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Optimizing Dough Proofing Conditions To Enhance Isoflavone Aglycones in Soy Bread

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Native β-glucosidase activity in soy bread can convert isoflavone glucosides to aglycones during proofing, and this study determined the time–temperature dependence of this process. Samples were taken every hour for 4 h during proofing at 22, 32, and 48 °C to determine β-glucosidase activity and isoflavone profiles of the dough. After 1–2 h, the β-glucosidase activity increased 43–84% achieving a plateau value at 22 °C but declining when proofed beyond 2 h at 32 °C and 48 °C. Large increases in aglycones and corresponding decreases in the simple glucosides were observed during proofing. The level of malonyl-glucosides decreased 3–15%, and acetyl-glucosides were fairly constant. The two higher temperatures drove more rapid conversion: 70–73% of simple glucosides in 2–4 h. The extent of conversion in the early proofing periods corresponded to β-glucosidase activity. The optimum time–temperature protocol was 2 h at 48 °C resulting in a rapid, high conversion.

KEYWORDS: Soy; bread; isoflavones; β-glucosidase

INTRODUCTION

Isoflavones in soy products are found as aglycones and their glucoside conjugates (malonyl-β-glucosides, acetyl-β-glucosides, and simple β-glucosides, **Figure 1**), whereas malonyl and simple glucoside conjugates are the predominant forms in unprocessed soybeans. Several studies have investigated whether conjugation affects bioavailability of these compounds. Clinical studies have found that isoflavone aglycones were absorbed faster and in greater amounts than their glucosylated forms (*1*, *2*), the opposite dependence (*3*, *4*) or no difference in bioavailability (*5*–*9*). Although several of these studies did not control the food matrix, dose, or involved too few subjects, it appears that bioavailability is unlikely to depend strongly on the initial chemical form of isoflavones. It is understood that soy isoflavones must be deglucosylated to the aglycone prior to absorption since no glucosides have been detected in human blood, urine, or tissues (*10*). Brush border β-glucosidase in the small intestine (*11*) and bacteria in the large intestine (*12*) can deglucosylate dietary isoflavone glucosides.

Despite the lack of evidence for effects of isoflavone conjugation on bioavailability, it is still possible that metabolism and bioactivity of isoflavones are altered by chemical form. Control of the isoflavone aglycone content in the same soy food would allow for determining the effect of isoflavone conjugation in a common food matrix. Isoflavones can be deglucosylated by β-glucosidases from a variety of sources. A study by Hessler et al. (*13*) showed that β-glucosidase in *Saccharopolyspora erythraea* was capable of converting genistin (β-glucoside of genistein) to genistein during fermentation of a soy-based media.

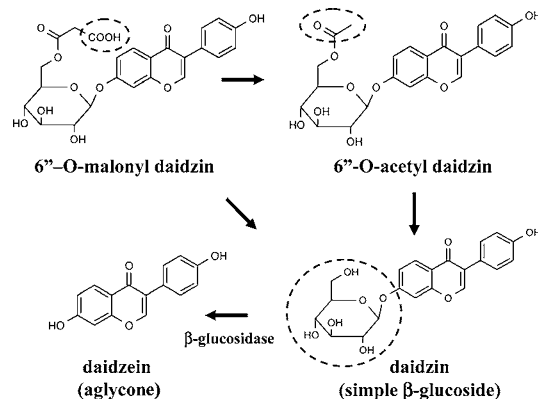

Figure 1. Chemical structures of soy isoflavones and typical transformations during food processing using daidzein as an example. Malonyl daidzin can be decarboxylated to produce the acetyl form usually requiring extreme heat and high moisture. The malonyl and acetyl functionalities can spontaneously hydrolyze to produce the simple glucoside, daidzin, under conditions of low pH or with heating. The simple glucoside daidzin can be deglucosylated to the aglycone daidzein through the action of β-glucosidase.

