemiological and clinical studies have shown several possible mechanisms for the health-promoting effects of isoflavones that include the alleviation of postmenopausal syndromes and prevention of osteoporosis, cancer, and cardiovascular disease (9-11). However, the studies indicated that these effects seem to depend on the

individual's ability to convert daidzein into equol. This conversion ability can vary, mainly due to the diversity of individual microbiota (12,13).

Equol (C₁₅H₁₄O₃) is a nonsteroidal estrogen of the isoflavonone class formed in the biotransformation of the soy isoflavones, namely, daidzin and daidzein (12). The literature describes that equol is exclusively produced by the action of the intestinal bacteria (11).

Equol can be produced in majority of the animals, including rodents. However, only 30-50% of humans who consume soy foods produce equol (14). Studies suggest that equol producers respond more favorably to diets that contain soy isoflavones, indicating that the metabolite equol has greater biological potency than all other isoflavones (12). For this reason, there is considerable interest in the development of bioprocesses capable of producing equol as a pharmaceutical or nutraceutical agent, including as a food additive that promotes health benefits.

Some studies have shown the potential benefits of biotransforming soy isoflavones into equol. Essentially, these works showed that enterobacteria fermentation processes can lead to conversions of soy isoflavones, leading to the higher bioavailability of these bioactive molecules and formation of equol (5,15). However, because of the nature of the microorganisms and enzyme sources applied, it is difficult to scale up the process.

Tannase (E.C. 3.1.1.20) is an extracellular enzyme produced in the

presence of tannic acid by bacteria, fungi, and yeast (16). Tannases have been largely characterized by their activity on complex polyphenols and their ability to hydrolyze the ester bond (galloyl ester of an alcohol moiety) and the depside bond (galloyl ester of gallic acid) of hydrolysable and condensed tannins, like tannic acid, epicatechin gallate, epigallocatechin gallate, and chlorogenic acid (17). However, recent tannase application studies conducted by our research group have indicated the additional potential of this enzyme to catalyze complex phenolic substrates including β -glucosidase activity, justifying the application of this enzyme to the proposed processes and thus potentializing the *Lactobacilli* action (18-20).

Based on this information, microbial and/or enzymatic processes were performed in this study to biotransform the soymilk isoflavones and increase aglycones and equol concentration to create a soy product with a higher biological potential. This product could be used as a functional and nutraceutical product for any person for whom phytoestrogens would be beneficial with higher chances of achieving the expected effects despite one's microbiota particularities.

Materials and Methods

Materials Soybean (*Glycine max*) was purchased at a local supermarket (Nattus, Campinas-SP, Brazil). Daidzin, genistin, daidzein, genistein, equol, 2,2-Azobis(2-amidinopropane) dihydrochloride (97%) (APPH), 2-diphenyl-1-picrylhydrazyl (DPPH), gallic acid, and tannic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). Trolox® (97%) was purchased from Acros Organics (Thermo Fisher Scientific, Waltham, MA, USA), and fluorescein was purchased from ECIBRA (São Paulo-SP, Brazil). The commercial cultures of lactic acid bacteria and bifidobacteria were donated by Chr. Hansen Ind. & Com. Ltd. (Valinhos-SP, Brazil). HPLC grade methanol was provided by J.T. Baker® (Mexico City, Mexico), and deionized water was used for all HPLC analyses. All other chemicals purchased were of commercial grade purity. The semi-purified preparation of tannase took place at the Bioprocess Laboratory (FEA-UNICAMP, Campinas-SP, Brazil).

Soymilk preparation Soymilk was prepared following the adapted processing technology as described by Mandarino and Carrão-Panizzi (21). Whole soybeans were soaked for 6 h at room temperature in distilled water. After decanting the soaking water, the beans were boiled in water for 5 min and then blended with distilled water (1:4 w/v) for 3 min. The resultant slurry was centrifuged at 9,630×g for 15 min at room temperature and the supernatant, known as soybean milk, was obtained and heated at boiling point for 10 min. The soymilk was dispensed into flasks and sterilized by heating at 121°C for 15 min before refrigeration.

