Contents and bioconversion of β‑glycoside isoflavones to aglycones in the processing conditions of soybean tempeh

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Abstract – The objective of this work was to evaluate the effect of the processing conditions of soybean tempeh on the contents of β‑glycoside isoflavones and on their bioconversion into aglycones. Different times of soaking (6, 12, and 18 hours), cooking (15, 30, and 45 minutes), and fermentation (18, 24, and 30 hours) with Rhizopus oligosporus at 37°C were evaluated for tempeh preparation. Grains from the cultivar 'BRS 267' were used, and the experiment was carried out according to a central composite design (2³). The response functions comprised the contents of genistin, malonyldaidzin, malonylgenistin, daidzein, and genistein, quantified by ultraperformance liquid chromatography (UPLC). Soaking, cooking, and fermentation times change the content, profile, and distribution of the different forms of isoflavones in tempeh. The highest bioconversion of glycoside isoflavones into aglycones occurred in 6-hour soaked soybean grains, whose cotyledons were cooked for 15 minutes and subjected to 18-hour fermentation.

Index terms: Glycine max, Rhizopus oligosporus, daidzein, fermented soybean, functional food, genistein.

Introduction

Tempeh is a traditional Indonesian food of high nutritional quality, obtained by fungal fermentation of dehulled, soaked, and cooked soybean grains (Starzyńska-Janiszewska et al., 2014). It is commercially available in different forms, such as fried, boiled, steamed or roasted. In general, the tempeh is obtained by fermentation of soybean grains with Rhizopus oligosporus, and it is an excellent protein source that has all the essential amino acids for human nutrition, in addition to dietary fibers, calcium, vitamin B12, folic acid, low sodium content, and unsaturated fats (United States Department of Agriculture, 2008). Moreover, fermented soybean foods contain several health beneficial bioactive compounds (Murooka & Yamashita, 2008) of which the most important are folate, vitamin B12, and isoflavones (Mo et al., 2013).

The preparation process of soy tempeh includes soaking soybean grains, cooking their cotyledons and fermentating them by the inoculation with Rhizopus
oligosporus; however, these processes are not standardized. Usually, soybean grains are soaked in water for 10 to 12 hours, at room temperature, in order to facilitate the removal of the hulls. Cotyledons are then cooked in boiling water, drained, cooled at 35 to 40°C, inoculated with microorganisms, and fermented at 37°C for 24 to 48 hours (Nout & Kiers, 2005). Rhizopus oligosporus is the most often used fungus for fermentation of soybean tempeh, due to its rapid growth between 30 and 42°C (Shurtleff & Aoyagi, 2001).

Fermentation is the major processing stage for obtaining tempeh, due to the changes that occur in the sensory characteristics and chemical composition of the food, mainly associated with the bioconversion of isoflavones (Chang et al., 2009). The fermentation process depends on soybean tempeh processing conditions, such as time, temperature, pH, inoculum amount, and relative moisture content (Nout & Kiers, 2005). In this stage, β-glycosidase hydrolyzes β-glycoside forms to aglycones, which are more bioactive and absorbable isoflavones in the intestine, compared to their glycosidic forms (Izumi et al., 2000; Liggins et al., 2000). Isoflavones, and mainly the aglycones forms, have drawn attention because of their ability to reduce the risk of cardiovascular diseases (Zhuo et al., 2004), to inhibit cancer cell growth (Sarkar & Li, 2003; Lund et al., 2004), to alleviate the menopause symptoms (Messina & Hughes, 2003), and to prevent diseases such as osteoporosis (Arjmandi et al., 1996). The bioconversion of β-glycoside forms into aglycones (daidzein, genistein and glycitein) requires hydrolysis by β-glycosidase.