In another study, glucosides were converted to their aglycones by β-glucosidase-producing Bifidobacteria in soy milk (*14*). Pandjaitan et al. (*15*) added almond β-glucosidase to soy protein isolate to convert most of its isoflavone glucosides to aglycones. Few studies have capitalized on the endogenous β-glucosidase activity present in whole soy ingredients (soy flour, soy milk powder) for the same purpose. When soybeans are soaked (*16, 17*) or stored under humid conditions for extended periods, a large portion of the glucose conjugates are hydrolyzed (*18*). This was largely attributed to nonenzymatic hydrolysis of the malonyl

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moiety followed by intrinsic glucosidase action. Similar conversions were witnessed by Zhang et al. (*19*) during proofing of soy bread dough; isoflavone aglycones increased approximately 5-fold. β-Glucosidase activity was present in extracts from wheat flour, soy ingredients (soy flour, soy milk powder), and active dry yeast (ADY, *Saccharomyces cerevisiae*), but the soy-based ingredients exhibited the highest activities. Baking increased the level of simple β-glucosides and decreased malonyl-β-glucosides but had no significant effect on aglycones. Isoflavones did not degrade significantly during proofing or baking.

The objective of this study was to identify temperature−time conditions for proofing of soy bread dough that would maximize conversion of isoflavone glucosides to aglycones in the shortest period of time.

MATERIALS AND METHODS

Experimental Design. Dough was proofed at 22, 32, or 48 °C for 1−4 h. Room temperature was chosen as a convenient low-temperature condition which might facilitate home preparation of the bread. We selected 32 °C since it is a common incubation temperature used in the food industry to support enzyme action and fermentation by yeast (*20*, *21*). In addition, Hsieh and Graham (*22*) found 30 °C was the optimum temperature of a soybean β-glucosidase. Proofing at 48 °C is part of the patent-pending process for soy bread (Coden: USXXCO US 2004071852 A1 20040415) as previously reported (*23*) and is near the maximum recommended temperature for yeast activity. Isoflavones and β-glucosidase activity were extracted from these samples before proofing and after each hour of proofing and then were quantified as described below.

Materials. *p*-Nitrophenol-β-D-glucopyranoside (*p*NPG), *p*-nitrophenol (*p*NP), sodium acetate, and sodium carbonate (for β-glucosidase assay) were purchased from Sigma Chemical Co. (St. Louis, MO), and isoflavone standard compounds (malonyl-glucoside, acetyl-glucoside, simple glucoside, aglycones) were purchased from LC Laboratories (Woburn, MA). All organic solvents and chemical reagents were HPLC grade from Fisher Scientific (Fair Lawn, NJ). The soy dough ingredients and formulation were as described by Zhang et al. (*19*, *23*) and included nontoasted defatted soy flour (Archer Daniels Midland, Decatur, IL) and soy milk powder (DevanSoy Farms, Carroll, IA).

Dough Preparation. Soy bread doughs were prepared using a patent-pending formula in which 60% of wheat flour in a wheat bread formula was replaced with soy flour and soy milk powder (*23*). The bread ingredients were combined and mixed in a 5-quart Kitchen Aid Mixer (KitchenAid Portable Appliance, St. Joseph, MI) to form doughs. The doughs were proofed at 22, 32, and 48 °C for 1−4 h using a CM2000 combination module proofer from InterMetro Industries Corp. (Wilkes-Barre, PA).

