



## Research Article

# An Approach to Processing More Bioavailable Chickpea Milk by Combining Enzymolysis and Probiotics Fermentation

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This research aimed to investigate an approach to processing more bioavailable chickpea milk by combining enzymolysis and probiotic bacterial fermentation. The regression model of three factors was established using Box–Behnken design (BBD), and the optimum technology of enzymolysis of isoflavone in specimens was determined. Moreover, the variations in isoflavone concentrations in chickpea milk processed with different enzymolysis conditions were explored during fermentation. The isoflavone content was the highest (246.18 mg/kg) when the doses of papain,  $\alpha$ -amylase, and  $\beta$ -glucosidase were 75.0 U/g protein, 69.0 U/g starch, and 11.0 U/g chickpea flour. In addition, the contents of isoflavone glucosides decreased and aglycones increased with the prolongation of fermentation. Compared with group C<sub>0</sub> (unhydrolyzed specimens), the isoflavone aglycone contents in groups treated with enzymolysis increased to varying degree. Particularly, the isoflavone aglycone contents in group C<sub>6</sub> (hydrolyzed with three compound enzymes) were the highest after 24 h fermentation, reaching  $56.93 \pm 1.61$  mg/kg (genistein),  $92.37 \pm 3.21$  mg/kg (formononetin), and  $246.18 \pm 2.98$  mg/kg (biochanin A). The data above indicated that compound enzymolysis coupled probiotic bacterial fermentation could promote the biotransformation of chickpea isoflavone glucosides into aglycones, which might be used as an effective approach to enhance the bioactivity and nutraceutical properties of chickpea milk.

## 1. Introduction

Chickpea (*Cicer arietinum* L.) is the second highest yielding pulse in the world [1]. It is also a cheap source of plant proteins with excellent functional features [2], including extraordinary emulsifying and foaming characteristics, trophic value, and wide practicality [3–5]. Chickpea milk is a new type of alimental drink with high carbohydrate, protein, and isoflavone contents and zero cholesterol [6, 7]. Unlike other plant-based milk, it does not cause allergies. Chickpea milk has received noticeable attention because it can alleviate the pressure of stock farming on the environment [8–10]. Hence, our team [7, 11] and others [6, 12] investigated an approach to prepare unfermented and/or fermented chickpea milk with sensory and physicochemical characteristics similar to those of its soymilk counterpart [1, 13, 14].

Isoflavones are one of the main active ingredients in chickpea milk. They are a class of natural flavonoids with phytoestrogen activity [15] and a secondary metabolic product of polyphenols. The isoflavones of chickpea milk exist mainly in four chemical forms including glucosides (genistin, ononin, and biochanin A- $\beta$ -D-glucoside) and aglycones (biochanin A, genistein, and formononetin) [2, 12]. Among them, biochanin A is the major isoflavone with the highest content [16], accounting for about 30% of the total isoflavones [12]. A number of previous studies have documented that isoflavones possess numerous health advantages, such as anticancer function; antioxidant activity; and prevention of atherosclerosis, cardiovascular disorders, and osteoporosis [15, 17–21]. According to the structure-activity relationship, the isoflavone structure itself is a limiting element for absorption in the gastric enteric canal [17, 22, 23]. The chemical forms of isoflavones

affect the absorption degree, with aglycones being more easily absorbed and more bioavailable compared with polar conjugated species [17, 22, 23]. Unfortunately, about 80% of natural isoflavones in legume products present as glucoside-combined forms with less bioactivity [15, 24, 25]. Only a small proportion are bioavailable and bioactive aglycones [15, 23, 26, 27]. Previous literature has revealed that it is feasible to change the glucoside/aglycone rate in legume food by using probiotic bacterial fermentation with  $\beta$ -glucosidase function [15, 25, 28, 29]. However, the influence of enzymolysis coupled fermentation on isoflavone profile in chickpea milk is poorly understood. Limited information is available about the variation of isoflavone contents and bioconversion in chickpea milk with enzymolysis coupled fermentation. Hence, the variations in isoflavone contents and bioconversion of chickpea milk with enzymolysis coupled fermentation needs to be explored.

The addition of enzymes affords a number of benefits in food processing: exceptionally pronounced matrix specificity; high response rate under mild response circumstances; and fast, continuous, and easily controlled response process with usually modest functional costs and investment [30]. Papain,  $\alpha$ -amylase, and  $\beta$ -glucosidase are usually utilized as hydrolase enzymes, which play a crucial role in enhancing the quality of chickpea milk [7, 30]. Generally, papain is used for the liquefaction of proteins to make products with good quality, and  $\alpha$ -amylase is used to accelerate starch degradation [30], both of which could promote the proliferation of probiotics and gut microbes with  $\beta$ -glucosidase activity [15, 25].  $\beta$ -Glucosidase possesses a capability for cleaving  $\beta$ -glycosidic bonds, thereby facilitating the bioconversion of isoflavone glucosides into aglycones with biological effects [15, 23, 31]. On this basis, chickpea milk was prepared with single and compound enzymes to determine the optimum technology of enzymolysis of specimens. The effects of enzymolysis coupled fermentation on isoflavone contents and bioconversion were explored for the first time. Hence, the aim of this research was to afford an optional and reasonable method for enhancing the bioactivity of plant-based milk utilizing chickpea as raw substance.

## 2. Materials and Methods

**2.1. Materials.** Untoasted chickpeas (Desi) were obtained from a local market (Zhengzhou, China). Papain ( $10^4$  U/g) was from Pang Bo Biotechnology Co., Ltd (Nanning, China).  $\alpha$ -Amylase ( $4 \times 10^4$  U/g) and  $\beta$ -glucosidase ( $3 \times 10^2$  U/g) were obtained from Jiangsu Rui Yang Biotechnology Co., Ltd (Jiangsu, China). Genistin, genistein, ononin, and formononetin standards were from Yuan Ye Biotechnology Co., Ltd (Shanghai, China). Biochanin A standard was from Aladdin. All other reagents were of chromatographic grade and obtained from Solarbio Co., Ltd (Beijing, China).

### 2.2. Chickpea Milk Enzymolysis Coupled Fermentation

**2.2.1. The Flow Diagram of the Treatment of Fermented Chickpea Milk (FCM).** The preparation methods were in accordance to our previous report [7] as follows:

Chickpea  $\rightarrow$  soaking overnight with a pulse water mass ratio 1:2  $\rightarrow$  triturating with 1:9 (w/v) water  $\rightarrow$  boiled for 12 min  $\rightarrow$  chickpea milk acquired  $\rightarrow$  enzymolysis at  $50^\circ\text{C}$  (30 min)  $\rightarrow$  enzyme inactivation at  $95^\circ\text{C}$  (5 min)  $\rightarrow$  cultivated at  $38^\circ\text{C}$  (24 h)  $\rightarrow$  refrigerating at  $4^\circ\text{C}$  (12 h).

**2.2.2. Key Points of Preparation.** The specimens were pre-treated based on our laboratory methods [7, 11] with several modifications. Chickpea milk was equally divided into seven groups. The first group comprised chickpea milk without enzymolysis and was labeled as  $C_0$ . Papain at a ratio of 80 U/g<sub>protein</sub> was added into the second group, and this specimen was treated by enzymolysis. Next, enzyme inactivation was conducted at  $95^\circ\text{C}$  (5 min), and this specimen was labeled as  $C_1$ .  $\alpha$ -Amylase at a ratio of 60 U/g<sub>starch</sub> was added into the third group, the remaining steps were as described above, and this specimen was labeled as  $C_2$ .  $\beta$ -Glucosidase at a ratio of 9 U/g<sub>chickpea flour</sub> was added into the fourth group, the remaining steps were as described above, and this specimen was labeled as  $C_3$ .  $\alpha$ -Amylase plus  $\beta$ -glucosidase at a ratio of 60 U/g<sub>starch</sub> and 9 U/g<sub>chickpea flour</sub> were added into the fifth group, the remaining steps were as described above, and this specimen was labeled as  $C_4$ . Papain plus  $\alpha$ -amylase and  $\beta$ -glucosidase mixture treatment (on the basis of the single-factor assay) at a ratio of 80 U/g<sub>protein</sub>, 60 U/g<sub>starch</sub>, and 9 U/g<sub>chickpea flour</sub> were added into the sixth group, the remaining steps were as described above, and this specimen was labeled as  $C_5$ . Papain plus  $\alpha$ -amylase and  $\beta$ -glucosidase mixture treatment (on the basis of response surface assay) at a ratio of 75 U/g<sub>protein</sub>, 69 U/g<sub>starch</sub>, and 11 U/g<sub>chickpea flour</sub> were added into the seventh group, the remaining steps were as described above, and this specimen was labeled as  $C_6$ .