Nakajima et al. (2005) concluded that the content of three isoflavones aglycones (daidzein, genistein and genistein) in soybean tempeh had increased about two times after 24 hours of fermentation. During soybean tempeh processing, glycosides isoflavones are converted to their corresponding aglycones, increasing the concentrations of daidzein and genistein, and decreasing the ones of some isoflavones (Haron et al., 2009). The bioconversion of glycosides isoflavones into aglycones occur during fermentation, with higher contents found after long fermentation times (Villaress et al., 2011). Ferreira et al. (2011) showed that the total glycoside forms (daidzin and genistin) in soybean cotyledons decrease, and that the malonyl forms, which react unsteadily to heat treatments, reduced greatly as a result of cooking. These authors also mention that aglycones forms (daidzein and genistein) increase after 24 hours of fermentation. Bavía et al. (2012) observed a 43% reduction in malonyl forms after cooking soybean grains, and a 50% increase in the aglycones levels after fermentation.

The objective of this study was to evaluate the effect of the processing conditions of soybean tempeh on the contents of β-glycosides isoflavones and on their bioconversion into aglycones.

Material and Methods

Soybean cultivar 'BRS 267' harvested in Londrina, PR, Brazil, in 2011/2012, was used to prepare the soybean tempehs. Lyophilized Rhizopus microsporus var. oligosporus was obtained from Intsoy (International Soybean Program, University of Illinois, EUA). Isoflavone standard solutions were prepared from 6''-O-acetylglucosides and 6''-O-malonylglucosides (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and from β-glucosides and aglycones (Sigma-Aldrich Co., St. Louis, MO, USA). The other reagents were of analytical grade and obtained from different sources.

To evaluate the effects of tempeh processing variables on the bioconversion of β-glycoside isoflavones into aglycones, a central composite design (2³) was used, with 3 replicates of central point, totaling 17 random assays. The tempehs were prepared according to the traditional method described by Wei (1991), with modifications. The following processing variables were investigated: X1 (soaking time, h), X2 (cooking time, min), and X3 (fermentation time, h). For each assay, 100 g of soybean grains (proportion 1:10, w/v) were soaked (X1), and grains were manually dehulled. Cotyledons were cooked in boiling water (X2), and then they were drained and cooled at 25°C. For the inoculation, 2 g of R. oligosporus were used per 100g of cooked and cooled cotyledons. After inoculation, the cotyledons were homogenized, packaged in perforated polypropylene bags, maintained at 37°C, and fermented (X3) according to the experimental design. Table 1 shows the coded and real levels of the independent variables. These levels were established in a preliminary test. The produced tempehs were lyophilised in a freeze-dryer, model Alpha 2-4 LD plus (Martin Christ, Osterode am Harz, Germany) and
stored at -26°C, for the extraction and quantification of isoflavone contents.

The response functions (Y) to different isoflavone forms in tempeh, Y1 (µmol genistin g⁻¹), Y2 (µmol malonyldaidzin g⁻¹), Y3 (µmol malonylgenistin g⁻¹), Y4 (µmol daidzein g⁻¹), and Y5 (µmol genistein g⁻¹), were determined according to the following mathematical model:

\[ Y = \beta_0 + \beta_1x_1 + \beta_2x_2 + \beta_3x_3 + \beta_1x_1^2 + \beta_2x_2^2 + \beta_3x_3^2 + \beta_{12}x_1x_2 + \beta_{13}x_1x_3 + \beta_{23}x_2x_3 + \varepsilon \]

in which: Y is the response function; x₁, x₂, and x₃ are coded variables; β is the estimated coefficient of the response surface model; and ε is the pure error. The response functions were evaluated by regression analysis and analysis of variance. The equation model was fitted to experimental data to propose the model. Response surface graphs and desirability parameters were generated for each response function, in order to obtain a tempeh with the maximum content of aglycones. All analyses, desirability parameters, and response surfaces were performed with the software Statistica, version 8.0 (StatSoft, Inc., Tulsa, OK, USA).

Table 1. Components of the central composite design (2³) and response functions (Y) to different isoflavone forms in tempeh produced with grains of soybean cultivar 'BRS 267'.