Tannase activity assay Tannase (EC: 3.1.1.20), obtained from *Paecilomyces variotii*, as described by Battestin and Macedo (22), was used in this study. The tannase activity was assayed according to

Sharma *et al.* (23) with modifications. The buffer used was 75 mM of potassium phosphate, pH 7.4, at an incubation temperature of 40°C. The enzyme activity was measured using a calibration curve of gallic acid in the phosphate buffer (75 mM, pH 7.4). The amount of gallic acid released per minute of reaction was defined as one unit of tannase activity.

Enzymatic biotransformation of isoflavonoids For the enzymatic hydrolysis using the tannase enzyme, the commercial standard controls of isoflavonoid solutions (daidzin, daidzein, genistin, genistein, and equol) were used as substrates. The standards of isoflavonoids (1 mg) were dissolved in 1 mL of phosphate buffer (75 mM pH 7.4,) and reacted with 0.18 U of tannase, for 30 min at 40°C. The biotransformation process was stalled by putting the reaction in an ice bath for 15 min.

Soymilk biotransformation processes

Process I-microbial biotransformation: The starter microorganisms used for the fermentation of soymilk were as follows: *Streptococcus* ssp. *Thermophilus* (YF-L811) and *Lactobacillus delbrueckii* ssp. *bulgaricus* (LB-340) and the probiotic lactic acid bacteria were *Bifidobacterium animalis* ssp. *lactis* (Bb-12) and *Lactobacillus acidophilus* (LA-05). All lyophilized cultures were recultivated individually in the sterilized soymilk at 37°C for 12 h in anaerobic conditions, using an anaerobic jar. The starter organisms were inoculated at a final concentration of 1% (v/v) and probiotic lactic acid bacteria of 5% (v/v), providing a count of between 10⁶-10⁷ CFU/mL (24).

The sterilized soymilk was aseptically simultaneously inoculated with all activated cultures. Inoculated soymilk was incubated at 37°C for 24 h in anaerobic conditions, using an anaerobic jar. The fermentation process was performed in triplicate.

Process II-microbial biotransformation associated with enzymatic biotransformation: A 1 mL aliquot of the fermented soymilk obtained in Process I was incubated with 1.8 U of tannase for 30 min at 40°C. The biotransformation process was stalled by putting the reaction in an ice bath for 15 min. The reactions were performed in triplicate.

Process III-enzymatic biotransformation: An 1 mL aliquot of sterilized soymilk was incubated with 1.8 U of tannase for 30 min at 40°C. The biotransformation process was stalled by putting the reaction in an ice bath for 15 min.

The biotransformation products from the isoflavone standard solutions were evaluated by HPLC-DAD, in triplicate. The products of the biotransformation process of soymilk were analyzed considering the total phenolic content and quantitative isoflavone profile by HPLC-DAD. They were also assayed to obtain the antioxidant capacity after dilution with 70% methanol solution for DPPH and phosphate buffer (pH 7.4, 75 mM) for ORAC assay.

Biotransformation characterization of the samples

Determination of total phenolic content: To evaluate the content of

total phenolics, a modified version of the Folin-Ciocalteu assay by Chandler and Dodds (25) was used. To evaluate the total phenolic content, the samples (1 mL) were diluted at a ratio of 1:5 using 70% methanol. Next, 5 mL of distilled water was added to each 1 mL of diluted extract sample or a blank (1 mL 70% methanol), followed by 0.5 mL of Folin-Ciocalteu reagent (1 M). The samples were vortexed and incubated for 5 min at room temperature. After this, 1 mL of 5% (w/v) Na_2CO_3 solution was added to each sample. The samples were vortexed again and then allowed to stand in the dark for 1 h at room temperature. After this, the samples were vortexed, and the absorbance was measured using a UV-visible spectrophotometer (DU® 640; Beckman Coulter, Brea, CA, USA) at 725 nm in triplicate. The concentrations were determined by comparing the calibration curve constructed using catechin with the absorbance of the samples. The results were expressed as μg of catechin mL soymilk.