Isoflavones Analysis. Isoflavones in soy bread doughs before and after proofing were extracted and analyzed for their isoflavone content and composition using the method described by Zhang et al. (*23*). Dough samples (0.5 g) were milled to a fine paste and were extracted with a mixture of 0.1 N HCl (2 mL), acetonitrile (10 mL), and water (3 mL) at room temperature for 2 h. Extracts were dried under nitrogen, and the residue was redissolved in methanol and was filtered through a 0.2 μm nylon syringe filter before injection. A Waters 2695 HPLC unit (Milford, MA) including a Waters 2996 photodiode array (PDA) detector was used to identify and quantify the isoflavones. Separation of isoflavones was achieved using a Waters Nova-Pak C_{18} reversed-phase column (3.9 × 150 mm; particle size 4 μm, pore size 60 Å). The PDA detector was set to monitor eluate absorption between 210 and 400 nm. The mobile phase consisted of 1.0% acetic acid in water (v/v) (solvent A) and 100% acetonitrile (solvent B). The elution condition was as follows: 0−5 min 15% B, 5−36 min 15−29% B, 36−44 min 29−35% B, 44−45 min 35−15% B, reequilibrate at 15% B for 5 min. Flow rate was 0.6 mL/min and the injection volume was 10 μL. The isoflavones from soy dough were identified by comparing their retention times and UV spectra with pure isoflavone compounds. The reliability of the extraction method was assessed by spiking extracts with known concentrations of pure isoflavones and determining their recovery. Recoveries for all test isoflavones exceeded 96%.

β-Glucosidase Assay. The β-glucosidase activity in soy bread dough was determined using a modified method based on that of McCue and Shetty (*24*). β-Glucosidase activity was extracted from dough by mixing two grams of dough with distilled water (15 mL), homogenizing for 1 min at 7000 rpm (Polytron, Kinemetica AG, Littau-Switzerland), and then centrifuging for 20 min at 10 000 rpm and 4 °C using a Sorvall RC5Cplus centrifuge with a SS-34 rotor (Ivan Sorvall, Inc., Norwalk, CO). The supernatant was collected and filtered through a 0.48 μm filter before analysis.

Test samples contained 0.1 mL *p*NPG (9 mM, pH 4.6) as substrate in sodium acetate buffer plus 0.8 mL sodium acetate buffer (pH 4.6) and 0.1 mL of the extract from soy bread dough. Blanks contained 0.1 mL of distilled water instead of the dough extract. The samples were incubated at 37 °C for 30 min, and the reaction was stopped by adding 1 mL of cold (4 °C) 100 mM sodium carbonate (pH = 8). The amount of *p*NP liberated was determined from the absorbance at 400 nm (UV−vis spectrophotometer, water blank) compared to a *p*NP standard curve. One unit of enzyme activity (U) was defined as 1 μmol of *p*NP released from *p*NPG per min under assay conditions. U of activity was divided by the mass of dough extracted (2 g) so that activity is reported as U per gram of dough.

Statistical Analysis. Each sample was analyzed in triplicate. All data were expressed as means unless otherwise indicated. Statistical analysis was performed using SAS software (SAS Inc., Cary, NC). Analyses of variance (ANOVA) using the general linear models (GLM) were conducted. Differences between the sample means were analyzed by Fisher's least significance (LSD) test at $\alpha = 0.05$.

RESULTS

Before proofing, soy bread dough contained 190 nmol/g of isoflavone aglycones, 924 nmol/g of isoflavone β-glucosides, 1215 nmol/g of malonyl-β-glucosides, and 164 nmol/g of acetyl-β-glucosides on a dry basis. In unproofed soy bread dough, the β-glucosidase activity was about 27.7 U/g.

Figure 2a shows the changes in isoflavone content in soy dough at a proofing temperature of 22 °C. Over 4 h of proofing, isoflavone aglycones doubled with nearly a linear dependence from 190 nmol/g to 380 nmol/g, while simple β-glucosides decreased by 89 nmol/g (9.4%). The increase in aglycones appears to arise from the breakdown of simple glucosides since little change was observed in malonyl- (decrease 35 nmol/g) or acetyl-glucoside levels (no significant change, $P > 0.05$). Relative β-glucosidase activity in the dough as shown in **Figure 3** increased 65% during the first hour of proofing but remained practically unchanged from the second hour of proofing through 4 h.

