STUDY OF HYDROLYSIS EFFICIENCY OF ISOFLAVONES FROM SOY GERM EXTRACTS BY CELLULASE ENZYME

NGHIÊN CỨU KHẢO SÁT HIỆU SUẤT THỦY PHÂN CÁC ISOFLAVONE TỪ DỊCH CHIẾT PHÔI ĐẬU TƯƠNG BẰNG ENZYME CELLULASE

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ABSTRACT

Soy germ is a rich source of isoflavones with many benefits for human health. Isoflavones have anti-oxidant, anti-cancer, anti-microbial, and anti-inflammatory properties, and improve female physiology. These benefits of isoflavones are determined by the aglycone form while the aglycone isoflavone content is only about 2 - 5% of the total isoflavones. This study deals with the use of cellulase to hydrolyze glucoside isoflavones into aglycone forms. Choosing the appropriate enzyme and regimen will help improve the hydrolysis efficiency of isoflavone glucosides. The result showed that with cellulase enzyme at pH 5, temperature 50°C, the content of enzyme is 1.5U/g for 5 hours, the efficiency of hydrolysis of β-glucosides; malonyl glucosides, acetyl glucosides are 95.84%; 64.82; 89.11%, respectively. Total algycones content at 5 hours was 35.91 ± 0.03μmol/g dried weight (including 16.67 ± 0.02μmol daidzein; 5.70 ± 0.02μmol glycitein; 13.54 ± 0.01μmol genistein).

Keywords: *Soy germ, hydrolysis, enzyme.*

TÓM TẮT

Phôi đậu tương là nguồn isoflavone dồi dào với nhiều lợi ích cho sức khỏe con người. Các isoflavone có khả năng chống oxy hóa, chống ung thư, chống vi sinh vật, chống viêm, cải thiện sinh lý nữ. Lợi ích này của các isoflavone là do các dạng aglycone quyết định trong khi hàm lượng các isoflavone aglycone chỉ chiếm khoảng 2 - 5% so với lượng isoflavone tổng số. Nghiên cứu này đề cập tới việc khảo sát chế độ thủy phân tối ưu khi sử dụng enzyme cellulase để thủy phân các isolavone dạng glucoside thành dạng aglycone. Kết quả cho thấy với enzyme cellulase tại pH 5, nhiệt độ 50 °C, nồng độ enzyme 1,5U/g; thời gian 5 giờ cho hiệu suất thủy phân dạng β-glucoside; malonyl glucoside, acetyl glucoside lần lượt là 95,84%; 64,82; 89,11%. Lượng các algycone tổng số thu được lớn nhất tại chế độ tối ưu là là 35,91 ± 0,03μmol/g CK (gồm 16,67 ± 0,02μmol daidzein; 5,70 ± 0,02μmol glycitein; 13,54 ± 0,01μmol genistein).

Từ khóa: *Phôi đậu tương, thủy phân, enzyme.*

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1. INTRODUCTION

Isoflavone is an isomer of flavone, which is chromone substituted with a phenyl group in the 2-position. In isoflavone, the phenyl group is in the 3-position [1, 2]. There are 4 kinds of isoflavones in soybean: aglycone isoflavones (genistein, daidzein, and glycitein), glucoside isoflavones (genistin, daidzin, glycitin), acetyl glucoside isoflavones (acetyl genistin, acetyl daidzin, and acetyl glycitin), and malonyl glucoside isoflavones (malonyl genistin, malonyl daidzin, and malonly glycitin) [3]. The chemical forms of these isoflavones would affect their biological activities. However, since the low content of aglycones in total isoflavones (2 - 5%), aglycones seem keep the role key, especially the genistein and daidzein exhibit higher biological activity than others. Isoflavone glucosides can convert to aglycones by acid HCl, HNO_3, enzymes and microorganisms. Enzymatic agents become more common and safer for environment than chemical agents [4]. Enzymes β-glucosidase from almond is very popular in hydrolysis isoflavone glucosides, now a day some reports showed that β-galactosidase and other enzymes which contain β-glucosidase, for example, cellulase can convert β-glucoside isoflavones too, but can not convert acetyl and malonyl forms [3]. In this study, all 12 isoflavones of soy germ extract were analyzed to clarify the hydrolytic effect of cellulase on all glucoside forms.