Chromatographic profile of the isoflavones: The most interesting products of the biotransformation processes tested (the aglycones daidzein and genistein and equol) as well as their most probable substrates (daidzin and genistin) were identified and quantified on all samples produced by HPLC-DAD.

The extraction of isoflavonoids from the soymilk samples was performed according to Aguiar *et al.* (26), with modifications. Aliquots of 1 mL from the biotransformed soymilk samples were mixed with 5 mL of 70% (v/v) methanol at 25°C for 60 min at 100 rpm. The mixture was then centrifuged for 10 min at 5,790 $\times g$, and the supernatants obtained were filtered through a membrane (0.45 μm) before undergoing reverse-phase HPLC analyses.

The chromatographic conditions used were previously described by Park *et al.* (27). A Dionex UltiMate 3000 (Sunnyvale, CA, USA) chromatography system equipped with a 4.6 $\times$ 150 mm, 5 μm , Waters C-18 Atlantis® column was used for separation at 30°C, which was maintained with a thermostat. The detection was performed using a UV/VIS (DAD-3000), and the mobile phase consisted of A: water/acetic acid, 95:5 v/v and B: methanol, set at a flow rate of 0.5 mL/min. The gradient elution was as follows: 20% B (0-15 min), 20-80% B (15-75 min), 80-100% B (75-80 min), 100-20% B (80-90 min), and 20% B (90-95 min). The injection volume was 20 μL , and all samples were analyzed in triplicate. The spectra were obtained at 190 and 480 nm and the chromatograms were processed at 254 nm. Individual isoflavonoids were identified by comparison with the retention time and absorbance spectrum of standard compounds: daidzin, genistin, daidzein, genistein, and equol. The isoflavonoids concentration of the samples was calculated by interpolation of the calibration curves prepared by various concentrations of the isoflavonoid standards. The isoflavonoid contents were expressed as g of each phenolic compound per mL of the sample (g/mL).

Antioxidant activity of biotransformed soymilk polyphenols

ORAC assay: ORAC assays were performed on a Fluostar Optima Microplate reader (FLUOstar OPTIMA; BMG Labtech, Ortenberg, Germany), which was equipped with fluorescence filters for an

excitation wavelength of 485 nm and an emission wavelength of 520 nm. The methodology was based on the previous paper by Macedo *et al.* (19), with slight modifications, using fluorescein (FL) as the fluorescent probe. The measurements were made in a Costar 96 plate at 37°C. The measurements were performed in triplicate. The differences between the area under the blank and the FL decay curve (net AUC) were defined as ORAC values. For all samples, the regression equations between net AUC and antioxidant concentration were calculated. ORAC-FL values were expressed as mol of Trolox equivalents per liter of soymilk (mol of TE/L) using the calibration curve of Trolox.

DPPH assay: For all soymilk samples, the antioxidant activity was also assessed by DPPH assay. The methodology was based on the previous paper by Macedo *et al.* (19). A linear regression equation determined by plotting the anti-radical activity of Trolox solutions of known concentrations was used to calculate the antioxidant activity. The measurements were performed in triplicate and DPPH values were expressed as mol of Trolox equivalents per liter of soymilk (mol of TE/L), using the calibration curve of Trolox.

Calculations and statistics The data were expressed as mean and standard deviation. The Tukey test was used to analyze the statistically significant differences between the groups' means and $p < 0.05$ was considered statistically significant.