<table>
<thead>
<tr>
<th>Assays</th>
<th>X₁(x₁)</th>
<th>X₂(x₂)</th>
<th>X₃(x₃)</th>
<th>Y₁</th>
<th>Y₂</th>
<th>Y₃</th>
<th>Y₄</th>
<th>Y₅</th>
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<tbody>
<tr>
<td>1</td>
<td>6(-1)</td>
<td>15(-1)</td>
<td>18(-1)</td>
<td>0.36</td>
<td>0.58</td>
<td>2.02</td>
<td>0.08</td>
<td>1.14</td>
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<tr>
<td>2</td>
<td>6(-1)</td>
<td>15(-1)</td>
<td>30(+1)</td>
<td>0.33</td>
<td>0.40</td>
<td>1.72</td>
<td>0.09</td>
<td>0.93</td>
</tr>
<tr>
<td>3</td>
<td>6(-1)</td>
<td>45(+1)</td>
<td>18(-1)</td>
<td>0.40</td>
<td>0.00</td>
<td>0.79</td>
<td>0.05</td>
<td>0.88</td>
</tr>
<tr>
<td>4</td>
<td>6(-1)</td>
<td>45(+1)</td>
<td>30(+1)</td>
<td>0.27</td>
<td>0.00</td>
<td>0.67</td>
<td>0.05</td>
<td>0.74</td>
</tr>
<tr>
<td>5</td>
<td>18(+1)</td>
<td>15(-1)</td>
<td>18(-1)</td>
<td>0.13</td>
<td>0.42</td>
<td>1.49</td>
<td>0.07</td>
<td>1.11</td>
</tr>
<tr>
<td>6</td>
<td>18(+1)</td>
<td>15(-1)</td>
<td>30(+1)</td>
<td>0.23</td>
<td>0.35</td>
<td>1.27</td>
<td>0.07</td>
<td>0.81</td>
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<tr>
<td>7</td>
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<td>45(+1)</td>
<td>18(-1)</td>
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<td>0.27</td>
<td>0.85</td>
<td>0.11</td>
<td>1.35</td>
</tr>
<tr>
<td>8</td>
<td>18(+1)</td>
<td>45(+1)</td>
<td>30(+1)</td>
<td>0.29</td>
<td>0.00</td>
<td>0.67</td>
<td>0.06</td>
<td>0.74</td>
</tr>
<tr>
<td>9</td>
<td>6(-1)</td>
<td>30(0)</td>
<td>24(0)</td>
<td>0.28</td>
<td>0.36</td>
<td>1.27</td>
<td>0.09</td>
<td>1.12</td>
</tr>
<tr>
<td>10</td>
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<td>30(0)</td>
<td>24(0)</td>
<td>0.28</td>
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<td>0.09</td>
<td>1.12</td>
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<tr>
<td>11</td>
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<td>24(0)</td>
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<td>0.99</td>
<td>0.06</td>
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</tr>
<tr>
<td>15</td>
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<td>30(0)</td>
<td>24(0)</td>
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<td>0.32</td>
<td>1.13</td>
<td>0.08</td>
<td>1.11</td>
</tr>
<tr>
<td>16</td>
<td>12(0)</td>
<td>30(0)</td>
<td>24(0)</td>
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<td>0.29</td>
<td>1.03</td>
<td>0.08</td>
<td>1.01</td>
</tr>
<tr>
<td>17</td>
<td>12(0)</td>
<td>30(0)</td>
<td>24(0)</td>
<td>0.22</td>
<td>0.39</td>
<td>1.15</td>
<td>0.08</td>
<td>1.11</td>
</tr>
</tbody>
</table>

Coded variables: x₁, x₂, and x₃. Real variables: X₁, hydration time (h); X₂, cooking time (min); and X₃, fermentation time (h). Y₁, µmol of genistin g⁻¹ of tempeh; Y₂, µmol of malonyldaidzin g⁻¹ of tempeh; Y₃, µmol of malonylgenistin g⁻¹ of tempeh; Y₄, µmol of daidzein g⁻¹ of tempeh; and Y₅, µmol of genistein g⁻¹ of tempeh.