**2.2.3. Fermentation Process of Specimens.** The culture conditions and means were carried out as per our previous literature [7]. The starter (constituents: *Lactocaseibacillus rhamnosus* CICC 20257, Beijing, China) was blended into the chickpea milk. Chickpea milk samples were incubated (DHS-500BS, Yue Jin, China) at  $38^\circ\text{C}$  for 24 h and then refrigerated at  $4^\circ\text{C}$  (12 h).

### 2.3. Single-Factor Assay

**2.3.1. Effect of Papain Dosage on the Contents of Isoflavone (IS) in FCM.** The utilization of selected temperature: time combination for hydrolysis, incubation, and inactivation for each enzyme were based on pre-experiment of our laboratory and the study of Yang [32]. Papain at a ratio of 20 U/g<sub>protein</sub>, 40 U/g<sub>protein</sub>, 60 U/g<sub>protein</sub>, 80 U/g<sub>protein</sub>, and 100 U/g<sub>protein</sub> was added into chickpea milk. Then, the

TABLE 1: Experimental results from process optimization for content of isoflavone.

| Run | Coded variable levels |                               |                                  | Content of isoflavone (mg/kg) |
|-----|-----------------------|-------------------------------|----------------------------------|-------------------------------|
|     | A<br>papain dosage    | B<br>$\alpha$ -amylase dosage | C<br>$\beta$ -glucosidase dosage |                               |
| 1   | 0 (70)                | 0 (60)                        | 0 (9)                            | 212.46                        |
| 2   | 1 (80)                | 0 (60)                        | 1 (11)                           | 201.85                        |
| 3   | 1 (80)                | 0 (60)                        | -1 (7)                           | 213.12                        |
| 4   | 0 (70)                | 0 (60)                        | 0 (9)                            | 202.39                        |
| 5   | -1 (60)               | 0 (60)                        | 1 (11)                           | 214.46                        |
| 6   | -1 (60)               | -1 (50)                       | 0 (9)                            | 239.79                        |
| 7   | 0 (70)                | 0 (60)                        | 0 (9)                            | 238.91                        |
| 8   | 0 (70)                | 0 (60)                        | 0 (9)                            | 225.38                        |
| 9   | 0 (70)                | 0 (60)                        | 0 (9)                            | 228.09                        |
| 10  | 0 (70)                | -1 (50)                       | -1 (7)                           | 215.00                        |
| 11  | 1 (80)                | -1 (50)                       | 0 (9)                            | 243.27                        |
| 12  | 0 (70)                | 1 (70)                        | 1 (11)                           | 214.07                        |
| 13  | 1 (80)                | 1 (70)                        | 0 (9)                            | 239.38                        |
| 14  | -1 (60)               | 1 (70)                        | 0 (9)                            | 234.38                        |
| 15  | 0 (70)                | -1 (50)                       | 1 (11)                           | 214.69                        |
| 16  | -1 (60)               | 0 (60)                        | -1 (7)                           | 239.38                        |
| 17  | 0 (70)                | 1 (70)                        | -1 (7)                           | 221.39                        |

specimens were hydrolyzed at 50°C for 30 min. Next, enzyme inactivation was performed at 95°C (5 min). The specimens were cooled to cultivate at 38°C (24 h). The effects of the additive amount of papain on the contents of IS (calculated with the concentrations of biochanin A) in specimens were investigated.

**2.3.2. Effect of  $\alpha$ -Amylase Dosage on the Contents of Isoflavone (IS) in FCM.**  $\alpha$ -Amylase at a ratio of 20 U/g starch, 40 U/g starch, 60 U/g starch, 80 U/g starch, and 100 U/g starch was added into chickpea milk. Then, the specimens were hydrolyzed at 50°C for 30 min. Next, enzyme inactivation was performed at 95°C (5 min). The specimens were cooled to cultivate at 38°C (24 h). The effects of the additive amount of  $\alpha$ -amylase on the contents of IS (calculated with the concentrations of biochanin A) in specimens were investigated.

**2.3.3. Effect of  $\beta$ -Glucosidase Dosage on the Contents of Isoflavone (IS) in FCM.**  $\beta$ -Glucosidase at a ratio of 3 U/g chickpea flour, 6 U/g chickpea flour, 9 U/g chickpea flour, 12 U/g chickpea flour, and 15 U/g chickpea flour was added into chickpea milk. Then, the specimens were hydrolyzed at 50°C for 30 min. Next, enzyme inactivation was performed at 95°C (5 min). The specimens were cooled to cultivate at 38°C (24 h). The effects of the additive amount of  $\beta$ -glucosidase on the contents of IS (calculated with the concentrations of biochanin A) in specimens were investigated.

**2.4. Response Surface Assay.** On the basis of single-factor experiments, a 17-run BBD including 5 axile points and 12 factorial points was utilized to analyze the optimal technological parameters according to the report of [33, 34]. Three variables including A (the additive doses of papain), B

(the additive doses of  $\alpha$ -amylase), and C (the additive doses of  $\beta$ -glucosidase) and three levels (coded 1, 0, and -1) are shown in Table 1. The second-order polynomial equation is as follows:

$$Y = \beta_0 + \sum_{i=1}^3 \beta_i X_i + \sum_{i=1}^3 \beta_{ii} X_i^2 + \sum_{i=1}^2 \sum_{j=i+1}^3 \beta_{ij} X_i X_j, \quad (1)$$

where  $Y$  is the response variable associated with different levels of each factor;  $\beta_0$ ,  $\beta_i$ ,  $\beta_{ii}$ , and  $\beta_{ij}$  are the regression coefficients representing intercept, linearity, square, and interactivity, separately;  $X_i$  and  $X_j$  represent independent variables ( $i \neq j$ ) [34, 35].

**2.5. Variation of Constituent and Content of IS in Specimens during Fermentation.** On the basis of single-factor and response surface assay, the optimum enzymolysis parameters of single and combined additive amounts of papain,  $\alpha$ -amylase, and  $\beta$ -glucosidase were obtained. Next, the specimens prepared according to the methods in Section 2.2 were withdrawn at 0, 3, 6, 9, 12, and 24 h to further investigate the variation of constituent and concentration of isoflavone.

## 2.6. Index Assay of FCM

### 2.6.1. Analysis of Biochanin a

**(1) Preparation of Specimens.** The isoflavones were extracted by serial procedure in line with the modified approach [25]. Briefly, a certain mass of FCM (5.0 g) was measured and blended with 50 mL of 90% methanol. Then, it was ultrasonically extracted (KQ-500B ultrasonic extractor with 300W power, Kunshan Instruments Co., Ltd) at 60°C for 20 min and centrifuged at 10,000g (10 min). The supernatant was concentrated in a rotational evaporator at 50°C, and

the residue was extracted twice with 90% methanol (50 mL). Next, the extracting supernatants were combined to concentrate at a final volume of 30 mL. This concentrated solution was transferred to a 50-mL volumetric flask and diluted with 90% methanol to volume. The above solution was filtered with a 0.45- $\mu\text{m}$  filtering membrane and utilized for determination [12, 36].