Results and Discussion

Bioprocess effect on content of total phenolics and antioxidant activity of soymilk samples The effects of fermentation and/or enzymatic biotransformation on the total phenolic content (TPC) and the antioxidant activities of soymilk, by ORAC and DPPH methods, are presented in Table 1. For all bioprocesses tested, significant differences ($p < 0.05$) were found for TPC, ORAC, and DPPH values.

The results showed that the total detectable phenolic content for all the biotransformed samples increased significantly ($p < 0.05$). After the fermentation process with lactic acid bacteria (Process I), the content of total phenolics of soymilk increased significantly from 101 to 254 $\mu\text{g/mL}$, an increase of nearly 2.5 times compared with the standard (soymilk). In Process II, this fact was also observed in case of the treatment of the fermented soymilk with tannase, in which the total phenolic content increased by about 3.8 times (101 to 387 $\mu\text{g/mL}$) compared with the standard. Process III showed the highest increase in total phenolic content (101 to 847 $\mu\text{g/mL}$) by about 8.4 times after the reaction of soymilk with tannase. These results suggest that the fermentation process and enzymatic biotransformation with tannase improve the release of phenolic compounds of soymilk matrix. Interestingly, the results seem to indicate that tannase has greater catalytic potential over the phenolics of the soymilk as substrates than over the substrates present in the fermented soymilk. In this case, tannase activity appeared to be more extensive than the biotransformation performed by the microbial process.

Table 1. Total phenolic content and antioxidant activities from ORAC and DPPH assays of soymilk before and after the biotransformations process

Process	Samples	TPC µg of catechin/mL	ORAC Trolox Eq µmol/L	DPPH Trolox Eq µmol/L
Standard	Soymilk	101 ^a ±6 ¹⁾	11,584 ^a ±1,022	821 ^a ±14
I	Fermented Soymilk	254 ^b ±11	25,987 ^b ±977	1,729 ^b ±27
II	Fermented Soymilk+Tannase	387 ^c ±9	31,801 ^c ±858	4,439 ^c ±39
III	Soymilk+Tannase	847 ^d ±7	45,758 ^d ±937	6,724 ^d ±36

¹⁾The results are expressed as mean±standard deviation ($n=3$). Means in the same column with different letters are significantly different ($p<0.05$).

There are two hypotheses for these observations. First, some tannase inhibitors may be released by reducing enzymatic activity in the fermentation process. The second and most probable one is that the bacterium is competing with the enzyme for substrates. Both ideas require further investigation.

In a study that produced a fermented soymilk using *Ker* cultures, McCue and Shetti (28) showed that the content of total phenolic, after 40 h of fermentation, increased from 232±32 µg to 418±13 catechin equivalents (CAE/mL) of yogurt sample. The authors hypothesized that the increase of phenolic content can be due the degradation of polymeric phenolic structures by β-glucosidase enzymes of the *Ker* cultures.

Nam *et al.* (29) showed that the fermentation process of *meju*, a fermented soybean derivative with *Aspergillus oryzae* NL 5, resulted in an increase in total phenolics, most likely due to the formation of free isoflavones. Tyug *et al.* (30) previously reported a wide range of phenolic constituents in soy by-products, which also demonstrated that the soybean variety and the processing conditions can affect the total phenolics content of soy-based products.

The antioxidant capacity of the samples, before and after the three biotransformation processes of soymilk, was assayed using two different *in vitro* methods: DPPH and ORAC. The results are presented in Table 1 and are expressed as Trolox[®] equivalent concentrations. In general, the results demonstrated that the antioxidant capacity of the biotransformed samples increased significantly in both methodologies ($p<0.05$), correlating with the TPC results.

