ISOFLAVONES AGLICONE OF TEMPE MALANG FRIED SLICES

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ABSTRACT

Soybean fermentation was carried out by using mixed culture Rhizopus oligosporus C and Rhizopus oryzae L16 (1:1) as inoculum. Incubation places in an incubator at 30 ^{o}C for 5 days and assessment of β-glucosidase activity daily was investigated. Isoflavones aglicone of tempe produced in laboratory scale, tempe commercial and fried slices tempe from Malang were analized by chromatography technique. Bioconversion of isoflavonoida in soybean to isoflavone aglicone compounds can be caused by extracelluler enzyme β -glucosidase. The observation showed that fresh tempe Malang was obtained genistein content 15.57 mg/ 100 g defatted tempe higher than tempe produced from laboratorium scale that was 5.45 mg/ 100 g defatted tempe. However, genistein content of fried slices tempe from Malang content decreased 25%, that was 11.45 mg/ 100 g defatted tempe. Tempe prepared at laboratory had the highest specific activity of β-glucosidase at day two that was 1693 U/g protein.

Keywords: *tempe, Rhizopus oligosporus C, Rhizopus oryzae L16, genistein.*

INTRODUCTION

Isoflavone compounds in tempe are very prospective to be used in the field of pharmaceutical and medicine among others thing as antioxidant, antihaemolisis, anticholesterol and anticancer. Isoflavonoids show a wide range of biological properties. The isoflavones with some known aglycone, form the largest group.The four commonest isoflavones are daidzein (7,4"-dihidroxyisoflavon) dan genistein (5,7,4"-trihidroxy-isoflavon), formononetin and biochanin [1].

Tempe is a popular Indonesian food which is palatable and easily prepared by fermentation of soybean with *Rhizopus* species usually *R.oligosporus* or *R.oryzae*. Over the last decade, the consumption of tempe outside Indonesia has increased significantly [2]. The presence of isoflavone components such as genistein, daidzein, genistin and daidzin in tempe was reported [3]. Genistein was able to stop the growth of new blood capillaries. These findings show that in the aglycone form, isoflavones are more active than the parent glycoside, an effect which is probably due to the increased number of hydroxyl groups in their molecules [4]. Isoflavone glucoside through hydrolysis become isoflavone aglicone (daidzein, genistein, glicytein) and glucose. The presence of enzyme β-glucosidase related with production of daidzein and genistein, which was increased during process of soybean soaked. Hydrolysis of isoflavone glucoside become isoflavone aglycone can be inhibited by glucono δ-lacton, competitive inhibitor for enzyme β-glucosidase [5].

Isoflavones were reported as having various activity, as anti-hemolytic [6], anti oxidant [7], antifungi [8], and anticancer [9]. The activities of isoflavone aglicones were reported stronger compared to their parent compounds. Many researchers have reported their investigations on various isoflavone contents in soybean and its product such as hydrolysed and unhydrolysed soybean meal. The previous analysis indicated that during fermentation with *R. oligosporus* and *R. oryzae*, soybean isoflavone glucosides i.e. daidzin, glycitin and genistin as well as their acetylated compounds were converted into their aglicones namely daidzein, glycitein and genistein [9-11].

The aims of this research was to evaluate the highest isoflavone aglicone content of various tempe especially commercial tempe such as tempe Malang, tempe plastic wrapped, tempe banana leaves wrapped compared with laboratory made tempe, which could produced as second generation tempe.

EXPERIMENTAL SECTION

Material

Dry soybeans used in tempe bought as Brazilia variety of *Glycine max* L which were derived from Bina Kimia Cooperatif–LIPI Bandung. Chemicals used as follows : ethanol 95%, acetic acid, n-hexane, chloroform, potato dextrose agar, ethil acetic, glycine carbonat buffer, H_2SO_4 p.a, Follin Ciocalteau, triton X-100, glycine buffer, daidzein, genistein standars from Sigma Chemicals Co. p-nitrophenyl-β-D-glucopyranoside used as substrate. All other chemicals used were analytical grade. Microorganism used in this experiment consist of *R. oryzae* L16 and *R. oligosporus* C, derived from RCChem collection.

Equipment

High Pressure Liquid Chromatographi Hitachi consist of colom L-7300, UV-VIS, detector L-7420,

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pump L-700 and integrator D-7500 used to determine isoflavone aglicone such as daidzein, genistein, factor-2 and glicytein. Spectrophotometer Spectronic 1201 Shimizu to determine intensity of colour.

