Immobilized soybean β-glucosidase application in commercial soy drink

Luciana C. Grade[1], Amanda A. Moreira[1], Geni S. Varea[1], José Marcos G. Mandarino[2], Josemeyre B. Silva[3], Elza I. Ida[3], Mara Lúcia L. Ribeiro[1,*]

[1]Universidade Estadual de Londrina, Departamento de Bioquímica e Biotecnologia, Campus Universitário, Caixa Postal 10.011, CEP 86057-970, Londrina, Paraná, Brasil. [2]Embrapa Soja, Rodovia Carlos João Strass, Acesso Orlando Amaral, Distrito de Warta, Caixa Postal 231, CEP 86001-970, Londrina, Paraná, Brasil. [3]Universidade Estadual de Londrina, Departamento de Ciência e Tecnologia de Alimentos, Campus Universitário, Caixa Postal 10.011, CEP 86057-970, Londrina, Paraná, Brasil. *Correspondent author Email: maraluciaribeiro@uel.br

Abstract

The objective of this study was to apply β-glucosidase immobilized on commercial soy drink to evaluate the bioconversion of β-glycosidic isoflavones in aglycones. The β-glucosidase was obtained from soybean cultivar BRS 213 and immobilized in chitosan beads activated with 2.5% glutaraldehyde.Two chitosan beads containing immobilized β-glucosidase were incubated with 1 mL of commercial soy drink from 0 to 60 min, pH 5.5 and at 50°C. The isoflavones content were quantified by High Performance Liquid Chromatography (HPLC). The initial content of aglycones in commercial soy drink was 32.20 $\mu g.mL^{-1}$. After 60 min of immobilized enzyme application in the beverage it was observed an increase of 24% in the aglycones content probably due to the action of β-glucosidase which convert β-glycosides into aglycones.
Therefore, application of β-glucosidase immobilized on chitosan in commercial soy drink was advantageous because the enzyme acted effectively in the bioconversion of isoflavones increasing the content of aglycones.

Key-words: immobilized β-glucosidase, beads chitosan, aglycones, soy products

Introduction

β-glucosidases (β-D-glucopyranoside glucohydrolases, E.C. 3.2.1.21) are enzymes that hydrolyzes β-glycosidic bonds to release glucose and hydrolyzes isoflavones to release aglycones. These enzymes are found widely in nature, can be synthetized by plants or produced by animals and microorganisms (Cairns and Esen, 2010). Soy has a high content of β-glycosides (daidzin, genistin and glycitin) which can be hydrolysed by β-glucosidases and then release aglycones (daidzein, genistein and glycitein). Soy isoflavones have biological properties associated with risk reduction or prevention of various chronic diseases, such as breast and prostate cancer (Liggins et al., 2000), osteoporosis, menopause symptoms (Levis et al., 2010), cardiovascular disease (Rimbach et al., 2008), improving in memory (Leparht et al., 2002), estrogenic and antioxidant activity (Liu et al., 2010; Ma et al., 2010). Aglycones forms exhibit higher biological activity and greater absorption properties than β-glycosides (Izumi et al.,

2000). However, soy contains 2% of the aglycones in relation of the total isoflavones (Kudou et al., 1991). Thus, application of endogenous β-glucosidase immobilized can be an alternative to promote the bioconversion of the β-glycosidic isoflavones to produce soy products with higher aglycones content. The immobilization of soybean β-glucosidase and its application has not been described.

Lately, immobilized enzymes have been developed in food industry for multiple utilization of enzymes to reduce operational costs (Chen et al., 2013). The selection of a matrix is an essential step for enzyme immobilization. The matrix must guarantee fixation and stability of enzyme, mechanical strength ability to cross-linking (Coelho et al., 2008). The matrix also must be readily and available materials, inexpensive, easy to operate on large scale and show high retention capacity (Canilha et al., 2006). Chitosan is a suitable matrix for the enzymes immobilization because is biocompatible, available in various forms (gel, membrane, fiber and film), nontoxic, biodegradable, and resistant to chemical modifications (Gomathi et al., 2010; Yi et al., 2009). Therefore, chitosan has potential for application as biomaterial (Arnaud et al., 2010).