(2) *Preparation of Biochanin A Standard and Establishment of Standard Curve.* According to the report of Wang [33], a certain quantity of biochanin A standard (5 mg) was measured and added into a 50-mL beaker. It was dissolved with 90% methanol by ultrasound and transferred to a 50-mL volumetric flask. Next, this standard solution was filled with methanol to volume and shaken up to prepare a standard solution of 100  $\mu\text{g}/\text{mL}$ .

About 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, and 2.0 mL of biochanin A standard solution (100  $\mu\text{g}/\text{mL}$ ) were drawn off and placed into a 10-mL volumetric flask. Then, these standard solutions were diluted with 90% methanol to volume and agitated well to prepare standard working solutions with concentrations of 6, 8, 10, 12, 14, 16, 18, and 20  $\mu\text{g}/\text{mL}$ . The absorbance value was measured at 261 nm with 90% methanol as blank control. The standard curve obtained was  $Y = 0.1797X + 0.0204$ ,  $R^2 = 0.998$ .

Using 90% methanol as blank, the absorbance of the extract acquired from specimens was determined with a UV spectrophotometer (TU-1910, PERSEE, Beijing) at 261 nm utilizing the same approach in Section 2.2.2. The contents of biochanin A in the specimens were calculated as follows:

$$X = C \times \frac{V}{m} \times \text{dilution ration}, \quad (2)$$

where  $X$  represents the content of biochanin A in the specimens (mg/kg);  $C$  represents the concentration of biochanin A acquired from the standard curve ( $\mu\text{g}/\text{mL}$ );  $V$  represents the final constant volume of specimen solution (mL); and  $m$  mass of the specimen (g).

2.6.2. *Determination of Isoflavones in FCM during Fermentation by HPLC.* According to the literature of [25, 37] with several modifications, quantification of five chickpea isoflavones in FCM during fermentation was carried out by HPLC. HPLC assay was performed with Alliance System (Waters, USA), utilizing a C-18 column (25.0 cm  $\times$  4.6 mm, 5.00  $\mu\text{m}$ , Phenomenex, Inc., USA) and UV detector. The mobile phase consisted of 0.1% acetic acid in water (A) and 0.1% acetic acid in acetonitrile (B). A was washed out following a gradient of 90% to 70% for 15 min, 70% to 60% for 10 min, 60% to 90% for 20 min, and steady at 90% for 10 min. The flow rate was 1.0 mL $\cdot\text{min}^{-1}$ , and the column oven temperature was 30.0°C. Detection was performed at 260 nm. Depending on the retention time of per isoflavone substance, the recognizable peaks were tracked on the HPLC diagrams, and the isoflavone contents were quantified utilizing the corresponding standard curves. The data were expressed as mg/kg.

2.7. *Statistical Assay.* SPSS 26.0 (Armonk, NY) was utilized for statistical assay. The data were expressed as mean  $\pm$  standard deviation ( $p < 0.05$ ). Origin 2021 and Design-Expert 12.0 were utilized to estimate the response surface results.

### 3. Results and Discussion

#### 3.1. Assay of Single-Factor Test

3.1.1. *Effect of Papain Dosage on the Concentration of Isoflavone in FCM.* Figure 1 shows the effect of papain dosage on the concentration of isoflavone in FCM. With the increase of papain dosage, the isoflavone concentration in FCM first increased and then decreased. The group added with 80 U/g<sub>protein</sub> of papain had the highest isoflavone concentration, reaching 161.2 mg/kg ( $p < 0.05$ ). Previous literature reported the isoflavone-protein compounds might be shaped by setting up hydrophobic interactions [37, 38], whereas isoflavones combined with chickpea protein could be released via appropriate enzymolysis by papain [39, 40]. However, when the additive amount was higher than 80 U/g<sub>protein</sub>, the content of hydrolyzed substrate (FCM) remained unchanged. Isoflavones had been completely dissolved and released due to papain dosage reaching saturation. There was no practical significance to further increase the dosage of papain. Hence, the optimum additive amount of papain was 80 U/g<sub>protein</sub>.

3.1.2. *Effect of  $\alpha$ -Amylase Dosage on the Concentration of Isoflavone in FCM.* Figure 2 shows that the effect of  $\alpha$ -amylase dosage on isoflavone concentration was similar to that of papain dosage. With the increase of  $\alpha$ -amylase dosage, the amount of isoflavone continued to increase from 105.62 mg/kg (0.0 U/g<sub>starch</sub> of  $\alpha$ -amylase) to 167.89 mg/kg (60.0 U/g<sub>starch</sub> of  $\alpha$ -amylase) in FCM. The rise in isoflavone concentration was attributable to the decomposition of large molecular substances (e.g., starch into small molecular compounds, i.e., oligosaccharides through  $\alpha$ -amylase, thus promoting the proliferation of *Lactobacillus rhamnosus* which had the ability to produce a high  $\beta$ -glucosidase functioning acquiring sufficient hydrolysis of glucoside isoflavones) [41]. As the  $\alpha$ -amylase dosage increased continuously (exceeding 60.0 U/g<sub>starch</sub>), the isoflavone concentration continued to decrease ( $p < 0.05$ ). It might be hypothesized that with the increase of  $\alpha$ -amylase dosage, the starch in specimens was completely hydrolyzed, generating small molecule cyclodextrin, which possessed the embedding effect on isoflavone. Moreover, the activity of  $\alpha$ -amylase decreased rapidly with decreasing degree of polymerization of the matrix [30], resulting in the reduction in isoflavone concentration.

3.1.3. *Effect of  $\beta$ -Glucosidase Dosage on the Concentration of Isoflavone in FCM.* Figure 3 shows that the isoflavone concentration in FCM first increased and then decreased when the  $\beta$ -glucosidase dosage increased. Interestingly, there was no remarkable change in isoflavone concentration when the  $\beta$ -glucosidase dosage increased from

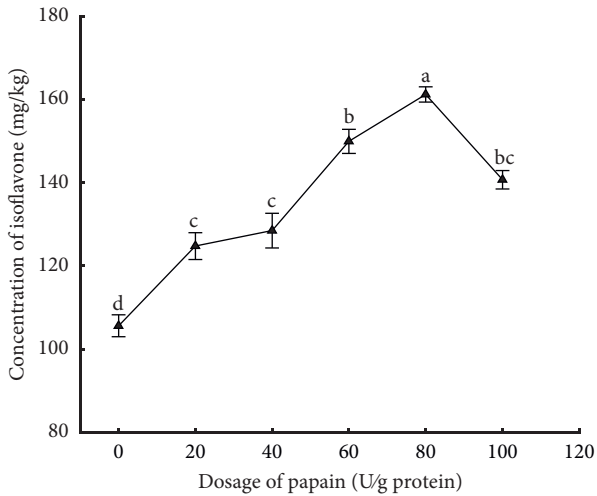


FIGURE 1: Effect of papain dosage on the content of isoflavone (mg/kg) in FCM. Means with different lowercase letters indicate significant differences of the same specimen at the different papain dosage ( $p < 0.05$ ).

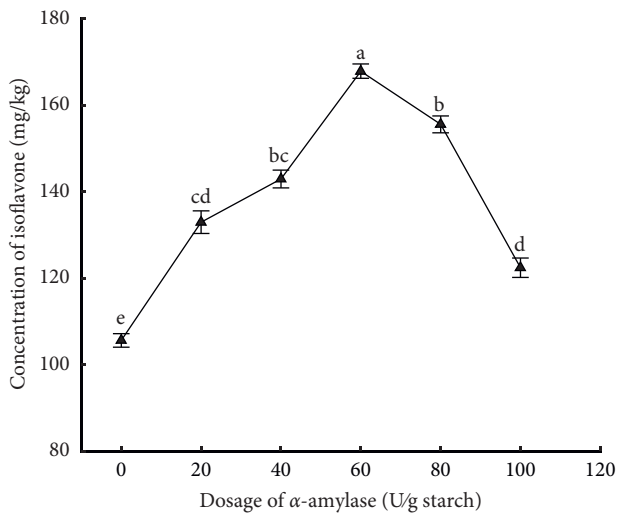


FIGURE 2: Effect of  $\alpha$ -amylase dosage on the content of isoflavone (mg/kg) in FCM. Means with different lowercase letters indicate significant differences of the same specimen at the different  $\alpha$ -amylase dosage ( $p < 0.05$ ).