2. MATERIALS AND METHODS

2.1. Materials

Soy germ with a size of 0.1mm was supplied by Vinanusoy Viet Nam Co. Ltd, cellulase from *Trichoderma* with a content of β-glucosidase 28U/ml was purchased from Sigma (C2730). Isoflavones standards (Wako Pure Chemical Industries Ltd.) were used for HPLC analysis. Other reagents were of analytical grade and were purchased from Fisher Scientific (USA).

2.2. Methods

Soy germ was defatted using 95% n-hexane at a solid/liquid ratio of 1:5 and was shaken at 180 rpm for 5

hours. The defatted soy germ with a moisture content of 5.79 ± 0.09% was packed in a dark glass grinder and stored at -4°C until further analysis. The content of total isoflavones in the defatted soy germ was 1748.01 ± 8.25mg/100g (40.80 ± 0.17µmol/g).

Preparation of total isoflavones extract from soy germ

The total isoflavones extraction was conducted as follows: defatted soy germ flours were added with 65% ethanol, at pH 9, and the solid/liquid ratio was 1:12; extraction time was 90 minutes. The liquid extract was separated from insoluble fractions by filtration. The extraction was then evaporated under a vacuum at 45 °C [5, 6].

Method of β-glucosidase enzyme activity analysis

β-glucosidase activity was determined according to Ghose [7] with some improvements by measuring the hydrolysis rate of 15mmol of cellobiose. One unit of enzyme activity was defined as the amount of β-glucosidase that releases 1µmol of glucose per minute. Glucose was measured using a D-glucose Assay Kit (GOPOD Format) - Megazyme.

Hydrolysis of soy germ extract by enzyme cellulase

The enzyme β-glucosidase hydrolyzed glycoside isoflavones to aglycone forms. The extract of total isoflavones from 2g of defatted soy germ flour was adjusted to pH 5 using 0.02N HCl and kept at room temperature for an hour. Then, this cloudy suspension was centrifuged at 6000rpm, at 4°C for 10 minutes to remove insoluble matter [3]. The cellulase was added in each sample and investigated at temperature 45, 50, 55, 60°C and pH 4.8; 5.0; 5.2; 5.4; 5.6; 5.8; 6.0 with various concentrations (0.5; 1.0; 1.5; 2.0U/g of defatted soy germ) for 4.0; 4.5; 5.0; 5.5; 6.0 hours. The enzyme hydrolysis conversion (%) was defined as follows:

$$\text{Enzymatic hydrolysis conversion (\%)} = \frac{M_0 - M_1}{M_0}.100\% \quad (1)$$

M_0: the content of glycoside forms before enzymatic hydrolysis (µmol/g); M_1: the content of glycoside form after enzymatic hydrolysis (µmol/g)

HPLC analysis

The HPLC analysis method was developed by the National Institute of Nutrition according to the Song method with some improvements [8].

Each test sample (0.5g or 2mL) was added with 10mL of acetonitrile and 2mL of 0.1M HCl, then added with 4mL of deionized water for the flour sample and 2mL of deionized water for the liquid sample with constant shaking on an orbital shaker at ambient temperature. Samples were sonicated for 10 minutes and shaken for two hours at temperatures below 25°C. Each mixture was centrifuged at 5000rpm for 30 minutes, then 1mL of the extract was placed in a test tube and blown dry with N_2. Standards were dissolved in a test tube with 1mL of methanol and sonicated for 10 minutes. Samples were shaken for 1 minute and then filtered through a 0.45µm PTFE membrane before being injected into the HPLC system.