The lyophilised and milled tempehs were defatted with hexane, in a 1:10 ratio (w/v), for 1 hour at room temperature by continuous and rotary agitation, followed by vacuum filtration. The isoflavone extraction was performed in triplicate with 0.1 g of the defatted sample, using 2 mL of an extraction solution containing ultrapure water, acetone and ethanol (1:1:1, v/v/v) (Yoshiara et al., 2012). The separation and quantification of isoflavones were performed in triplicate, using an ultraperformance liquid chromatography (UPLC) system, as described by Handa et al. (2014). Since different isoflavone forms have different molecular masses, in order to improve the comparisons, the isoflavone concentrations were expressed in micromoles of isoflavones per grams of lyophilized and defatted tempeh.

Results and Discussion

Grains of 'BRS 267' soybean cultivar had the following isoflavone components quantified by UPLC: 1.48 µmol β-glucoside isoflavones g⁻¹ (1.12 µmol genistin g⁻¹, and 0.36 µmol daidzin g⁻¹), 4.25 µmol malonylglucosides g⁻¹ (3.04 µmol malonylgenistin g⁻¹, and 1.21 µmol malonyldaidzin g⁻¹), and only 0.33 µmol genistein g⁻¹. The aglycone forms were present in lower concentrations than the other isoflavone forms (Carrão-Panizzi et al., 2003). However, this same cultivar, harvested at different times and with isoflavones quantification by high performance liquid chromatography (HPLC), showed the following contents (Silva et al., 2009; Bavía et al., 2012): 2.16 µmol daidzin g⁻¹, 1.71 µmol genistin g⁻¹, 2.33 µmol malonyldaidzin g⁻¹, 1.70 µmol malonylgenistin g⁻¹, 0.24 µmol daidzein g⁻¹, and 0.80 µmol genistein g⁻¹. This differences in isoflavone contents can be attributed to the genetic variability and to different environmental conditions (Wang & Murphy, 1996; Carrão-Panizzi et al., 2003).

The X₁ variable (soaking time, h) showed a significant linear negative effect (β₁x₁ = −0.05x₁, p<0.05) on the Y₁ response function (µmol of genistin per g of tempeh). The effects of the X₂ (cooking time, min) and X₃ (fermentation time, h) were not significant (p>0.05). Based only in the significant variable, the mathematical model ̃Y₁ = 0.24 − 0.05x₁, was developed, indicating that the genistin level in tempeh is independent of the cooking and fermentation times.
The lack-of-fit of the model was not significant (at 95% confidence interval), and 77% of the experimental data were properly adjusted (R²) to the model. The response surface (Figure 1 A) and the desirability parameters (Figure 1 B) showed a region where Y₁ was maximum (Ŷ₁ = 0.40 µmol g⁻¹), and obtained when the following conditions were used: x₁ = -1 or X₁ = 6 h of soaking; x₂ = 0.5 or X₂ = 22.5 min of cooking; and x₃ = -1 or X₃ = 18 h of fermentation with *Rhizopus oligosporus*. These processing conditions of soybean tempeh agreed with the assay 3, in which the experimentally obtained Y₁ was 0.40 µmol of genistin per g of tempeh.

Genistin content in the tempeh was 36% lower (0.40 µmol g⁻¹) than in the grains (1.12 µmol g⁻¹), and daidzin content was neglectable (while its contents in the grains were 0.36 µmol g⁻¹).

For the Y₂ response function (µmol of malonyldaidzin per g of tempeh), the X₂ (cooking time, min) and X₃ (fermentation time, h) variables showed significant linear negative effects (β₂x₂ = -0.46x₂ and β₃x₃ = -0.09x₃, respectively). The interaction between X₁ and X₂ variables had a significant linear positive effect (β₁₂x₁x₂ = 0.13x₁x₂) (p<0.05). The other effects were not significant, and the mathematical model Ŷ₂ = 0.32 − 0.21x₂ − 0.05x₃ + 0.07x₁x₂ was developed, indicating that low levels of the three variables (X₁, X₂, and X₃) are recommended, in order to prepare tempeh with the maximum malonyl content. The lack-of-fit of the model was not significant (at 95%), and 99% of the experimental data were properly adjusted (R²) to the model. The response surface (Figure 2 A and C) and desirability parameters (Figure 2 B and D) suggested a region where Y₂ (0.58 µmol malonyldaidzin g⁻¹) and Y₃ (2.01 µmol malonylgenistin g⁻¹) were maximal, with the following conditions: x₁ = -1 or X₁ = 6 h soaking time; x₂ = -1 or X₂ = 15 cooking time; and x₃ = 0 or X₃ = 24 h fermentation time. These conditions coincided with the assay 1, in which Y₂ (0.58 µmol malonyldaidzin g⁻¹)