Figure 2b shows the changes in isoflavone content in soy dough proofed at 32 °C. Isoflavone aglycones more than doubled in the first 2 h from an initial value of 190 nmol/g and nearly doubled in the last 2 h for a net change 662 nmol/g. During the 4 h proofing at 32 °C, simple β-glucosides decreased by 668 nmol/g (66.7%) almost linearly. The isoflavone malonyl-β-glucosides decreased by 187 nmol/g (14.4%) while acetyl-β-glucosides did not change significantly ($P > 0.05$). Both simple and malonyl-glucosides were likely sources for the rise in aglycones although simple glucosides were the main source. Relative β-glucosidase activity increased 43.3% during the first 2 h of proofing and then decreased to near 50% of the initial value during the last 2 h of proofing (**Figure 3**).

Figure 2c shows the changes in isoflavone content in soy dough proofed at 48 °C. Isoflavone aglycones increased by 660 nmol/g in the first 2 h of proofing and had increased by 768 nmol/g by 4 h. During 4 h of proofing at 48 °C, simple β-glucosides decreased by 687 nmol/g (74.3%), with most loss occurring within the first 2 h. There is a striking symmetry

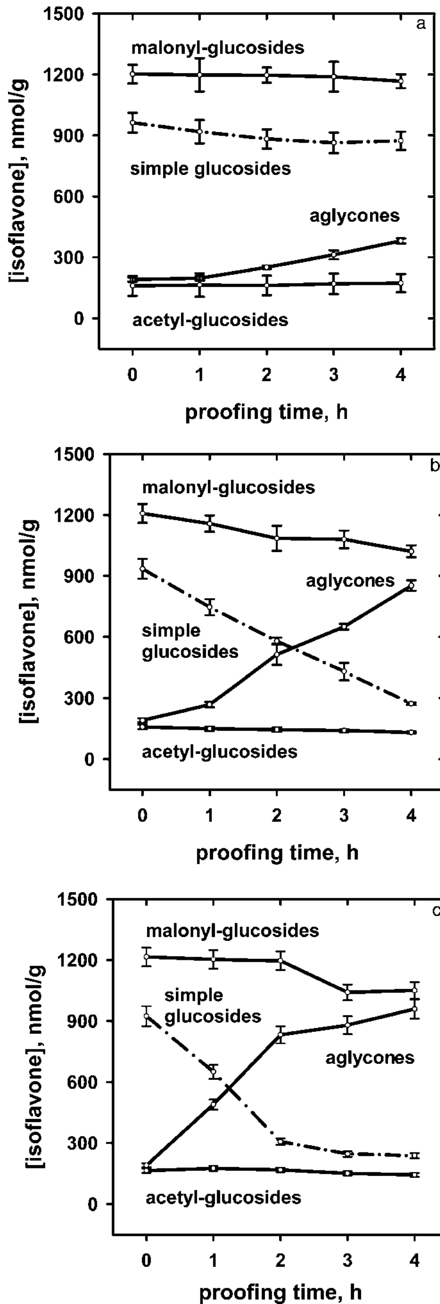

Figure 2. Isoflavone content in soy bread dough during proofing at (**a**) 22, (**b**) 32, and (**c**) 48 °C as a function of proofing time. Isoflavone classes are as indicated representing the sum of daidzein, genistein, and glycitein families. Values are means ± SD of three independent determinations.

between the rise in aglycone levels and the decrease in simple β-glucosides although malonyl-glucosides also decreased by 165 nmol/g (13.6%) and may have in part contributed to aglycone changes. Similar to the result at the lower temperatures, the acetyl-β-glucosides did not change significantly with time ($P > 0.05$). As at the lower temperatures, relative β-glucosidase activity increased early in proofing but had increased by 83% by 1 h and then decreased during the last 3 h of proofing to undetectable levels (**Figure 3**).