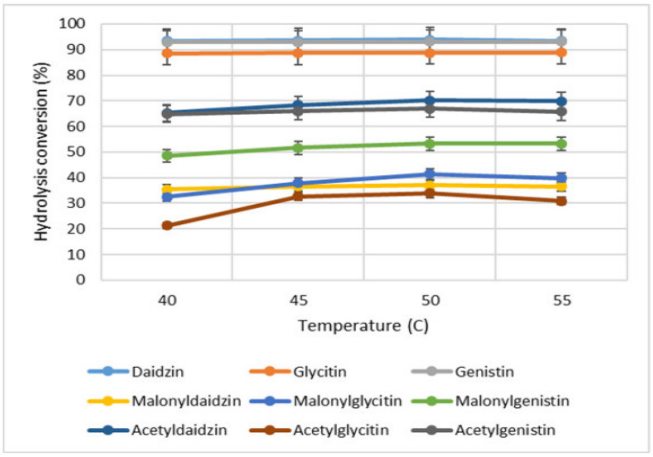
Isoflavones were analyzed by Alliance System, Waters, USA equipped with a Zorbax SB-C18 (5µm × 4.6mm × 150mm). The HPLC conditions were set at a column temperature of 35°C, detection wavelength of 260nm, mobile phases A - 0.1% acetic acid and B - acetic acid/acetonitrile 20/80, flow rate of 1.0mL/min. The detection was carried out under linear gradient elution with mobile phase percentage changing from A 88%, B 12% to A 60%, B 40% and was completed at A 88%, B 12%. The quantification of each isoflavone was performed by integrating the chromatographic peak areas into the calibration curves.

Statistical analysis: All measurements were conducted in triplicate and were statistically analyzed using the analysis of variance (ANOVA). Duncan's multiple range test using the SPSS software program version 25 (SPSS Inc., Chicago, IL, USA) were performed. The significance of the difference was defined at $p < 0.05$.

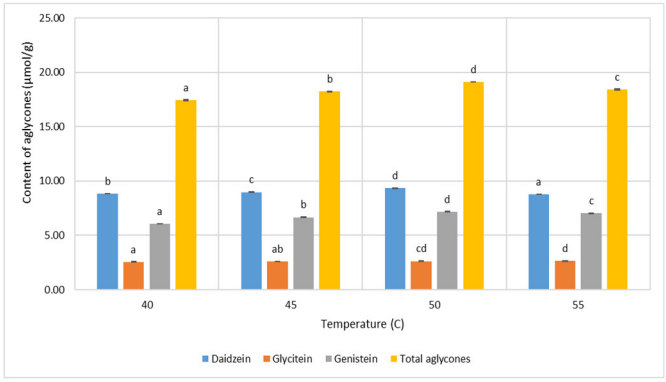
3. RESULTS AND DISCUSSION

3.1. The effect of temperature hydrolysis reaction

The effect of temperature hydrolysis reacion was showed in Figs. 1a and 1b.



(a) The effect of temperature on the hydrolysis rate of isoflavone glucosides



(b) The effect of temperature on the content of isoflavone aglycones (Different letters on the bar are significant at $p<0.05$)

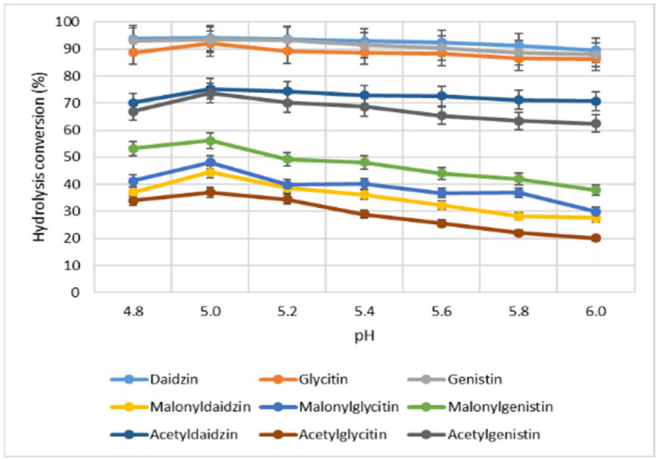
Figure 1. The effect of temperature on isoflavones

