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Evaluation of enzymic potential for biotransformation of isoflavone phytoestrogen in soymilk by *Bifidobacterium animalis*, *Lactobacillus acidophilus* and *Lactobacillus casei*

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Abstract

Three strains of *Lactobacillus acidophilus*, two of *Lactobacillus casei* and one of *Bifidobacterium* were screened for β-glucosidase activity using ρ-nitrophenyl-β-D-glucopyranoside as a substrate and their potential for the breakdown of isoflavone glucosides to the biologically active aglycones in soymilk. Isoflavones quantification with HPLC and β-glucosidase activity were performed after 0, 12, 24, 36, and 48 h of incubation in soymilk at 37 °C. All six micro-organisms produced β-glucosidase, which hydrolysed the predominant isoflavone β-glucosides. There was a significant increase and decrease ($P < 0.05$) in the concentration of isoflavone aglycones and glucosides, respectively, in fermented soymilk. Based on the concentration of isoflavones during peak β-glucosidase activity, the hydrolytic potential was calculated. *L. acidophilus* 4461 had the highest aglycone concentration of 76.9% after 24 h of incubation, up from 8% in unfermented soymilk (at 0 h). It also had the best isoflavone hydrolytic index of 2.01, signifying its importance in altering the biological activity of soymilk.

Keywords: *Bifidobacterium*; *Lactobacillus*; β-Glucosidase activity; Isoflavones; Aglycone

1. Introduction

For many centuries, soy has been a common part of the diet in many countries. As a result, isoflavones, naturally occurring phytoestrogens components of soy has been consumed in substantial quantities by those populations whose soy intake is high. Asian populations, with their high intake (50–70 mg/d) of soy-derived isoflavones, are known to have a low incidence of osteoporosis, menopausal symptoms and mortality from cardiovascular disease (Nagata, Takatsuka, Kurisu, & Shimizu, 1998). On the other hand, isoflavone intake is generally less than 2 mg/d in Western countries (De Kleijn, van der Schouw, & Wilson, 2001; Jones, Price, & Fenwick, 1989).

There have been many reports in the recent literature describing soybean isoflavones from epidemiological-, biochemical-, mechanistic-, pharmacological-, and toxicological perspectives. There have been, however, no reports of adverse effects from consumption of soymilk (Munro et al., 2003). Studies investigating the metabolic properties of isoflavones have indicated that they are readily absorbed, metabolised, and excreted, although individual and sex-related differences have been reported (Munro et al., 2003). The phytoestrogens found abundantly in soybeans consist of the diphenolic, isomeric family of compounds known as isoflavones. There are three structural "families" of the isoflavones found in soy foods, namely, the aglycones, glucosides, malonyl-, and acetyl-glucosides (King & Bignell, 2000).

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Generally, the processing of soybeans for the manufacture of soy containing food products increases the hydrolysis of isoflavone glucosides, resulting in higher concentrations of aglycones (Hutchins, Slavin, & Lampe, 1995; Zhou & Erdman, 1997). The biological activity and metabolic fate of dietary soy isoflavones differ depending on their chemical forms (Cassidy, 1996; Cassidy, Bingham, & Setchell, 1994; Kelly, Nelson, Waring, Joannou, & Reeder, 1993). Since the structure itself is a limiting factor for absorption from gastrointestinal tract (Hendrich, Wang, & Lin, 1999), the chemical forms of the isoflavone and their metabolites influence the extent of absorption, with aglycones more readily absorbed and more bioavailable than highly polar conjugated species (Setchell, 2000).