It was hypothesized that soy β-glucosidase effects the conversion of glucosides to aglycones during proofing of dough (*19*). To test this notion, the isoflavone turnover can be compared to the glucosidase activity during proofing. The glucosidase activities (**Figure 3**) were determined at 37 °C as specified in the protocol (*24*). This temperature was selected to maximize assay sensitivity (increased rate of turnover) while avoiding enzyme denaturation which would occur at higher temperatures. The activities shown in **Figure 3** reflect the amount of intact enzyme present in the dough at a given time. Matsuura et al. have shown that the rate of glucosidase-catalyzed hydrolysis of glucosides increases with increasing temperature (*16*, *17*) to a maximum around 50 °C above which activity is rapidly lost. Since the assay was conducted at 37 °C, the true potential for glucoside turnover is expected to have been less at 22 °C, slightly less at 32 °C, and much greater at 48 °C. The rate of hydrolysis of β-aryl glucosides as catalyzed by soybean β-glucosidase approximately doubles from 22 °C to 32 °C and doubled again from 32 °C to 48 °C (*16*). Thus, the effective glucosidase activity (EGA, **Figure 5a**) in dough proofed at 22 °C was estimated by dividing values obtained at 37 °C by 2 and multiplying the activity in 48 °C-proofed dough by 2 to compare values directly to those for 32 °C-proofed dough.

$$\mathrm{EGA}(T) = (\text{activity at } 37\ °\mathrm{C})/(\text{correction factor})$$

where correction factors are 2 at 22 °C and 0.5 at 48 °C. To

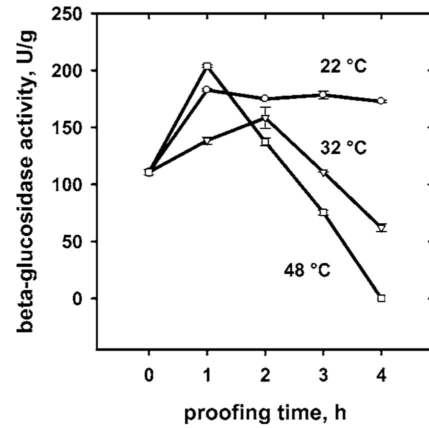

Figure 3. β-Glucosidase activity extracted from soy bread dough as a function of proofing time (relative to time zero) assayed at 37 °C.

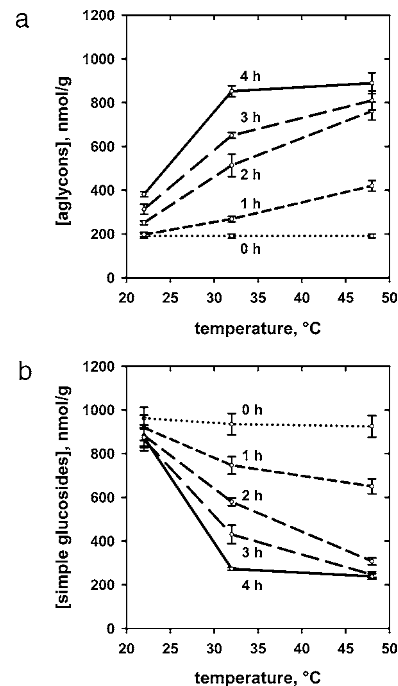

Figure 4. (**a**) Isoflavone aglycone and (**b**) simple glucoside levels as function of proofing temperature; data as in **Figure 2a**, **b**, **c** to emphasize the agreement between aglycones produced and simple glucosides lost.