From 40 - 55°C, all conjugated forms including β-glucoside, malonyl-glucoside, and acetyl-glucoside were

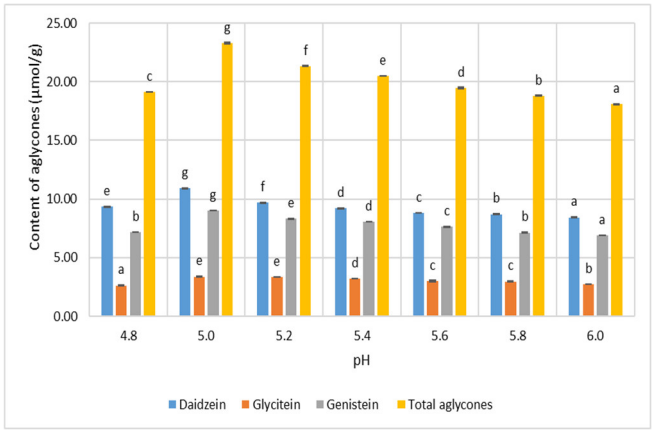
hydrolyzed with different efficiencies. The hydrolysis efficiency of β-glucoside forms is quite high, ranging from about 90%, the highest hydrolysis efficiency at a temperature of 50°C reaches 92.29% with the corresponding hydrolysis efficiency of daidzin, genistin and glycitin being at 85%; 88.71%; 93.10%, respectively. The hydrolysis efficiency of total glucoside forms reaches its maximum value at 50 °C, which is 74.87%. At 50°C, the total aglycones formed reaches a maximum value of 19.12 ± 0.02 (µmol/g) total aglycones includes 9.33 ± 0.02µmol daidzein; 2.62 ± 0.02µmol glycitein and 7.17 ± 0.02µmol genistein.

3.2. The effect of pH hydrolysis reaction

The effect of the pH hydrolysis reaction from 4.8 to 6.0 on isoflavones was showed in Figure 2. In the pH range from 4.8 to 6.0, all glucoside forms are hydrolyzed, the hydrolysis efficiency of glucoside forms reaches the maximum value at pH 5.0 of 78.06%. At this pH, the forms β-glucoside, malonyl-glucoside, and acetyl-glucoside also have the highest hydrolysis efficiency of 93.35%, 53.18%; 73.39% respectively.



(a) The effect of pH on the hydrolysis rate of isoflavone glucosides



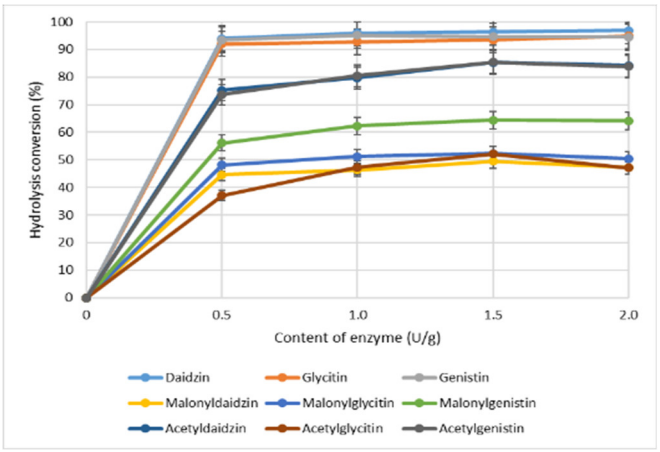
(b) The effect of pH on the content of isoflavone aglycones
(Different letters on the bar are significant at $p < 0.05$)