Following ingestion, the acetyl and malonyl derivatives of genistin and daidzin are metabolised to genistin and daidzin, which are then hydrolysed in the large intestine by bacteria, resulting in the removal of sugar moiety to produce their respective aglycone daidzein and genistein (Izumi et al., 2000; Kelly et al., 1993). Following absorption of the aglycones, these compounds and their metabolites are readily conjugated in the liver with glucuronic acid and/or sulfate, circulate enterohepatically with potential metabolism and reabsorption in the intestine, and are excreted in the unconjugated forms in faeces (Adlercreutz et al., 1995). The glucuronide fraction, the predominant conjugate, representing up to 90% of circulating isoflavones in both rats and humans (Doerge, Chang, Churchwell, & Holder, 2000), is considered biologically inactive, whereas the free and the sulfated fraction, present at much lower concentration, are generally thought to be biologically active. Therefore, bioavailability of isoflavones (like most nutreceuticals) is usually evaluated in terms of plasma concentrations and/or urinary excretion. In terms of absorption into the plasma and urinary excretion, isoflavone glucosides are not absorbed intact through the intestinal epithelium, and glucoronides are the principal form found in plasma. That is, the glucoside forms must be first hydrolysed by β-glucosidases of gut microflora to be absorbed in vivo (Hendrich & Murphy, 2001; Setchell et al., 2002). This knowledge (and some in vivo results showing superior estrogenic effects of genistein over its glucosides) has led to the development of aglycon-enriched products, directly by β-glucosidase treatment (Park, Aguiar, Alencar, Mascrenhas, & Scamparini, 2002, 2003) or by fermentation with bifidobacteria (Tsangalis, Ashton, McGill, & Shah, 2003).

Bifidobacterium and *Lactobacillus* are the predominant members of the intestinal microflora and being classified as probiotics, which are defined as live microbial feed supplement that provides beneficial effects on the host. Probiotics can be used to improve intestinal microbial balance. Due to the health benefits, bifidobacteria are widely used in dairy preparations in conjunction with *Lactobacillus acidophilus* (Shah, 2000). *Bifidobacterium*, *L. acidophilus* and *Lactobacillus casei* grow slowly in soymilk during product manufacture (Otieno, Ashton, & Shah, 2005; Tsangalis, Ashton, McGill, & Shah, 2002, 2003). Therefore, the established practice is to incorporate yogurt cultures (ie. *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*) along with probiotic cultures. It is reasonable to assume that the beneficial effects of probiotic bacteria can be expected only when viable cells are ingested (Shah, 2000). The physiological function of the viable cells can be best determined by analysing the enzymic functions of these cells (Otieno et al., 2005).

The objectives of this study were to evaluate the hydrolytic potential of β-glucosidase of *Bifidobacterium*, *L. acidophilus* and *L. casei* for biotransformation of isoflavone glucosides in soymilk and to establish a set of criteria for selection of probiotic micro-organisms based on biotransformation of isoflavones in soymilk during incubation at 37 °C.

2. Materials and methods

2.1. Bacteria

Pure cultures of *L. casei* ASCC 290, *L. casei* 2607, *L. acidophilus* 4962, *L. acidophilus* 33200, and *L. acidophilus* 4461 were obtained from the Victoria University Culture Collection (Werribee, Vic., Australia). *Bifidobacterium animalis* BB12 was obtained from Chr Hansen Pty. Ltd. (Bayswater, Vic., Australia). The purity of cultures was checked through gram staining and the micro-organisms were stored at −80 °C in 40% glycerol.

2.2. Bacterial growth media

Rehydrated de Mann Rogosa Sharpe (MRS) broth (De Mann, Rogosa, & Sharpe, 1960), pH adjusted to 6.7 using 5 M sodium hydroxide, was prepared according to the manufacturer's instructions (Oxoid Ltd., West Heidelberg, Vic., Australia) and autoclaved at 121 °C for 15 min.

2.3. Soymilk manufacture

Soy protein isolate (SPI; SUPRO 590), supplied by Solae Co. (Chatswood, NSW, Australia), was used in the production of soymilk at 40 g/l using ultra-pure distilled water. After reconstitution, the soymilk was dispensed in six 250 ml glass bottles, and autoclaved at 121 °C for 15 min. After cooling to room temperature, the pH was adjusted in the laminar flow to 6.7 using 5 M sodium hydroxide.

2.4. Assay for β-glucosidase activity in soymilk

The six micro-organisms were individually inoculated in soymilk and β-glucosidase activity was determined at 12, 24, 36 and 48 h of incubation. The strains were activated first by growing in MRS broth (De Mann et al., 1960) and the incubation was carried out at 37 °C for 20 h. Subsequently, 10 ml of active culture was inoculated in triplicate into 250 ml of each of the batches of soymilk