The objective of this study was to apply β-glucosidase immobilized in chitosan on commercial drink soy to evaluate the bioconversion of β-glycosidic isoflavones in aglycones.

Material and Methods

Material

Soybean cultivar BRS 213, developed by Embrapa Soybean Londrina, Brazil, was used for the extraction of β-glucosidase. The cotyledons were grinded in a knife mill (100 mesh) (TE 631, Tecnal, Brazil) to obtain a flour with fine granulometry.

Commercial soy drink was purchased at local market (Londrina, Brazil) and used for immobilized enzyme application and isoflavones determination.

Extraction and Fractionation of β-glucosidase

Conditions for β-glucosidase extraction were as described by Matsuura and Obata (1993), with some modifications. 60 g of soy cotyledon flour and 100 mM sodium phosphate buffer, pH 6.6, in a 1:10 proportion (w/v) were slowly shaken for 1 h at 4°C and then centrifuged at 4.000 *x* g at 4°C for 15 min. The supernatant was acidified with 100 mM HCl to pH 5.0, and the samples were centrifuged again under the same conditions. The supernatant obtained (crude extract) was precipitated by ammonium sulfate at 4 °C according to Santos et al. (2012). The crude extract was first precipitated by ammonium sulfate at 4°C and 40% saturation. After centrifugation at 4.000 *x* g at 4°C for 15 min, ammonium sulfate was added to the supernatant until 85 % saturation was achieved and centrifuged again under the same conditions. The precipitated was resuspended in a 50 mM citrate phosphate buffer, pH 5.0 and dialysed with the same buffer for 14 h at 4°C.

Immobilization of β-glucosidase

Chitosan beads in the concentration of 1% (w/v) (Sigma-Aldrich) were prepared according to Kumar et al. (2009). Chitosan solution was prepared in MilliQ water with 1.5% acetic acid and heated in a water bath at 60°C. KOH solution 1M at 37°C was

added to the chitosan solution to form the beads. Subsequently, the beads were activated with 2.5% glutaraldehyde at pH 7.5 and 8 hours agitation. For β-glucosidase immobilization were used 24 mg protein (182 UA)/4 beads, pH 6.5 and 20 h incubation at 4°C. After incubation, the beads were washed with 100 mM Tris-acetate buffer, pH 7.6 and stored in the same buffer at 4°C until to be used.

β-glucosidase activity

β-glucosidase activity was determined using ρ-nitrophenyl-β-D-glucopyranoside (ρ-NPG) substrate according to the procedures described by Matsuura and Obata (1993). A standard curve of ρ-nitrophenol (0.04-0.32 µmol) was prepared, and β-glucosidase activity unit (UA) was defined as the amount of enzyme needed to liberate 1 µmol of ρ-nitrophenol.min^{-1} under assay conditions. The soluble protein content was quantified according to Lowry et al. (1951) with a bovine serum albumin standard solution (40-400 µg.mL^{-1}).

Application of immobilized β-glucosidase in commercial soy drink

For β-glucosidase immobilized application two granules of the enzyme were used and incubated with 1 mL of commercial soy beverage at pH 5.5 and 50°C. The incubation time varied from 0 to 60 min.

Isoflavones content in the soy drink were quantified by High Performance Liquid Chromatography (HPLC), and after application the immobilized enzyme could be reused.