Figure 1. Response surface (A) and desirability parameters (B) for the response function Y₁ (µmol of genistin g⁻¹ of tempeh).
and $Y_3$ (2.02 µmol malonylgenistin g$^{-1}$) were experimentally obtained. In the grains, the contents of malonylglucosides were 4.25 µmol g$^{-1}$, with 3.04 µmol malonylgenistin g$^{-1}$.

The $X_2$ variable (cooking time, min) showed a significant linear negative effect ($\beta_{2x_2} = -0.01x_2$) on the $Y_4$ response function (µmol of daidzein per g of tempeh). The $X_1$ and $X_3$ variables showed a significant quadratic effect ($\beta_{1x_1^2} = 0.01x_1^2$ and $\beta_{3x_3^2} = -0.01x_3^2$, respectively). The interaction between $X_1$ (soaking time, h) and $X_2$ showed a significant linear positive effect ($\beta_{12x_1X_2} = 0.01x_1x_2$), and the interactions between

![Figure 2](image)

**Figure 2.** Response surface (A and C) and desirability parameters (B and C) for the response functions $Y_2$ (µmol malonyldaidzin g$^{-1}$) and $Y_3$ (µmol malonylgenistin g$^{-1}$).
X₁ and X₃ (fermentation time, h) and X₂ and X₃ showed significant linear negative effects (β₁x₁x₃ = −0.01x₁x₃ and β₂x₂x₃ = −0.01x₂x₃, respectively) (p<0.05). The other effects were not significant, and the mathematical model Ŷ₄ = 0.08 + 0.01x₁² − 0.01x₁ − 0.01x₃² + 0.01x₁x₂ − 0.01x₁x₃ − 0.01x₂x₃ was developed. The lack-of-fit of the model was not significant (at 95%), and 90% of the experimental data were properly adjusted (R²) to the model. The response surface (Figure 3 A) and desirability parameters (Figure 3 B) suggested a region where Ŷ₄ (Ŷ₄ = 0.11 µmol g⁻¹) was maximal, when obtained by using the following conditions: x₁ = −1 or X₁ = 6 h soaking time; x₂ = −1 or X₂ = 15 min cooking time; and x₃ = +0.5 or X₃ = 30 h fermentation.

Figure 3. Response surfaces (A and C) and desirability parameters (B and D) for the response functions Y₄ (µmol daidzein g⁻¹) and Y₅ (µmol g⁻¹ genistein g⁻¹)
time. This processing conditions did not differ from
the assay 7, in which the experimentally obtained Y 4
was 0.11 µmol of daidzein per g of tempeh (Table 1).
Therefore, according to the equation Ŷ4 in order to
obtain the maximum daidzein content in the tempeh, it
is recommended to use low levels of x1 (−1) or X1 (6 h
of soaking); for x2 (−1) or X2 (15 min of cooking); for
x3 (± 0.5) or at least X3 (30 h of fermentation).