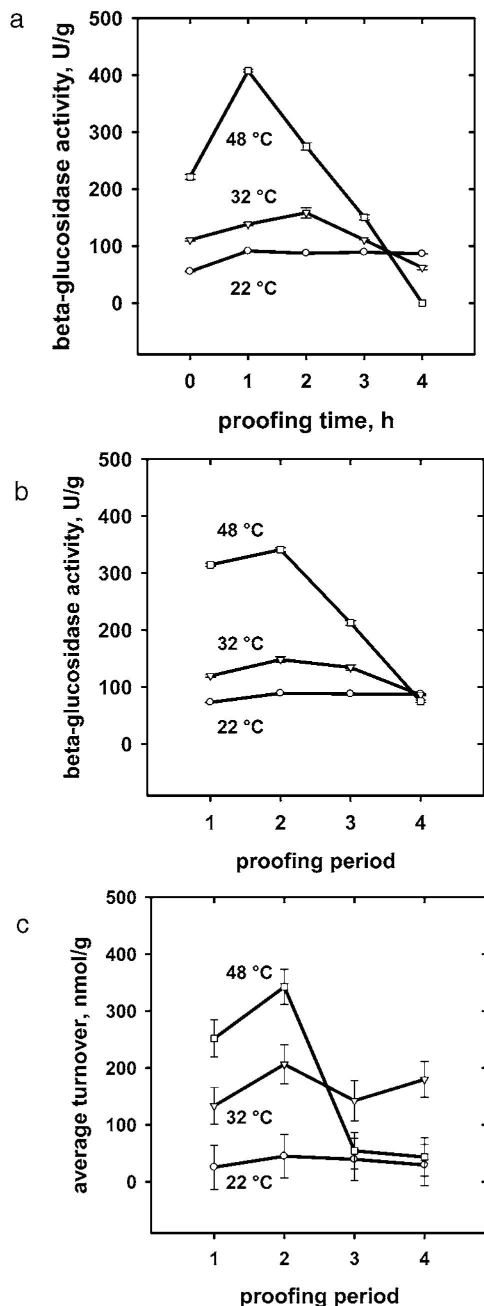

Figure 5. (**a**) Effective glucosidase activity extracted from soy bread dough as a function of proofing time, determined using nitrophenol glucoside as substrate. Estimates of activity during proofing were obtained by adjusting values in **Figure 3** for proofing temperature; values at 22 °C were divided by 2 and values at 48 °C were multiplied by 2. (**b**) Effective glucosidase activity averaged over each hour of proofing and (**c**) measured isoflavone turnover (difference between successive time points in **Figure 2**) as average of isoflavone aglycones produced and simple glucosides consumed. Isoflavones determined by HPLC with UV detection at appropriate absorption maxima, 250–260 nm.

compare EGA to turnover, the EGA was averaged over each proofing period (**Figure 5b**).

Isoflavone turnover was taken as the average of aglycone produced (product) and glucoside consumed (substrate) during a given proofing hour since both elementary reactions should reflect the reaction mediated by glucosidase. The simple glucosides and malonyl-glucosides were considered as possible substrates for glucosidase since only their levels changed significantly during proofing ($p < 0.05$). However, the rising aglycone levels shown as a function of temperature in **Figure 4a** corresponded remarkably well with decreasing simple glucosides (**Figure 4b**). Thus, simple glucosides appear to have been the main substrate for glucosidase action and the slight decrease in malonyl-glucosides was likely due to nonenzymatic hydrolysis. The average of simple glucosides consumed and aglycones produced, average turnover (AT), was calculated as follows:

$$\mathrm{AT} = ([\mathrm{AG}]_t - [\mathrm{AG}]_{t-1} + [\mathrm{SG}]_t - [\mathrm{SG}]_{t-1})/2$$

where AG is aglycone, SG is simple glucoside, and t represents a particular time period, hour 1, 2, 3, or 4. The trend of AT with proofing period is presented in **Figure 5c**.