Figure 2. The effect of pH on isoflavones

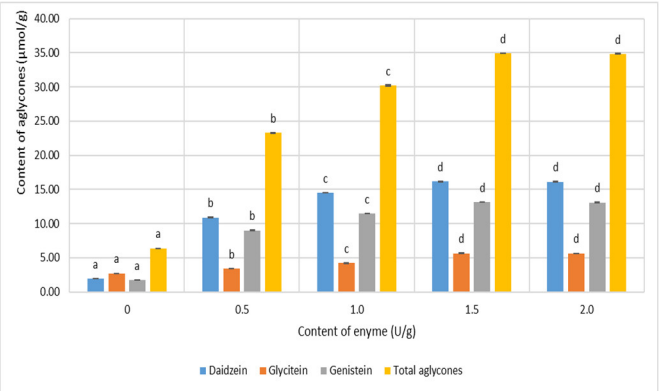
At pH 5.0 the total aglycones formed to reaches a maximum value of 23.29 ± 0.06 (µmol/g) includes 10.90 ± 0.03µmol daidzein; 3.38 ± 0.02µmol glycitein and 9.01 ± 0.02µmol genistein.

3.3. The effect of enzyme concentration

The effect of enzyme concentration on isoflavones was showed in Figure 3. In the investigated enzyme concentration range from 0.5 - 2.0U/g, all glucoside forms were hydrolyzed, the hydrolysis efficiency of glucoside forms reached the maximum value at the enzyme concentration of 1.5U/g. When the enzyme concentration increases from 0 - 1.5U/g, the amount of component aglycone and total aglycone (daidzein, glycitein, genistein) increases and reaches the maximum value at the enzyme concentration of 1.5U/g. Total aglycone content reached 34.94 ± 0.04 (µmol/g CK) including 16.15 ± 0.03µmol daidzein; 5.66 ± 0.03µmol glycitein; 13.12 ± 0.01µmol genistein.



(a) The effect of the content of enzyme on the hydrolysis rate of isoflavone glucosides



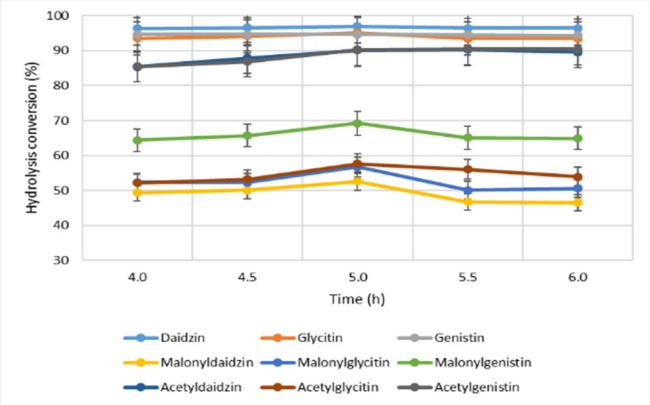
(b) The effect of the content of enzyme on isoflavone aglycones
(Different letters on the bar are significant at p <0.05)

Figure 3. The effect of enzyme concentration on isoflavones

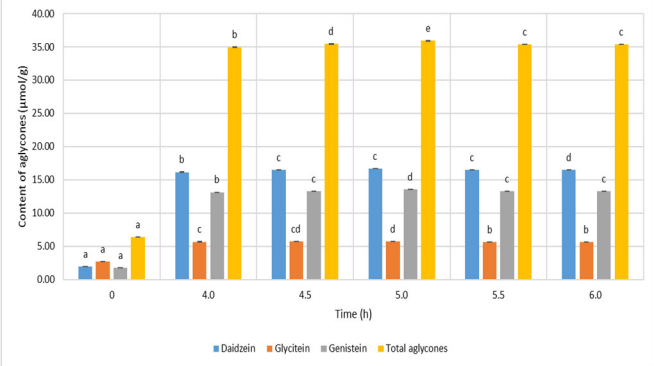
3.4. The effects of incubation time on glucoside conversion

Hydrolysis of total phytoestrogen extract with reaction time conditions of 4.0; 4.5; 5.0; 5.5, and 6.0 (hours). The results of hydrolysis of glucosides and conjugated forms of β-glucoside, malonyl-glucoside, acetyl-glucoside in total isoflavones extracts are as follows: During the survey period from 0 to 4 hours, the hydrolysis efficiency of β-glucoside, malonyl-glucoside, and acetyl-glucoside increased rapidly. After 4 hours, the hydrolysis efficiency of β-glucoside

reached over 95%; malonyl reaches over 60% and acetyl-glucoside reaches over 80%. The content of total algycones at 5 hours reached a maximum value of 35.91 ± 0.03µmol/g (including 16.67 ± 0.02µmol daidzein; 5.70 ± 0.02µmol glycitein; 13.54 ± 0.01µmol genistein).