For the Y5 response function (µmol of genistein
per g of tempeh), only the X3 variable (fermentation
time, h) showed significant linear negative effect
(β3x3 = − 0.17x3) (p<0.05). Based only in this significant
variable, the mathematical model Ŷ5 = 1.07 − 0.17x3
was developed. The lack-of-fit of the model was not
significant (at 95%), and 86% of the experimental data
were properly adjusted (R2) to the model. The response
surface (Figure 3 C) and desirability parameters
(Figure 3 D) suggested that the maximum genistein
content (Ŷ 5 = 1.35 µmol g⁻¹) was obtained when using
the following conditions: x1 = +1 or X1 = 18 h of soaking
time; x2 = 0 or X2 = 20 min of cooking time; and x3 = − 1
or X3 = 18 h of fermentation time. This estimated result
of the maximum genistein content coincided with
the assay 7 (Y5 = 1.35 µmol g⁻¹) (Table 1). Therefore,
according to the equation Ŷ5, in order to obtain a higher
genistein content in tempeh, it is recommended to use
the lower level condition (x3 = − 1 or 18 h fermentation
time), which was independent of the X1 or X2 variables.

Based on the confidence interval (CI) of the assay
7 (CI = 1.03 to 1.49), and on the response functions
of the assays 1, 5, 9, 10, 11, 13, 16, and 17 where
x3 = − 1, all response functions were within the interval
of the assay 7, confirming that X3 (soaking time) and
X2 (cooking time) were independent variables to
obtain tempehs with maximum genistein content. In
the grains, 'BRS 267' showed only 0.33 µmol g⁻¹. For
tempeh obtained in the assay 7, the aglycone levels
(0.11 µmol daidzein g⁻¹ and 1.35 µmol genistein g⁻¹)
increased by 4.4 times, mainly due to the fermentation
of cotyledons.

The processing variables for tempeh preparation
changed the level, profile, and distribution of the
different isoflavone forms (Table 1). However, when
comparing tempeh with cooked soybean grains,
tempeh showed a 57% reduction in the content of
glucosides, and the aglycone levels increased 6.5 fold
due to the action of β-glucosidase produced by the
fungus during fermentation (Wang & Murphy, 1996).

The cooking of soybean grains does not change the
profile or distribution of the isoflavones, but it causes a
drastic reduction in malonylglucosides. The isoflavone
malonyl forms are unstable at high temperatures and
are converted into their respective β-glycosides forms,
due to the cleavage of malonyl ester groups, to daidzin
and genistin forms. Kudou et al. (1991) reported that
the content of malonylglucosides increased, after 12 to
24 h of fermentation. Bavia et al. (2012) observed an
increase of 78% in the content of isoflavone aglycones
(from 27.22 to 82.72 mg 100g⁻¹ of tempeh) in tempehs
prepared from different soybean cultivars. The authors
also observed an increase of 15.01 to 26.74 mg 100g⁻¹
in tempeh by soaking for 17 h, cooking for 30 min,
and fermenting for 26 h. In the present work, the
fermentation for 24 h resulted in the conversion of
glycosides isoflavones and in the increase of the
concentration of the corresponding aglycones, which
agrees with other studies (Nakajima et al., 2005; Haron
et al., 2009; Ferreira et al., 2011; Villares et al., 2011).
However, in the process of fermentation, despite the
nonsignificant losses of isoflavones, the β-glucosidase
enzyme hydrolyzes β-glucosides forms to aglycones,
which have high biological activity and are the
isoflavones readily available to humans (Izumi et al.,
2000).

Therefore, the models established the following
conditions to obtain tempeh with maximum content
of different isoflavone forms, which would favor its
classification as a functional food: x1 (− 1), for genistin;
x1, x2, and x3 (− 1, − 1, and 0), for malonyl; x1, x3, and
x3 (− 1), for daidzein; and x3 (− 1), for genistein. To
achieve the maximum content of different isoflavone
forms, including aglycones, it is recommended to
use the minimal amount of time for the processing
variables, which corresponded to the assay 1, as
follows: 6 h of soaking, 15 min of cooking and 18 h of
fermentation.

Conclusions
1. In the preparation of soybean tempeh, the
processing variables including soaking, cooking, and
fermentation times with Rhizopus oligosporus change
the level, profile, and distribution of the different
isoflavone forms.
2. The highest bioconversion of β-glycoside
isoflavones into aglycones occurs when soybean grains
are soaked for 6 h, cotyledons are cooked for 15 min,
and the fermentation with R. oligosporus lasts for 18 h.

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