There was qualitative agreement between the average EGA and AT as turnover increased with temperature and time within the first 2 h of proofing and then decreased at extended times at 32 °C and 48 °C. The turnover at the two higher temperatures was larger relative to that at 22 °C (1:4:6) than predicted by the EGA (1:1.5:4), that is, actual turnover was more sensitive to temperature than glucosidase activity would predict. One would not expect perfect agreement between the two trends since the actual reaction velocity depends on several other variables besides enzyme concentration. Assuming typical Michaelis–Menten enzyme kinetics, these variables would include concentrations of substrate and product as well as values of k_{cat} and K_m (which might also depend on temperature). Soy and yeast β-glucosidases have similar K_m values, typically 0.1–0.3 mM toward *p*NPG (*25*, *26*), and the K_m for wheat β-glucosidase was reported as 1.70 mM (*27*). Immediately after mixing the dough, simple glucosides were about 1 mM which is 3–10 times the expected K_m value for soy and yeast glucosidase, that is, near saturating conditions. However, as the reaction progressed, substrate concentration fell well below saturation, and consequently, the turnover rate should decrease. In addition, depletion of substrate and accumulation of product would slow the reaction at extended proofing times which explains the seemingly premature loss in turnover by 3 and 4 h. Yeast metabolites and general matrix effects might also alter glucosidase action. The agreement between trends in turnover and effective enzyme activity was fairly good considering the complexity of the system. Taken together, the rise in aglycones most likely originated from β-glucosidase-catalyzed deglucosylation of simple isoflavone glucosides.

DISCUSSION

β-Glucosidase activity in the dough increased over the first 2 h of proofing at all three proofing temperatures. The additional glucosidase was most likely produced by baker's yeast in the dough as inducible aryl-β-glucosidase activity has been described for some strains of *Saccharomyces cerevisiae* (*28*–*31*). Commercial strains of *S. cerevisiae* used in baking can grow over a range of proofing temperatures (20–40 °C) but optimally near 26 °C while fermentative activity as indicated by gas production is greater between 27 and 38 °C with optimum around 35 °C (*20*). These trends suggest baker's yeast might produce more glucosidase at 22 °C and 32 °C than at 48 °C, yet the rise in activity was largest at 48 °C (83% increase), slightly smaller at 22 °C (68% increase), and smallest and slowest to rise at 32 °C (40%, 2 h) (**Figure 3**). It is also possible that the level of glucosidase did not change but metabolites produced by the yeast affected the activity such as ethanol which can activate glucosidase at low concentrations (*32*) and inactivate at high concentrations.

The profile of β-glucosidase activity at 32 °C and 48 °C with time was affected by the competing process of enzyme inactivation. β-Glucosidase activity in dough decreased when proofing at 32 °C and 48 °C beyond 2 and 1 h, respectively. The decrease in enzyme activity was most likely due to thermal denaturation. Matsuura et al. (*16*, *17*) found that the maximum turnover of isoflavone glucosides by soybean glucosidase was evident around 50 °C. The same authors showed that at temperatures at and above 50 °C most of the enzyme activity was lost within 5 min. Yeast β-glucosidases are not very heat stable and 45 °C conditions can usually inactivate glucosidase within 30 min (*33*). Thus, loss of activity over 4 h of proofing at 48 °C was not surprising. However, the rate of glucosidase inactivation was more rapid at 32 °C than expected (**Figure 3**). This might be explained by a difference in glucosidase synthesis by yeast.

Despite losses at extended proofing times, when temperature dependence of reaction rate was considered (**Figure 5a**), the effective glucosidase activity showed the greatest turnover potential at 1 h and 48 °C, less than half that value at 2 h and 32 °C, and about half that again at 1 h and 22 °C. The high rate of conversion at 32 °C and 48 °C indicates that the apparent synthesis of glucosidase by yeast and the effect of temperature on the reaction kinetics compensated for inactivating processes during proofing, providing sufficient activity for conversion.

By 4 h at 48 °C, the increase in aglycones represented conversion of 73% of the simple glucosides, the presumed main substrate. Complete conversion of simple β-glucosides to the aglycone forms was not observed in dough proofed at any of the three temperatures studied. Pandjaitan et al. (*15*) achieved complete conversion of the simple glucosides of genistein, genistin, to genistein after adding an exogenous β-glucosidase to soy protein concentrate. In the current study, the incomplete conversion was likely due to the already described premature loss of enzyme activity. Turnover could have been further limited by substrate depletion (simple glucosides), product inhibition (glucose, aglycone), and accumulation of inhibitory fermentation products, that is, acids and alcohols (*34*).