(a) The effect of incubation time on hydrolysis rate of isoflavone glucosides



(b) The effect of incubation time on the content of isoflavone aglycones (Different letters on the bar are significant at $p < 0.05$)

Figure 4. The effect of incubation time on isoflavones

Using enzymes to hydrolyze isoflavone glucosides in soybeans is safer and more effective than using acid or base agents. Hydrolysis of isoflavone glucosides with 4N HCl acid, or 37% HCl takes place at high temperatures of 80 - 105°C, for a long time from 5 to 12 hours to maximize the efficiency of β-glucoside metabolism. When the hydrolysis time is prolonged, it can lead to a change in the product's odor (sour product phenomenon). In addition, published works on acid hydrolysis of phytoestrogen glucosides did not identify malonyl and acetyl derivative groups, which in fact account for a high proportion in soybeans and seed embryos [9, 10].

Based on the results of hydrolysis of isoflavone glucosides with two enzymes β-glucosidase from almond and cellulase *Trichoderma reesei*, show that using pure enzymes from plants is not as effective as using enzymes from microorganisms. According to Le, at the concentration of β-glucosidase almond is 4U/g, pH 5, 37°C, incubation time of 5.5 hours could reach the hydrolysis isoflavone glucosides at 77.31%. Cellulase *Trichoderma reesei* is seem very effective in hydrolyzing acetyl-glucosides, thereby increasing the total hydrolysis efficiency of glucoside forms by more than 10% [11].

Cellulase is very popular in food isdustry and cheaper than β-glucosidase from almonds. Enzyme from plant can reach high purity at 99% [12], but enzymes from microorganisms often consist of a combination of multiple enzymes [13]. In cellulase from *Trichoderma reesei*, the main enzyme endoglucanase is with activity > 700U/ml; β-glucosidase activity was 28U/ml, and exoglucanase had negligible content. In-depth research on the composition of cellulase enzymes shows that endoglucanase includes 8 types with symbols from EGI-EGVIII, exoglucanases with 2 types: CBH I and CBH II, and 7 β-glucosidase enzymes with symbols from BG I to BG VIII [14]. The relatively rich spectrum of β-glucosidase enzyme from *Trichoderma reesei* is also an advantage for using this enzyme to hydrolyze glucosides. Thus, it can be seen that with the substrate isoflavone glucosides, it is assumed that endoglucanases and exoglucanases also participate in the hydrolysis reaction first, creating conditions for the enzyme β-glucosidase to work, so when using cellulase enzyme with a concentration of β-glucosidases is only 1.5U/g lower than the almond β-glucosidase concentration of 4U/g, we also obtain a larger aglycones content, especially glycitein content.

4. CONCLUSION

The result of this paper can be listed as follows:

1. All conjugated forms including β-glucosides, malonyl-glucosides, and acetyl-glucosides were hydrolyzed with different efficiencies.

2. Cellulase enzymes from *Trichoderma reesei* is very effective in hydrolyzing isoflavone glucosides, and can even hydrolyze glucosides more effectively than using almond β-glucosidase enzyme. Optimal conditions for the enzymatic hydrolysis reaction are temperatures at 50 °C, pH 5 with the content of enzyme of 1.5U/g, incubation time of 5 hours.

3. The content of total algycones at 5 hours reached a maximum value of 35.91 ± 0.03µmol/g (including 16.67 ± 0.02µmol daidzein; 5.70 ± 0.02µmol glycitein; 13.54 ± 0.01µmol genistein).

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