Isoflavones content determination by HPLC

The isoflavones were extracted according to Carrão-Panizzi et al. (2002) and quantified by the method of Berhow (2002) using a liquid chromatograph (HPLC) (Model 2690, Waters, USA) with a reverse phase column ODS C18 (YMC-Pack ODS-AM S-5 mm, 120 a, with a diameter of 4.6 mm and length 250 mm) and a diode array detector (model 996, Waters, USA) adjusted to a wavelength of 254 nm. A linear binary gradient system with methanol, trifluoroacetic acid and ultrapure deionized water was used for separation. The initial gradient was 20%, reaching 80% at 35 min and returned to 20% at 40 min. The mobile phase flow rate was of 1 mL.min^{-1}, and the temperature during the separation was kept constant at 25°C. Quantitation was performed with the external standard calibration curves of daidzin, genistin, glycitin, daidzein, genistein, glycitein, malonyldaidzin, malonylgenistin, malonylglycitin, acetyldaidzin, acetylgenistin and acetylglycitin purchased from Sigma Chemicals Co. (St. Louis, E.U.A.), and the results were expressed as µg isoflavones.mL^{-1} of commercial soy drink.

Results and Discussion

β-glucosidase immobilized on chitosan beads activated with 2.5 % glutaraldehyde was added to commercial soy drink and the isoflavones content were quantified (Table 1). Commercial soy drink without β-glucosidase immobilized showed a total isoflavones content of 169.40 µg .mL^{-1} of soy milk (109.30 µg of β-glucosides.mL^{-1}, 27.90 µg of malonyl-glucosides.mL^{-1} of soy milk and 32.20 µg of aglycones.mL^{-1} of soy milk). After 60 min incubation with β-glucosidase, it was observed an increase of 24 % in the

aglycones content probably due the β-glucoside activity that bioconverted the β-glucosides forms in aglycones. The β-glucosides and malonyl-glucosides contents decreased 18.8 % and 6.4% respectively. Thus, addition of immobilized β-glucosidase to commercial soy drink was efficient to increase the aglycones content.
No reports about soybean β-glucosidase immobilization on chitosan beads and its addition to soy drinks were found.

Table 1. Isoflavones content (ug.mL^{-1}) in commercial soy drink with the application of soybean β-glucosidase immobilized on chitosan beads activated with 2.5% glutaraldehyde*.

Incubation time (min)	β-Glycosides	Malonyl-glycosides	Aglycones
0	109.30a	27.90bc	32.20d
15	100.20c	27.50c	31.70d
30	102.40b	29.30ª	35.30bc
45	98.20d	29.20ª	36.10b
60	88.70g	26.10d	40.00a

*values obtained are an average of two repeats. Means followed by equal letters in the same column do not differ by Tukey's test at 5 % probability.

Conclusion

Application of soybean β-glucosidase immobilized on chitosan beads activated with 2.5% glutaraldehyde and incubated for 60 min was effective for isoflavones bioconversion in commercial soy drink increasing the aglycones content. .

Acknowledgments

To Araucaria Foundation for the financial support and CAPES for graduated scholarships.

References

Arnaud, T.M.S., Barros-Neto, B., Diniz, F.B. Chitosan effect on dental enamel de-remineralization: An in vitro evaluation. **J. Den.**, 38, 848-852, 2010.
Berhow, M.A. Moderm analytical techniques for flavonoid determination. In: BUSLIG, B.S.; MANTHEY, J.A. (ed.). Flavonoids in the living cell. New York: Klusher Academic, 2002, 61-76.
Cairns, J.K., Esen, A. β-glucosidases. **Cell. Mol. Life Sci**., 67, 3389-3405, 2010.
Canilha, L., Carvalho, W., Almeida-Silva, J.B. Biocatalizadores imobilizados. Uso de células e enzimas imobilizadas em processos biotecnológicos. **Biotec. Ciên. & Desenvol**., 36, 48-57, 2006.
Carrão-Panizzi, M.C., Favoni, S.P.G, Kikuchi, A. Extraction time for soybean isoflavone determination. **Braz. Arch. Biol. Techn**., 45, 4, 515-518, 2002.