Soybeans and soy-based products are nutritionally rich foods that contain various phytochemicals (isoflavones, phytosterols, saponins, phenolic acids, phytic acid, and trypsin inhibitors), that show functional, antioxidant, and radical scavenging activities (31). The phenolic compounds of soybeans have free radical-scavenging properties (32), which may explain the corresponding increase of total phenolic content with antioxidant activity, as shown in Table 1. These results indicate that the fermentation process of soymilk (Process I) resulted in a significant increase of the antioxidant capacity by around 2.2 and 2.1 times by the ORAC and DPPH assays, respectively, as shown in the Table 1. The fermentation followed by enzyme biotransformation (Process II) resulted in a significant increase about 2.7 times of the antioxidant capacity by the ORAC assay and an increase of approximately 5.4 times by the DPPH method. Similarly, in the biotransformation of soymilk, when

catalyzed only by tannase (Process III), the antioxidant capacity by the ORAC and DPPH assays increased significantly around four and eight times, respectively.

The improvement in the soluble phenolic content of a complex sample after biotransformation processes, followed by higher antioxidant capacity results, has been observed in previous studies. The application of tannase from *P. variotii* in polyphenol-rich food matrices has been reported in others studies, resulting in the enhancement of the functional activity of the products compared with the original samples (18,19).

The application of tannase from *P. variotii* to improve the functional activity of polyphenol-rich food matrices has also been reported in others studies, resulting in the improvement of the functional activity of the food matrix compared to the original samples.

According to Macedo *et al.* (19), the antioxidant capacity of yerba mate and green tea after enzyme treatment with tannase from *Paecilomyces variotii*, increased by 55 and 43%, respectively, using the ORAC method, while the results using the DPPH method showed an increase of 23 and 17%, respectively.

Similar results were also found in a study developed by Ferreira *et al.* (18) in which the antioxidant capacity of orange juice increased significantly after the biotransformation process by tannase from *P. variotii*; 50% increase in the ORAC method and 70% increase in the DPPH method. In this study the tannase from *P. variotii* was shown to be able to remove the glycosides of naringin and hesperidin and this enzymatic biotransformation led to an enhancement of the bioactivity of orange juice. Similarly, in the present study the enzymatic treatment with the tannase was able to hydrolyze the glycoside forms of isoflavones in soymilk, and the aglycone forms contributed to the increase of the antioxidant capacity of soymilk, since the aglycone isoflavones are known to be more bioactive than their respective glycosides (7).

Cho *et al.* (6) presented results that are consistent with our observations. These authors evaluated the changes in the total phenolics and antioxidant capacity (by DPPH assay) during the fermentation of *cheonggukjang*, a fermented soybean paste used in Korea, for 60 h at 37°C. By the end of the fermentation process, the total phenolics had increased markedly from 253 to 9586 g/kg, and the level of DPPH had increased from 54.5 to 96.2%.

The consensus among the authors is that hydrolysis of complex phenolics, generating aglycones, and smaller phenolics from

Table 2. Concentration of isoflavone isomers and equol ($\mu\text{g/mL}$) in soymilk before (standard) and after the biotransformation process

Process	Samples	Isoflavone contents (μg/mL)				
		Glycoside		Aglycone		Equol
		Daidzin	Genistin	Daidzein	Genistein	
Standard	Soymilk	313.0 ^a ±2.0 ¹⁾	393.8 ^a ±3.2	25.6 ^a ±0.6	10.2 ^a ±0.5	0.08 ^a ±0.00
I	Fermented Soymilk	194.2 ^b ±5.7	202.5 ^b ±5.9	91.4 ^b ±1.9	52.3 ^b ±2.0	0.31 ^b ±0.00
II	Fermented Soymilk+Tannase	ND ²⁾	ND	717.2 ^c ±11.0	251.4 ^c ±6.9	0.63 ^c ±0.01
III	Soymilk+Tannase	ND	ND	944.5 ^d ±0.9	321.6 ^d ±0.8	0.82 ^d ±0.01

¹⁾The results are expressed as mean±standard deviation ($n=3$). Means in the same column with different letters are significantly different ($p<0.05$).