6 U/g chickpea flour to 12 U/g chickpea flour. The highest isoflavone concentration (157.87 mg/kg) was found in the group with  $\beta$ -glucosidase dosage of 9 U/g chickpea flour. However, as the  $\beta$ -glucosidase dosage exceeded 9 U/g chickpea flour, the isoflavone content in the FCM specimen significantly decreased ( $p < 0.05$ ). This finding indicated that hydrolysis with appropriate amount of  $\beta$ -glucosidase could accelerate cell wall rupture, thus promoting the dissolution of isoflavones in specimens. However, when the amount of  $\beta$ -glucosidase added was too high, the excessive concentration of enzyme could result in excessive isoflavone degradation, which reduced the yield of isoflavones. This result was similar to the finding by Cho et al. [17]. Hence, the optimum additive amount of  $\beta$ -glucosidase was about 12 U/g chickpea flour.

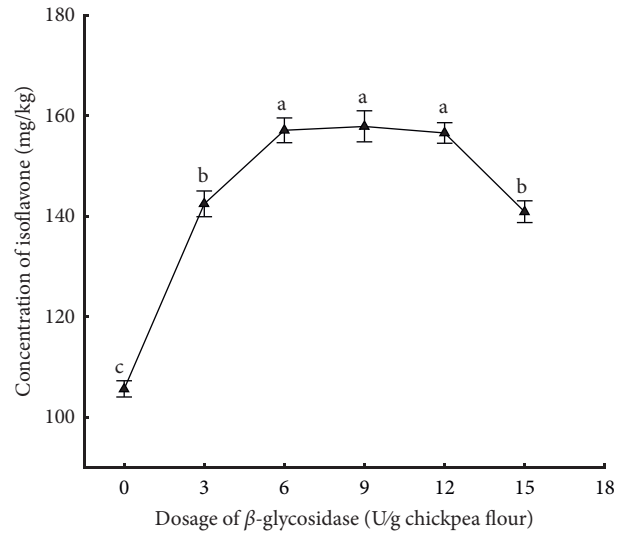


FIGURE 3: Effect of  $\beta$ -glucosidase dosage on the content of isoflavone (mg/kg) in FCM. Means with different lowercase letters indicate significant differences of the same specimen at the different  $\beta$ -glucosidase dosage ( $p < 0.05$ ).

### 3.2. Establishment of the Response Surface Model and Optimization Assay

3.2.1. *Establishment of the Response Surface Model and Quadratic Regression Fitting.* Design-Expert 12.0 was utilized to perform the three-factor and three-level experimental design. The additive amounts of papain (A),  $\alpha$ -amylase (B), and  $\beta$ -glucosidase (C) were adopted as independent variables, and the concentration of biochanin A (Y) was adopted as the response value. The experimental results are shown in Table 1. Quadratic multiple regression assay was conducted on the test data in Table 2, and the regression equation was as follows:

$$Y = 119.57 + 0.3888A + 0.5125B + 0.7463C + 0.355AB - 0.6925AC + 1.57BC - 4.3A^2 - 3.29B^2 - 1.11C^2, \quad (3)$$

where Y represents the response variable associated with each factor at different levels (the concentration of biochanin A); A, B, and C represent the additive amount of papain,  $\alpha$ -amylase, and  $\beta$ -glucosidase, respectively.

3.2.2. *Variance Analysis of the Response Surface Model.* The significance analysis of the regression model (Table 2) shows that the variance assay of this model reached an extremely significant level ( $F = 83.55$  and  $p < 0.0001$ ). The lack of fit was 0.3068, indicating that the fitting effect of this model was appropriate. The determination coefficient of this model ( $R^2$ ) was 0.9908, which represented that the theoretical value fitted by Box-Behnken design could reflect 99.08% of the real value, suggesting that the regression model was statistically significant. The modified  $R^2_{Adj}$  and predicted correlation coefficient  $R^2_{Pred}$  were 0.9789 and 0.9607, respectively, and the results were similar, indicating that the predicted values possessed a good correlation with

TABLE 2: Assay of variance for the fitted quadratic model of isoflavone content.

| Source                        | Sum of squares | Df | Mean square | F value | P-value   |
|-------------------------------|----------------|----|-------------|---------|-----------|
| Model                         | 160.19         | 9  | 17.8        | 83.55   | <0.0001** |
| A                             | 1.21           | 1  | 1.21        | 5.68    | 0.0487*   |
| B                             | 2.10           | 1  | 2.10        | 9.86    | 0.0164*   |
| C                             | 4.46           | 1  | 4.46        | 20.91   | 0.0026**  |
| AB                            | 0.5041         | 1  | 0.5041      | 2.37    | 0.1679    |
| AC                            | 1.92           | 1  | 1.92        | 9.00    | 0.0199*   |
| BC                            | 9.86           | 1  | 9.86        | 46.28   | 0.0003**  |
| A <sup>2</sup>                | 77.95          | 1  | 77.95       | 365.91  | <0.0001** |
| B <sup>2</sup>                | 45.58          | 1  | 45.58       | 213.96  | <0.0001** |
| C <sup>2</sup>                | 5.21           | 1  | 5.21        | 24.47   | 0.0017**  |
| Residual                      | 1.49           | 7  | 0.2130      |         |           |
| Lack of fit                   | 0.2790         | 3  | 0.0930      | 0.3068  | 0.8204    |
| Pure error                    | 1.21           | 4  | 0.3031      |         |           |
| Cor total                     | 161.68         | 16 |             |         |           |
| R <sup>2</sup>                | 0.9908         |    |             |         |           |
| R <sup>2</sup> <sub>Adj</sub> | 0.9789         |    |             |         |           |
| CV (%)                        | 0.3997%        |    |             |         |           |
| Adeq precision                | 22.9923        |    |             |         |           |

Note. significant at  $p < 0.01$  for \*\*, significant at  $p < 0.05$  for \*.

the experimental values. The signal-to-noise ratio (Adeq precision = 22.9923) was large, and the variation coefficient (CV = 0.3997%) was low, which indicated that the model could be used to predict the isoflavone concentration in specimens in practice. Moreover, according to variance analysis, the order of influence of the additive amount of three enzymes on isoflavone concentration was  $C > B > A$ .

**3.2.3. Two-Factor Interaction.** The significance analysis of regression model coefficients (Table 2) showed that the interaction terms AC and BC were significant ( $p < 0.05$ ); that is, the pairwise interaction of the additive amounts of papain and  $\beta$ -glucosidase,  $\alpha$ -amylase, and  $\beta$ -glucosidase had a significant effect on the isoflavone concentration in FCM. Contour lines were oval and dense, and the response surface of interaction is presented in Figure 4.

The effects of papain and  $\beta$ -glucosidase dosage on isoflavone concentration in FCM are illustrated in Figures 4(a) and 4(b). Within a certain dosage range (75.0 U/g protein of papain and 11.0 U/g chickpea flour of  $\beta$ -glucosidase), the isoflavone concentration in FCM specimens increased with the increase of papain and  $\beta$ -glucosidase dosage. However, the isoflavone concentration decreased with the continuous increase of papain and  $\beta$ -glucosidase dosage. We hypothesized that the synergistic effect of  $\beta$ -glucosidases and papain could not only destroy the cell wall, but also make the solvent quickly penetrate into the cell membrane, increasing the mass transfer rate and promoting the transformation and release of isoflavones [30]. However, an excessive additive amount of these two enzymes would degrade the dissolved isoflavones into smaller fragments and reduce their contents [25].