Chen, K.I., Lo, Y.C., Su, N.W., Chou, C.C., Cheng, K.C. Enrichment of two isoflavones aglycones in black soymilk by immobilised β-glucosidase on solid carriers. **J. Agr. Food Chem.**, 60, 51, 12540-12546, 2013.
Coelho, M.A.Z., Salgado, A.M., Ribeiro, B.D. Tecnologia enzimática. Rio de Janeiro: EPUB, 2008.
Gomathi, P., Ragupathy, D., Choi, J. H., Yeum, J. H., Lee, S. C., Kim, J. C., Lee, S. H., Ghim, H. D. Fabrication of novel chitosan nanofiber/gold nanoparticles composite towards improved performance for a cholesterol sensor. **Sens. Actuators** B, doi: 10.1016/j.snb.2010.10.005, 2010.
Izumi, T., Piskula, M.K., Osawa, S., Obata, A., Tobe, K., Saito, M., Kataoka, S., Kubota, Y., Kikuchi, M. Soy isoflavone aglycones are absorbed faster and in higher amounts than their glucosides in humans. **J.Nutrition**, 130, 1695-1699, 2000.
Kumar, S., Dwevedi, A., Kayastha, A. M. Immobilization of soybean (Glycine max) urease on alginate and chitosan beads showing improved stability: analytical applications. **J. Mol. Catal. B: Enzymatic**, 58, 138-145, 2009.
Kudou, S., Fleury, Y., Welti, D., Magnolato, D., Uchida, T., Kitamura, K., Okudo, K. Malonil isoflavones glycosides in soybean seeds (*Glycine max.* Merril). **Agricul. Biol. Chem.,** 55, 2227-2233, 1991.
Liggins, J., Bluck, L. J. C., Runswick, S., Atkinson, C., W. A., Coward, W. A., Bingham, S. A. Daidzein and genistein contents of vegetables. **Brit. J. Nutr.**, 84, 717-725, 2000.
Levis, S., Strickman-Stein, N., Doerge, D. R., Krischer, J. Design and baseline characteristics of the Soy Phytoestrogens As Replacement Estrogen (SPARE) study — A clinical trial of the effects of soy isoflavones in menopausal women. **Contemporary Clinical Trials**, 31, 293-302, 2010.
Lephart, E.D., West, T.W., Weber, K.S., Rhees, R.W., Setchell, K.D.R., Adlercreutz, H., Lund, T.D. Neurobehavioral effects of dietary soy phytoestrogens. **Neurotoxicol.Teratol.,** 24,1, 5-16, 2002.
Liu, Z., Kanjo, Y., Mizutani, S. A review of phytoestrogens: Their occurrence and fate in the environment. **Water Research**, 44, 567-577, 2010.
Lowry, O.H., Rosenbrough, N.J., Farr, A. L., Randal, R.J. Protein measurement with the folin phenol reagent. **J. Biol.Chem.**, 193, 265-275, 1951.
Ma, W., Yuan, L., Yu, H., Ding, B., Xi, Y., Feng, J., Xiao, R. Genistein as a neuroprotective antioxidant attenuates redox imbalance induced by β-amyloid peptides 25–35 in PC12 cells. **Int. J. Develop. Neurosc**., 28, 289-295, 2010.
Matsuura, M., Obata, A. β-glucosidases from soybeans hydrolyze daidzin and genistin. **J. Food Sci**., 58, 1, 144-147, 1993.
Rimbach, G., Boesch-Saadatmandi,C., Frank, J., Fuchs, D., Wenzel, U., Daniel, H., Hall, W. L., Weinberg, P. D. Dietary isoflavones in the prevention of cardiovascular disease – A molecular perspective. **Food Chem.Toxicol.,** 46, 1308-1319, 2008.
Santos, R.F., Oliveira, C.F., Varéa, G.S., Orradi da Silva, M.L.C., Ida, E.I., Mandarino, J.M.G., Carrão-Panizzi, M.C., Ribeiro, M.L. L. P. Purification and characterization of soy cotyledons beta-glucosidase. **J.Food Bioch**., 37, 3, 302-312, 2012.
Yi, S., Noh, J., Lee, Y. Amino acid modified chitosan beads: improved polymers supports for immobilization of lipase from Candida rugosa. **J Mol. Catalysis B: Enz.**, 57, 123-129, 2009.