From the glucosidase assay, the activity in freshly mixed dough was ~100 U/g. Therefore, the dough had the capacity to convert ~100 μmol of substrate per minute per gram of dough. If one considers the initial concentration of simple glucoside (1 μmol/g), one might surmise that all simple glucoside should have been converted to aglycone in 1/100 of a minute or 0.6 s. The actual conversion required several hours, thus approximately 4 orders of magnitude slower. Keep in mind that the dough and assay conditions are not the same. The assay is conducted in dilute solution such that substrate and enzyme diffuse rapidly and collide more frequently than in a viscous dough matrix. We believe slow diffusion of reactants in the dough accounts for the poor conversion rate. As the trend of conversion coincides with enzyme activity during proofing, limited diffusion rates seem to be a constant factor during proofing.

The decrease of malonyl-glucosides in dough proofed at 32 °C and 48 °C was slow and only represented a 14−15% loss. The malonyl forms likely hydrolyzed spontaneously to liberate the simple glucoside. Hydrolysis of the malonyl groups is common when processing soy with heat if the product is sufficiently hydrated (*15*, *18*). It follows that the simple glucosides thus liberated could have served as substrate for glucosidase to yield aglycones. If such a process occurred in this study, it was a minor contributor to aglycone levels.

After 4 h of proofing at the two higher temperatures, the aglycone levels reached more than 900 nmol/g, a conversion of about 73% of simple glucosides. After 2 h at 48 °C, 67% of simple glucosides had been converted to aglycones, and this combination of time and temperature was deemed optimum for this study. The relatively small increase in conversion from 2 to 4 h was considered cost-prohibitive, and extending proofing beyond 4 h at 48 °C would not improve conversion since there was no residual glucosidase activity by 4 h. Similar time considerations made extended proofing at 32 °C impractical. To sustain glucosidase activity and achieve greater aglycone yield, one might use a proofing temperature near 22 °C and longer proofing time, but this approach would require proofing for more than 14 h to achieve similar conversion.

Other strategies might be investigated to further accelerate glucoside conversion or simply increase the proportion of aglycones in soy dough: (1) utilize soy ingredients with isoflavones predominantly in their aglycone form, (2) supplement dough with β-glucosidase, (3) if yeast are synthesizing glucosidase, optimize proofing conditions to favor glucosidase production, or (4) find a means to access the malonyl glucoside pool of the current dough. To this last point, half of the glucosides in soy dough after proofing are malonyls using the current protocol. One could heat treat well-hydrated soy ingredients to drive hydrolysis of malonyl-glucosides to simple glucosides before fermenting the dough. Alternatively, one might directly hydrolyze the malonyl form to release aglycones using malonyl-specific β-glucosidase as found in soybean seedling roots (*22*). Several of these options are currently being explored in our laboratory.

Endogenous β-glucosidase activity likely mediated the conversion of simple glucosides to their aglycone form in soy bread dough and to a much greater extent at higher proofing temperatures. Conversion at higher temperatures was hampered at extended proofing times by loss of glucosidase activity, likely because of heat denaturation or inactivation of the enzyme. Of the three proofing temperatures studied, 22, 32, and 48 °C, proofing at 48 °C proved to be most effective yielding 67% conversion of simple glucosides to aglycones within 2 h.

ABBREVIATIONS USED

HPLC, high-performance liquid chromatography; LSD, least significant difference; PDA, photodiode array; *p*NP, *p*-nitrophenol; *p*NPG, *p*-nitrophenol-β-D-glucopyranoside; SD, standard deviation; U, units of enzyme activity.

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