Methodology

Microorganism Culture

Potato dextrose agar slants were sterilized at 121 °C, 1 atm for 20 min. Then, streak the agar slant each other with *R. oryzae* L16 and *R. oligosporus* C from stock source. Fungi were incubated at 30 °C for 7 days. Every 2 weeks, transferred to the new Potato dextrose agar slant.

Soybean Tempe

Fried slices tempe and fresh tempe Malang (*Mendoan*) were purchased from Malang, meanwhile tempe which made of mixed culture *R. oligosporus* C and *R. oryzae* L16 in ratio (1:1) prepared at our Laboratory. Tempe banana wrapped and plastic wrapped was purchased from Bandung traditional market.

Making Soybean Tempe

Tempe was made in laboratory as follows : dried soybean (1 kg) were soaked in boiled water containing 0.01% acetic acid overnight at room temperature. The water ajusted of pH 5. The soaking water was discarded and soybean was dehulled by hand in excess water. After dehulling, soybean were steamed for 30 min, and drained. After cooling, the cooked beans were inoculated with 2% inoculum (mixed culture *R.oligosporus* C and *R.oryzae* L16 in ratio 1:1) . The inoculated material was packed into perforated clear plastic bag (20x10) and place in an incubator at 30 °C for 36 h.The tempe produced was sliced and dried in an oven at 50 °C for 12 h prior to grinding. The assesment of β-glucosidase enzyme activity was carried out for tempe with incubation time such as : 1, 2, 3, 4 and 5 days respectively.

Analysis

Chemistry analysis include by assesment of protein content by using Lowry methods. The protein concentration in enzyme fractions was determined with bovine serum albumin as standard. Measurement of β-glucosidase enzyme by using Simmon Gatt methods. The total spores were determined with total plate count. Fat content, water content determined by using AOAC standard method.

Assay of β–Glucosidase Activity

The procedure applied for this purpose was explained by Gatt [12], in which p-nitrophenyl-β-d-glucopyranoside was treated as the substrate. The reagen used as follows : acetate buffer (1 M, pH 5.0), p-nitrophenyl-β-D-glucopyranoside (50 mM, glycine-carbonate (0.25, pH 10), triton X-100 (0.5% in chloroform-methanol 2:1), sodium taurocholate (0.5% in chloroform-methanol 2:1) and trichloroacetic acid (2.75%). The enzyme of β-glucosidase was extracted from tempe, produced from various fermentation times (0, 1, 2, 3, 4 and 5 days) with the following procedure.

First, each sample (5 g) was mixed with 0.1% tween 80 (50 mL), this mixture was the ground. The resulted suspension was poured into a sterile erlenmeyer and shaked for 1 h at 200 rpm. The formed filtrate was centrifuged for 30 min at 3000 rpm, the temperature during this period was maintained at 25 °C. After pH of the resulted supernatant was measured, it was then kept at -20 °C until required.

The prepared extract of the crude enzyme (0,5 mL) was placed into incubation tubes containing acetate buffer (0.1 mL), substrate (0 .05 mL) , triton X-100 (0,2 mL), taurocholate (0.1 mL) and water (0.5 mL). After 1 h incubation at 37 °C, 1,5 mL of trichloro acetic acid was added and the mixture was centrifuged. The supernatant was then decanted prior to the addition of 0.9 mL of 0.5 N NaOH and 1,5 mL of the glycine carbonate buffer. The intensity of the colour was determined using UV-VIS recording spectrophotometer Spectronic 1201 at 420 nm.The above method gave data on the activity of crude β-glucosidase.The specific enzyme activity was calculated by dividing those data with the protein content, measured with Lowry method of each sample.

Analysis of Isoflavones Aglicone

About 45 g ground tempe was first wrapped carefully in a piece of filter paper. The packed sample was placed in a soxhlet apparatus. The solvent used for extraction was hexane and the extraction was carried out for 8-12 h until the whole fat has been removed or tempe powder defatted. After this period, the powder was placed in a fume cupboard until traces of solvent had evaporated. Thirty g defatted tempe powder was mixed (1: 10 w/v) with 80% aqueous methanol and kept in a refrigerator 4 °C overnight. The mixture was then filtered, and the solvent was evaporated under reduced pressure at 40-50 °C. The resulting residue was disolved in methanol (120 mL), any insoluble material removed by centrifugation and discarded. The methanol supernatant was reduced by evaporation to about half of its original volume and then chilled at -20 °C for 20 min. The formed precipitate was removed by filtration (Whatman no : 42). The filtrate was evaporated under reduced pressure at 40 °C and the residue formed was diluted to 10 mL with methanol pro analysed and used for HPLC analysis.