The effects of  $\alpha$ -amylase and  $\beta$ -glucosidase dosage on isoflavone concentration in FCM are illustrated in Figures 4(c) and 4(d). The isoflavone contents in FCM

specimens increased significantly in a certain dosage range of  $\alpha$ -amylase and  $\beta$ -glucosidase (60.0 U/g starch of  $\alpha$ -amylase and 11.0 U/g chickpea flour of  $\beta$ -glucosidase).  $\alpha$ -Amylase combined with  $\beta$ -glucosidase could synergistically destroy the cell wall, degrade starch and cellulose, and accelerate the fermentation process, which was beneficial to the transformation and release of isoflavone. However, when the additive amount of  $\alpha$ -amylase and  $\beta$ -glucosidase exceeded saturation, the content of isoflavone decreased.

Through deciphering the equation of regression and analyzing the response surface contour plots, the optimal process conditions for FCM enzymatic hydrolysis were 75.06 U/g protein of papain, 68.59 U/g starch of  $\alpha$ -amylase, and 11.06 U/g chickpea flour of  $\beta$ -glucosidase, at which the highest isoflavone concentrations were obtained, reaching 246.19 mg/kg. To validate the sufficiency of the model equation, the verification test was performed under optimal actual conditions (75.0 U/g protein of papain, 69.0 U/g starch of  $\alpha$ -amylase, and 11.0 U/g chickpea flour of  $\beta$ -glucosidase). As a result, an actual isoflavone content of 246.18 mg/kg was acquired, which was not remarkably different from the forecasted value. Hence, the model is appropriate for the prediction of isoflavone content.

**3.3. Isoflavone Biotransformation.** On the basis of single-factor and response surface tests, the enzymolysis of isoflavone from glucosides to aglycones was observed during chickpea milk fermentation, in which the additive amounts of enzymes were referenced to the same method in Section 2.2.2. Substrate content variations were analyzed, and data are presented in Tables 3–9.

As demonstrated in Table 3, with longer incubation, the contents of genistin and ononin decreased continuously, and the contents reduced to 47.95 and 49.94 mg/kg after 24 h fermentation, respectively. However, the contents of genistein, formononetin, and biochanin A continued to increase, and the contents increased to 37.96, 39.96, and 112.37 mg/kg after 24 h fermentation, separately. Among these, formononetin was a type of aglycone isoflavone. During chickpea milk fermentation, *Lactobacillus rhamnosus* with  $\beta$ -glucosidase function could convert the glycoside isoflavone into aglycone [41]. Hence, formononetin decreased at 12 h in comparison to 24 h. We speculated that  $\beta$ -glucosidase was secreted by the metabolism and proliferation of lactic acid bacteria. Glucosidase isoflavones were continuously degraded into corresponding aglycone isoflavones via enzymolysis. This result was agreeing with the research of [12].

As shown in Table 4, the variation trend of genistin and ononin contents in group C<sub>1</sub> was similar to that in group C<sub>0</sub>, the contents of which were lower than those in group C<sub>0</sub> after 24 h fermentation. For group C<sub>1</sub>, the contents of genistein, formononetin, and biochanin A increased continuously; 4.15%, 31.48%, and 33.60% increases were observed after 24 h incubation compared with group C<sub>0</sub>. This phenomenon may be due to the fact that proteins possess the capacity to bond isoflavone substances [30]. On the basis of enzymolysis of chickpea milk, the conjugated compound of



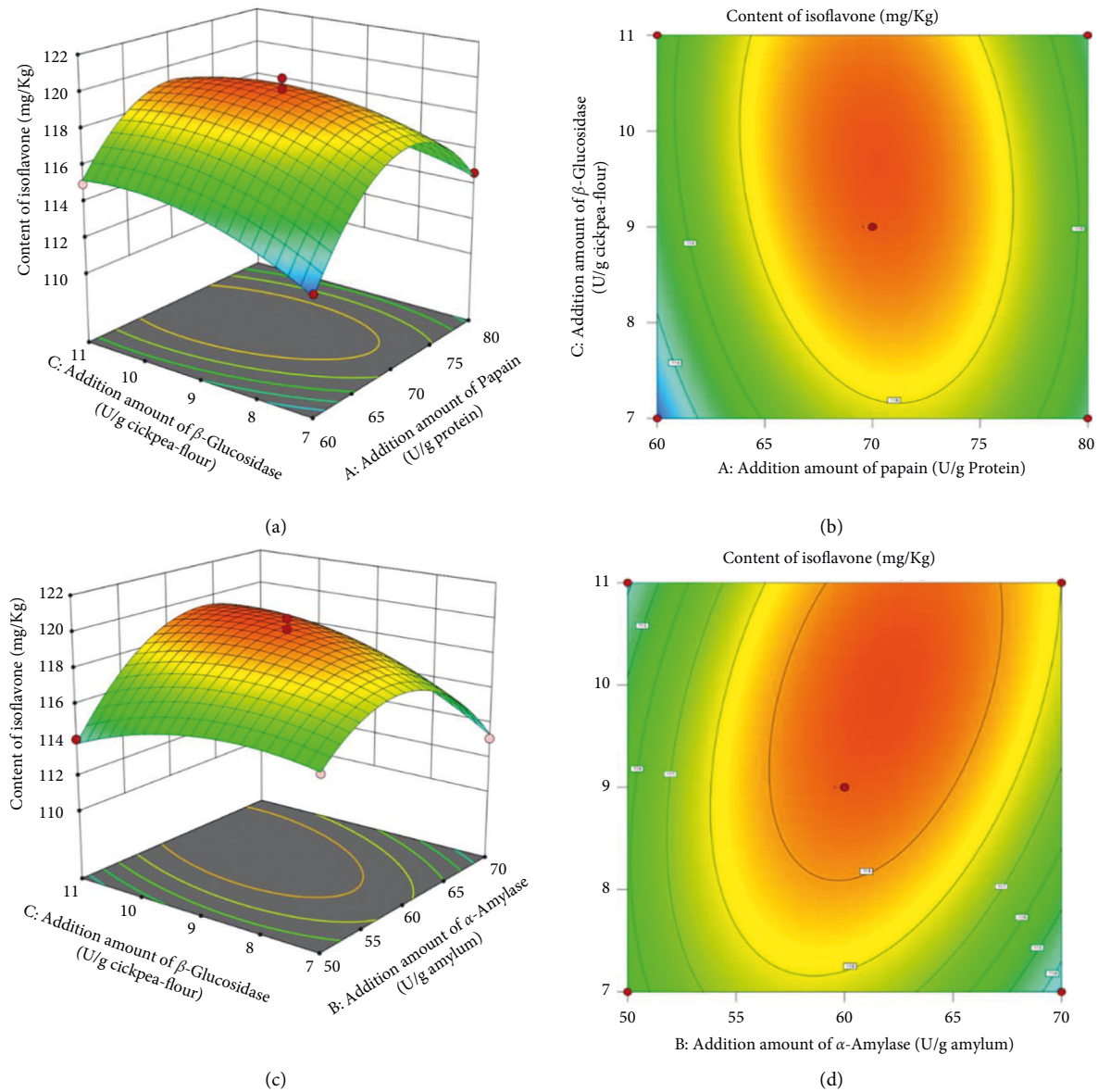


FIGURE 4: Interaction of different enzyme additions on the content of isoflavone. A, addition amount of papain ( $U/g_{\text{protein}}$ ); B, addition amount of  $\alpha$ -amylase ( $U/g_{\text{starch}}$ ); C, addition amount of  $\beta$ -glycosidase ( $U/g_{\text{chickpea flour}}$ ). (a) Response surface diagram of interaction between papain and  $\beta$ -glycosidase addition levels. (b) Contour map of interaction between papain and  $\beta$ -glycosidase addition levels. (c) Response surface diagram of interaction between  $\alpha$ -amylase and  $\beta$ -glycosidase addition levels. (d) Contour map of interaction between  $\alpha$ -amylase and  $\beta$ -glycosidase addition levels.

isoflavone protein retained by protein might be liberated over the course of fermentation, and further produce oligopeptides and amino acids, which could promote the transformation of isoflavone glucosides into aglycones. This finding was in alignment with the research of [25].