²⁾ND, not detected, under detection limit for this methodology and equipment

polymers, results in higher antioxidant capacity in the samples. In light of this acquired knowledge, Process III, or the enzymatic biotransformation of the soymilk, demonstrated the potential for the production of bioactive soy extract.

Bioactive isoflavones profile analysis by HPLC-DAD According to the methodology of Aguiar *et al.* (26), using HPLC-DAD (Table 2), major bioactive isoflavones in soymilk were identified and quantified before and after the three biotransformation processes. The targets of the analysis were the glycosides daidzin and genistin, and the aglycone forms of daidzein and genistein. Specific attention was also given to equol quantification.

The results demonstrated that the bioprocesses that were developed liberated significantly different amounts of genistein and daidzein from their respective glycosides. As shown in Table 2, the glycoside isoflavones were the predominant forms in the soymilk and fermented soymilk samples. However, the concentrations of glycosides daidzin and genistin in fermented soymilk (Process I) decreased by about 1.6 and 2 times, respectively, and the aglycones daidzein and genistein increased by about 3.5 and 5 times, respectively, when compared to the standard. This result therefore suggests that because of the fermentation process, the glycoside isoflavones were transformed into aglycones forms and that other substrates were also converted into daidzein and genistein. This could be partly due to the β -glucosidase activity inherent in the microorganisms used to carry out the fermentation process as previously documented (33).

As observed in Fig. 1, after Processes II and III, the glycoside forms (daidzin and genistin) were not detected, indicating that these forms were converted into their respective aglycone forms, as shown by the significant increase in the levels of daidzein and genistein after these bioprocesses. The concentrations of aglycone forms of fermented soymilk after Process II significantly increased by about 28 times (from 25.6 to 717.2 $\mu\text{g/mL}$) for daidzein, and 25 times (from 10.2 to 251.4 $\mu\text{g/mL}$) for genistein, compared to soymilk. In Process III, the aglycone forms increased markedly compared to soymilk by about 37 times (from 25.6 to 944.5 $\mu\text{g/mL}$) for daidzein, and 32 times (from 10.2 to 321.6 $\mu\text{g/mL}$) for genistein. These results indicate that the enzymatic reaction with tannase (Process III) showed the highest increase on the aglycone forms of isoflavones, correlating with the

total phenolic content and the ORAC and DPPH assays. The fact that the processes that involved a tannase catalytic action (II and III) showed a higher final concentration of the aglycone forms than the initial soymilk concentration of the glycosylated isoflavons (daidzin and genistin), is evidence that the tannase does not only act with β -glucosidase activity. The enzymatic processes would most likely convert other daidzein and genistein precursors present in the soymilk.

Ferreira *et al.* (18) demonstrated that the polyphenolic composition of orange juice was changed by the action of the tannase enzyme from *P. variotii*, and that the antioxidant capacity of the biotransformed samples increased significantly by both the ORAC and DPPH methods.

The ability of the tannase enzyme to biotransform the commercial standards of daidzin and genistin (glycoside isoflavones), was proven in the tests presented in Fig. 2. After 30 min at 40°C, the chromatograms obtained the reaction of standard daidzin (A) and genistin (B) with the tannase enzyme; the retention time and the absorption spectrum of the reaction products are shown in Fig. 2.

As can be seen in Fig. 2, the interaction between the tannase and the glycoside isoflavones (daidzin and genistin), leads to the formation of the respective aglycones daidzein (A) and genistein (B). Further studies are needed to identify which other tannase substrates from soymilk are necessary to produce daidzein and genistein. Similar observations concerning the production of equol by the enzymatic processes were possible.

Observing the results in Table 2, the concentration of equol after the fermentation of soymilk with lactic acid bacteria (Process I) increased 3.9 fold. In Process II, the enzymatic treatment of fermented soymilk resulted in a 7.9 fold increase in equol content when compared to native soymilk in Process III. After enzymatic treatment of soymilk (Process III), equol concentration increased by a factor of about 10.