As illustrated in Table 5, the variation trend of genistin and ononin contents in group  $C_2$  was similar to those of groups  $C_0$  and  $C_1$ , the contents of which decreased to 39.41 and 44.90 mg/kg, respectively. The contents of genistein, formononetin, and biochanin A increased to 41.90, 54.87, and 163.62 mg/kg, respectively, which were higher than those of aglycone isoflavones in groups  $C_0$  and  $C_1$ . This is

because  $\alpha$ -amylase degrades starch in chickpea milk and generates oligosaccharides, promoting the metabolism and proliferation of lactic acid bacteria to release  $\beta$ -glucosidase, and accelerating the conversion isoflavone glucosides into aglycones.

As shown in Table 6, with longer fermentation (24 h), the contents of aglycone isoflavones in group  $C_3$  were substantially higher than those in groups  $C_0$ ,  $C_1$ , and  $C_2$ . We hypothesized that the cell walls were damaged via  $\beta$ -glucosidase during hydrolysis, resulting in solvent penetrating into the cell membrane more rapidly, improving the mass transfer rate, and promoting the bioconversion and liberation of glucoside isoflavone to aglycone.

TABLE 3: Variations in isoflavone (IS) concentrations (mg/kg) of group C<sub>0</sub> during fermentation process.

| IS/(mg/kg)   | Fermentation time         |                            |                            |                             |                            |                            |
|--------------|---------------------------|----------------------------|----------------------------|-----------------------------|----------------------------|----------------------------|
|              | 0 h                       | 3 h                        | 6 h                        | 9 h                         | 12 h                       | 24 h                       |
| Genistin     | 73.79 ± 5.50 <sup>a</sup> | 70.08 ± 0.08 <sup>ab</sup> | 62.91 ± 4.22 <sup>bc</sup> | 53.99 ± 5.76 <sup>cd</sup>  | 50.93 ± 1.53 <sup>d</sup>  | 47.95 ± 2.83 <sup>d</sup>  |
| Genistein    | 9.97 ± 0.02 <sup>c</sup>  | 12.52 ± 3.55 <sup>de</sup> | 19.97 ± 0.02 <sup>cd</sup> | 24.99 ± 7.02 <sup>bc</sup>  | 31.45 ± 2.04 <sup>ab</sup> | 37.96 ± 2.82 <sup>a</sup>  |
| Ononin       | 77.29 ± 3.68 <sup>a</sup> | 70.08 ± 0.08 <sup>b</sup>  | 62.91 ± 4.25 <sup>c</sup>  | 58.99 ± 1.52 <sup>cd</sup>  | 53.92 ± 1.54 <sup>de</sup> | 49.94 ± 0.01 <sup>e</sup>  |
| Formononetin | 19.95 ± 0.04 <sup>c</sup> | 28.03 ± 2.80 <sup>bc</sup> | 32.45 ± 6.36 <sup>ab</sup> | 37.49 ± 3.46 <sup>ab</sup>  | 34.96 ± 7.14 <sup>ab</sup> | 39.96 ± 0.01 <sup>a</sup>  |
| Biochanin A  | 79.78 ± 0.16 <sup>d</sup> | 86.59 ± 9.10 <sup>cd</sup> | 94.86 ± 7.08 <sup>bc</sup> | 104.97 ± 6.87 <sup>ab</sup> | 109.84 ± 0.26 <sup>a</sup> | 112.37 ± 3.51 <sup>a</sup> |

Note. all the values were expressed as mean ± SD ( $n = 3$ ). different minuscule letters indicated significant difference of five group standards in comparison to the same line of values at different fermentation time ( $p < 0.05$ ). C<sub>0</sub>, fermented chickpea milk without enzymolysis (control specimen).

TABLE 4: Variations in isoflavone (IS) concentrations (mg/kg) of group C<sub>1</sub> during fermentation process.

| IS/(mg/kg)   | Fermentation time         |                            |                             |                            |                            |                            |
|--------------|---------------------------|----------------------------|-----------------------------|----------------------------|----------------------------|----------------------------|
|              | 0 h                       | 3 h                        | 6 h                         | 9 h                        | 12 h                       | 24 h                       |
| Genistin     | 71.32 ± 2.10 <sup>a</sup> | 66.97 ± 1.39 <sup>b</sup>  | 58.89 ± 1.36 <sup>c</sup>   | 55.28 ± 0.56 <sup>c</sup>  | 48.96 ± 1.35 <sup>d</sup>  | 43.54 ± 2.14 <sup>c</sup>  |
| Genistein    | 10.47 ± 0.71 <sup>d</sup> | 15.49 ± 2.13 <sup>d</sup>  | 21.96 ± 2.84 <sup>c</sup>   | 26.90 ± 4.30 <sup>bc</sup> | 30.97 ± 1.45 <sup>ab</sup> | 39.53 ± 2.11 <sup>a</sup>  |
| Ononin       | 75.82 ± 5.67 <sup>a</sup> | 68.97 ± 1.39 <sup>ab</sup> | 61.89 ± 2.87 <sup>bc</sup>  | 54.77 ± 6.90 <sup>cd</sup> | 52.45 ± 3.47 <sup>cd</sup> | 47.54 ± 3.52 <sup>d</sup>  |
| Formononetin | 24.94 ± 7.05 <sup>c</sup> | 32.99 ± 4.25 <sup>bc</sup> | 37.93 ± 2.85 <sup>abc</sup> | 42.84 ± 9.97 <sup>ab</sup> | 44.96 ± 7.12 <sup>ab</sup> | 52.54 ± 3.51 <sup>a</sup>  |
| Biochanin A  | 82.30 ± 3.50 <sup>c</sup> | 94.96 ± 7.10 <sup>d</sup>  | 117.28 ± 3.43 <sup>c</sup>  | 131.97 ± 3.87 <sup>b</sup> | 142.37 ± 3.35 <sup>a</sup> | 150.13 ± 0.07 <sup>a</sup> |

Note. all the values were expressed as mean ± SD ( $n = 3$ ). different minuscule letters indicated significant difference of five group standards in comparison to the same line of values at different fermentation time ( $p < 0.05$ ). C<sub>1</sub>, fermented chickpea milk with papain hydrolysis.

TABLE 5: Variations in isoflavone (IS) concentrations (mg/kg) of group C<sub>2</sub> during fermentation process.

| IS/(mg/kg)   | Fermentation time         |                            |                            |                            |                            |                            |
|--------------|---------------------------|----------------------------|----------------------------|----------------------------|----------------------------|----------------------------|
|              | 0 h                       | 3 h                        | 6 h                        | 9 h                        | 12 h                       | 24 h                       |
| Genistin     | 70.03 ± 0.09 <sup>a</sup> | 64.33 ± 1.82 <sup>a</sup>  | 56.88 ± 4.11 <sup>b</sup>  | 45.96 ± 5.64 <sup>c</sup>  | 41.35 ± 0.73 <sup>c</sup>  | 39.41 ± 0.63 <sup>c</sup>  |
| Genistein    | 12.00 ± 2.81 <sup>c</sup> | 19.95 ± 0.09 <sup>bc</sup> | 24.96 ± 4.29 <sup>b</sup>  | 33.97 ± 5.66 <sup>a</sup>  | 40.85 ± 1.44 <sup>a</sup>  | 41.90 ± 1.49 <sup>a</sup>  |
| Ononin       | 72.53 ± 3.63 <sup>a</sup> | 66.83 ± 4.54 <sup>ab</sup> | 59.88 ± 0.13 <sup>bc</sup> | 51.96 ± 2.84 <sup>cd</sup> | 47.33 ± 3.55 <sup>d</sup>  | 44.90 ± 7.14 <sup>d</sup>  |
| Formononetin | 27.01 ± 4.21 <sup>d</sup> | 34.89 ± 6.89 <sup>cd</sup> | 38.92 ± 1.32 <sup>bc</sup> | 46.96 ± 1.40 <sup>ab</sup> | 49.82 ± 0.03 <sup>ab</sup> | 54.87 ± 6.95 <sup>a</sup>  |
| Biochanin A  | 87.53 ± 3.43 <sup>c</sup> | 104.74 ± 7.54 <sup>d</sup> | 124.76 ± 7.34 <sup>c</sup> | 139.89 ± 0.04 <sup>b</sup> | 154.44 ± 6.95 <sup>a</sup> | 163.62 ± 5.33 <sup>a</sup> |

Note. all the values were expressed as mean ± SD ( $n = 3$ ). different minuscule letters indicated significant difference of five group standards in comparison to the same line of values at different fermentation time ( $p < 0.05$ ). C<sub>2</sub>, fermented chickpea milk with  $\alpha$ -amylase treatment.

TABLE 6: Variations in isoflavone (IS) concentrations (mg/kg) of group C<sub>3</sub> during fermentation process.

| IS/(mg/kg)   | Fermentation time         |                            |                            |                            |                            |                            |
|--------------|---------------------------|----------------------------|----------------------------|----------------------------|----------------------------|----------------------------|
|              | 0 h                       | 3 h                        | 6 h                        | 9 h                        | 12 h                       | 24 h                       |
| Genistin     | 66.89 ± 4.11 <sup>a</sup> | 60.92 ± 1.37 <sup>ab</sup> | 55.97 ± 1.50 <sup>b</sup>  | 43.49 ± 2.01 <sup>c</sup>  | 35.99 ± 5.56 <sup>cd</sup> | 33.52 ± 4.92 <sup>d</sup>  |
| Genistein    | 15.36 ± 0.84 <sup>d</sup> | 21.47 ± 2.10 <sup>c</sup>  | 26.49 ± 2.16 <sup>c</sup>  | 38.99 ± 1.32 <sup>b</sup>  | 45.50 ± 3.66 <sup>a</sup>  | 48.03 ± 2.88 <sup>a</sup>  |
| Ononin       | 68.36 ± 0.79 <sup>a</sup> | 62.92 ± 4.28 <sup>ab</sup> | 54.97 ± 4.32 <sup>b</sup>  | 43.50 ± 2.23 <sup>c</sup>  | 44.00 ± 5.77 <sup>c</sup>  | 36.02 ± 2.80 <sup>c</sup>  |
| Formononetin | 29.72 ± 0.27 <sup>c</sup> | 36.95 ± 4.21 <sup>bc</sup> | 42.48 ± 3.47 <sup>b</sup>  | 54.99 ± 0.14 <sup>a</sup>  | 55.99 ± 5.51 <sup>a</sup>  | 61.04 ± 1.47 <sup>a</sup>  |
| Biochanin A  | 99.08 ± 0.89 <sup>e</sup> | 114.85 ± 7.14 <sup>d</sup> | 134.94 ± 7.27 <sup>c</sup> | 149.98 ± 0.38 <sup>b</sup> | 165.00 ± 7.51 <sup>a</sup> | 175.10 ± 6.91 <sup>a</sup> |

Note. all the values were expressed as mean ± SD ( $n = 3$ ). different minuscule letters indicated significant difference of five group standards in comparison to the same line of values at different fermentation time ( $p < 0.05$ ). C<sub>3</sub>, fermented chickpea milk with  $\beta$ -glucosidase treatment.

Table 7 shows that when the incubation time was up to 24 h, the isoflavone glucoside contents in group C<sub>4</sub> decreased, whereas the isoflavone aglycone contents increased remarkably. This was probably due to the fact that the viscosity of chickpea milk decreased (liquefaction) via synergistic hydrolysis by  $\alpha$ -amylase plus  $\beta$ -glucosidase [30] and that starch and cellulose were degraded into small molecular substances (i.e., oligosaccharides and dextrans). The exposure of glycosidic bonds increased the probability of substrate in contact with two compound enzymes, promoted the cleavage of glycosidic bonds, and further accelerated the bioconversion of isoflavone glucoside to the aglycone.

A comparison between Tables 8 and 9 revealed that with the prolongation of fermentation, the isoflavone glucoside contents in group C<sub>5</sub> were higher than those in group C<sub>6</sub>, whereas the aglycone isoflavone contents were lower than those in group C<sub>6</sub>. The data indicated that through the synergistic hydrolysis of three compound enzymes, increasing the dosage of  $\alpha$ -amylase and  $\beta$ -glucosidase could accelerate the penetration of solvent, hence promoting the cleavage of glycosidic bonds, and increasing the conversion of glycosidic isoflavones to aglycones. As previously reported, the anticancer and antioxidation activities of legumes have been ascribed to isoflavones, particularly



TABLE 7: Variations in isoflavone (IS) concentrations (mg/kg) of group C<sub>4</sub> during fermentation process.

| IS/(mg/kg)   | Fermentation time           |                             |                            |                            |                            |                            |
|--------------|-----------------------------|-----------------------------|----------------------------|----------------------------|----------------------------|----------------------------|
|              | 0 h                         | 3 h                         | 6 h                        | 9 h                        | 12 h                       | 24 h                       |
| Genistin     | 63.43 ± 0.82 <sup>a</sup>   | 57.98 ± 2.87 <sup>a</sup>   | 48.95 ± 1.40 <sup>b</sup>  | 36.47 ± 4.97 <sup>c</sup>  | 34.49 ± 2.11 <sup>cd</sup> | 29.99 ± 0.02 <sup>d</sup>  |
| Genistein    | 16.98 ± 1.38 <sup>d</sup>   | 21.99 ± 2.81 <sup>d</sup>   | 32.97 ± 0.01 <sup>c</sup>  | 45.96 ± 2.85 <sup>b</sup>  | 47.49 ± 3.56 <sup>ab</sup> | 51.98 ± 1.37 <sup>a</sup>  |
| Ononin       | 64.93 ± 7.18 <sup>a</sup>   | 59.97 ± 0.04 <sup>ab</sup>  | 49.95 ± 0.01 <sup>bc</sup> | 39.96 ± 0.02 <sup>cd</sup> | 37.49 ± 3.52 <sup>d</sup>  | 33.98 ± 5.63 <sup>d</sup>  |
| Formononetin | 34.95 ± 7.00 <sup>d</sup>   | 44.98 ± 7.03 <sup>cd</sup>  | 52.44 ± 3.54 <sup>bc</sup> | 62.44 ± 3.49 <sup>ab</sup> | 64.99 ± 7.04 <sup>ab</sup> | 69.97 ± 0.05 <sup>a</sup>  |
| Biochanin A  | 118.85 ± 12.49 <sup>d</sup> | 133.44 ± 4.85 <sup>cd</sup> | 144.85 ± 7.09 <sup>c</sup> | 167.34 ± 3.64 <sup>b</sup> | 192.46 ± 3.62 <sup>a</sup> | 199.92 ± 0.16 <sup>a</sup> |

Note. all the values were expressed as mean ± SD ( $n = 3$ ). different minuscule letters indicated significant difference of five group standards in comparison to the same line of values at different fermentation time ( $p < 0.05$ ). C<sub>4</sub>, fermented chickpea milk with  $\alpha$ -amylase plus  $\beta$ -glucosidase mixture treatment.