Observing Fig. 1, the results indicate that tannase treatment showed higher hydrolytic catalysis of isoflavonoid substrates in native soymilk substrates compared to fermented soymilk. It also resulted in a greater increase in the content of equol in fresh soymilk compared to fermented soymilk samples. Once again, the fermentation process seemed to compete with the enzyme for the equol precursors (besides daidzin) present in the soymilk; substrates that tannase

Fig. 1. HPLC-DAD chromatograms of the (A) raw soymilk, (B) fermented soymilk, (C) fermented soymilk reacted with 1.8 U of tannase, and (D) raw soymilk reacted with 1.8 U of tannase for 30 min at 40°C. HPLC-DAD chromatogram of the (E) time of retention, and (F) the absorption spectrum of the equol.

could convert to equol, but that the fermentation could not.

The results in Table 2 show that the content of aglycone forms (daidzein and genistein) increased significantly after both the fermentation and tannase process. Furthermore, these bioprocesses lead to the production of equol. The next experimental effort must be to try to identify which tannase substrate found in soymilk can be converted into equol. The assay with daidzin as the commercial standard and the tannase extract demonstrated clearly that equol does not come from and cannot be produced from this interaction (Fig. 2).

The literature affirms that the gut microflora transform the isoflavones into equol (11,12). A number of bacteria in intestinal flora have also been reported to be responsible for this conversion (14). However, the formation of equol during soymilk fermentation without the presence of bacteria from intestinal microflora, has also been considered in some studies that correlate the equol formation with the use of enzymes (15,34,35).

The equol metabolite was found in the study developed by Tsangalis *et al.* (35) that evaluated the process of transforming isoflavones to equol, in soymilk, by the β -glucosidase activity from five strains of bifidobacterias. The highest concentration of equol (0.521 mg/100 mL) was synthesized after 24 h of incubation with *Bifidobacterium animalis*. This strain showed the highest β -glucosidase activity among the strains studied. Di Cagno *et al.* (15) found similar results in a study in which soymilk with food-related lactic acid bacteria contained 0.9 mg/100 mL of equol after 96 h of fermentation. The authors proposed correlations between the growth characteristics of the bifidobacteria in soymilk, their β -glucosidase activity, and the conversion of isoflavone glycosides into aglycone forms.

Although there is a need for more analytic studies for the characterization of the changes observed in the polyphenolic soymilk composition after the biotransformation by the tannase enzyme, it can be observed that the action of the tannase extract was not limited to the deglycosylation of isoflavones (Table 2). The tannase

Fig. 2. HPLC-DAD chromatograms of the tannase reaction at 30 min at 40°C of (A) daidzin and (B) genistin.

was also able to increase the content of equol. From observing the data, it is clear that the reduction in concentration of the isoflavones daidzin and genistein at the end of Process III was smaller than the increase in concentration of the aglycone forms of the isoflavones, added to the amount of equol that appeared after the reaction process. This clearly indicates that the tannase is able to catalyze the production of these interesting bioactive compounds from other substrates, other than simply daidzin and genistein, present in the soymilk. Hydrolysis reactions of tannase over more complex polyphenols have yet to be characterized, however this phenomenon is very interesting since the production yield of isoflavones and equol with greater bioactivity is increased in the process due to the extensive hydrolase range demonstrated by this enzyme.

These results show that tannase can act in different ways in the biotransformation process, making this enzyme a very interesting option for application on soy-based foodstuffs, given its rich composition of polyphenols.

Based on the results of the bioprocesses proposed in this study, there was a significant increase in aglycones and equol concentration in the soymilk, thus a soy product with a higher biological potential was created. This is the first work that shows the possibility of

significantly increasing equol concentration in soymilk using enzymatic processing without microbial action. The results suggest a new and effective biotechnological process with great commercial potential, capable of producing a bioactive soy extract that intends to be “functional for everyone.”

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