TABLE 8: Variations in isoflavone (IS) concentrations (mg/kg) of group C<sub>5</sub> during fermentation process.

| IS/(mg/kg)   | Fermentation time          |                             |                             |                            |                            |                            |
|--------------|----------------------------|-----------------------------|-----------------------------|----------------------------|----------------------------|----------------------------|
|              | 0 h                        | 3 h                         | 6 h                         | 9 h                        | 12 h                       | 24 h                       |
| Genistin     | 59.71 ± 0.08 <sup>a</sup>  | 54.42 ± 2.12 <sup>a</sup>   | 47.93 ± 2.87 <sup>b</sup>   | 32.52 ± 2.20 <sup>c</sup>  | 29.91 ± 0.01 <sup>cd</sup> | 24.94 ± 4.19 <sup>d</sup>  |
| Genistein    | 21.39 ± 2.08 <sup>c</sup>  | 24.96 ± 1.41 <sup>c</sup>   | 34.95 ± 2.79 <sup>b</sup>   | 48.52 ± 2.01 <sup>a</sup>  | 49.35 ± 2.10 <sup>a</sup>  | 53.38 ± 4.85 <sup>a</sup>  |
| Ononin       | 62.20 ± 3.60 <sup>a</sup>  | 46.43 ± 4.94 <sup>b</sup>   | 40.94 ± 1.45 <sup>b</sup>   | 29.01 ± 1.35 <sup>c</sup>  | 24.92 ± 7.04 <sup>c</sup>  | 19.96 ± 0.03 <sup>c</sup>  |
| Formononetin | 42.29 ± 3.46 <sup>c</sup>  | 54.92 ± 7.06 <sup>d</sup>   | 59.91 ± 0.06 <sup>cd</sup>  | 70.03 ± 0.16 <sup>bc</sup> | 74.78 ± 7.07 <sup>ab</sup> | 82.32 ± 3.39 <sup>a</sup>  |
| Biochanin A  | 130.36 ± 1.57 <sup>d</sup> | 144.79 ± 7.06 <sup>cd</sup> | 157.77 ± 11.15 <sup>c</sup> | 185.07 ± 6.66 <sup>b</sup> | 214.35 ± 7.11 <sup>a</sup> | 221.53 ± 2.45 <sup>a</sup> |

Note. all the values were expressed as mean ± SD ( $n = 3$ ). different minuscule letters indicated significant difference of five group standards in comparison to the same line of values at different fermentation time ( $p < 0.05$ ). C<sub>5</sub>, fermented chickpea milk with papain plus  $\alpha$ -amylase and  $\beta$ -glucosidase mixture treatment (on the basis of one-way assay).

TABLE 9: Variations in isoflavone (IS) concentrations (mg/kg) of group C<sub>6</sub> during fermentation process.

| IS/(mg/kg)   | Fermentation time          |                            |                            |                            |                             |                            |
|--------------|----------------------------|----------------------------|----------------------------|----------------------------|-----------------------------|----------------------------|
|              | 0 h                        | 3 h                        | 6 h                        | 9 h                        | 12 h                        | 24 h                       |
| Genistin     | 57.93 ± 1.26 <sup>a</sup>  | 52.46 ± 3.46 <sup>b</sup>  | 44.43 ± 2.04 <sup>c</sup>  | 29.02 ± 1.36 <sup>d</sup>  | 25.00 ± 1.47 <sup>d</sup>   | 23.47 ± 0.62 <sup>c</sup>  |
| Genistein    | 22.97 ± 1.47 <sup>c</sup>  | 25.98 ± 1.45 <sup>c</sup>  | 36.94 ± 1.48 <sup>b</sup>  | 52.04 ± 2.92 <sup>a</sup>  | 57.00 ± 2.70 <sup>a</sup>   | 56.93 ± 1.61 <sup>a</sup>  |
| Ononin       | 59.92 ± 0.16 <sup>a</sup>  | 44.96 ± 7.01 <sup>b</sup>  | 39.94 ± 0.07 <sup>b</sup>  | 27.52 ± 3.49 <sup>c</sup>  | 14.99 ± 7.04 <sup>d</sup>   | 9.99 ± 0.03 <sup>d</sup>   |
| Formononetin | 39.95 ± 0.11 <sup>c</sup>  | 64.96 ± 7.15 <sup>b</sup>  | 62.40 ± 3.65 <sup>b</sup>  | 72.55 ± 3.67 <sup>b</sup>  | 85.01 ± 7.26 <sup>a</sup>   | 92.37 ± 3.21 <sup>a</sup>  |
| Biochanin A  | 155.82 ± 8.89 <sup>d</sup> | 164.88 ± 6.85 <sup>d</sup> | 184.71 ± 7.40 <sup>c</sup> | 215.15 ± 7.46 <sup>b</sup> | 231.01 ± 1.94 <sup>ab</sup> | 246.18 ± 2.98 <sup>a</sup> |

Note. all the values were expressed as mean ± SD ( $n = 3$ ). different minuscule letters indicated significant difference of five group standards in comparison to the same line of values at different fermentation time ( $p < 0.05$ ). C<sub>6</sub>, fermented chickpea milk with papain plus  $\alpha$ -amylase and  $\beta$ -glucosidase mixture treatment (on the basis of response surface assay).

genistein, one type of aglycone isoflavone [15]. Treatment of genistein illustrated cancer repression by lessening cell multiplication, migration, and intrusion and eliciting apoptosis [15, 42]. Also, lots of researches have been conducted on the fermentation of legume foods to improve the bioavailability of legume-isoflavones utilizing probiotic bacteria, for example, *L.acidophilus*, *L. casei*, *Lactobacillus plantarum*, *Lactobacillus fermentum*, *Bifidobacterium animalis* subsp. *lactis* and *Bifidobacterium longum*  $\beta$ -glucosidases of harboring endogenous [15, 43, 44]. Therefore, the bioactivity and bioavailability of isoflavones could be enhanced through probiotic bacterial fermentation coupled with compound enzymolysis [15].

#### 4. Conclusion

On the basis of single-factor assay and Box–Behnken design, the regression model of enzymatic treatment was established using three factors of papain,  $\alpha$ -amylase, and  $\beta$ -glucosidase dosages as the independent variables and isoflavone content

as the dependent variable. The optimum technology of enzymolysis of isoflavone in specimens was determined. Moreover, the variations in isoflavone concentrations in chickpea milk processed with different enzymolysis conditions were explored during fermentation. The isoflavone content was the highest (246.18 mg/kg) when the doses of papain,  $\alpha$ -amylase, and  $\beta$ -glucosidase were 75.0 U/g protein, 69.0 U/g starch, and 11.0 U/g chickpea flour. In addition, the contents of isoflavone glucosides decreased and aglycones increased with the prolongation of fermentation. Compared with group C<sub>0</sub> (unhydrolyzed specimens), the isoflavone aglycone contents in groups treated with enzymolysis increased to varying degree. Particularly, the isoflavone aglycone contents in group C<sub>6</sub> (hydrolyzed with three compound enzymes) were the highest after 24 h fermentation, reaching 56.93 ± 1.61 mg/kg (genistein), 92.37 ± 3.21 mg/kg (formononetin), and 246.18 ± 2.98 mg/kg (biochanin A). The above results inferred that compound enzymolysis coupled with probiotic fermentation could provide a reference for the industrial processing of chickpea milk.

## Data Availability

The data that support the findings of this study can be obtained from the corresponding author upon reasonable request.

## Ethical Approval

This study does not involve any human or animal testing.

## Conflicts of Interest

The authors declare no conflicts of interest.

## Authors' Contributions

Xue Zhang conceptualized (equal) the study, curated the data (equal), investigated (equal) the study, and wrote, reviewed, and edited the original draft (lead); Shuangbo Liu wrote the original draft (equal) and curated the data (equal). Bijun Xie developed the methodology (supporting). Zhida Sun supervised (lead) the study.

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## Supplementary Materials

In the manuscript, Graphical abstract was used in the supplementary materials. (*Supplementary Materials*)

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