RESULT AND DISCUSSION

The β-glucosidase enzyme may be naturally present in the soybean material or present from

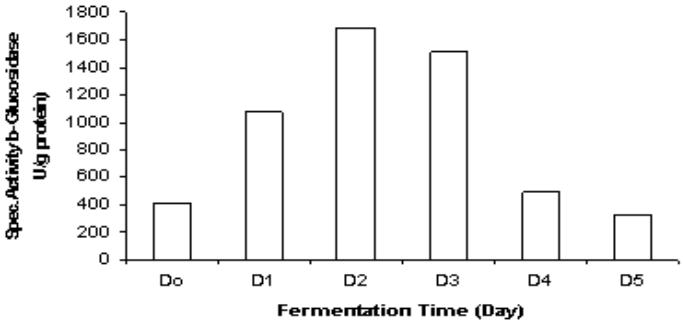

Figure 1. The Specific Activity of β-glucosidase excreted from tempe mixed of *R.oligosporus* C dan *R.oryzae* L16 (1:1).

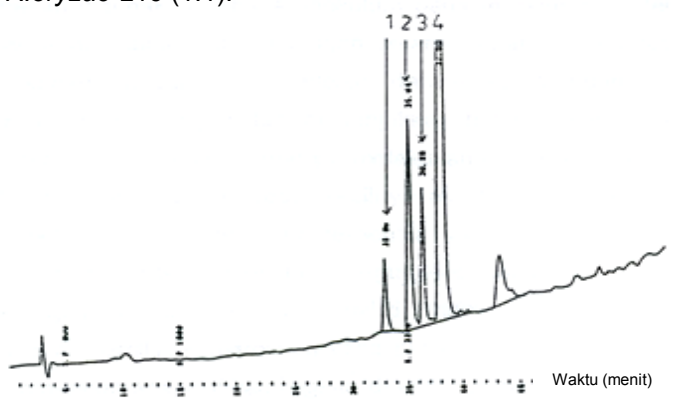

Figure 2. HPLC profile of standard isoflavones Peak 1 : faktor-2; peak 2=daidzein; peak 3=glycitein; peak 4= genistein.

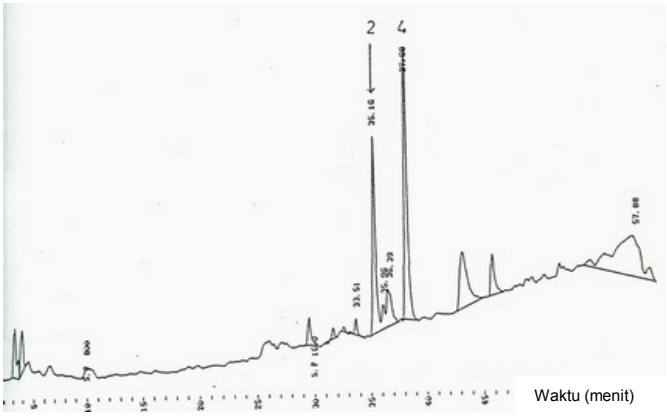

Figure 3. HPLC profile of tempe Malang

microbial growth. The amount of enzyme sufficient to perform the conversion of isoflavone glucoside to be isoflavone aglicones. The enzyme activity of β-glucosidase from tempe mixed culture as shown in figure 1.

During fermentation process by mixed fungal, showed that at day two or 48 hr fermentation had the highest specific activity of enzyme β-glucosidase that was 1693 U/g protein. Meanwhile, β-glucosidase at day 0 already shows specific activity 415 U/g protein, caused by enzyme in soybean which might activated by soaking process in hot water. This enzyme will hydrolyze isoflavone glycoside into isoflavone aglycone [9]. The specific activity of β-glucosidase of tempe Malang was 2486 U/g protein at day two, tempe plastic wrapped were 1844 U/g protein, and tempe banana wrapped 2053 U/g protein. To know isoflavones content of the famous soybean fermented which was purchased in the traditional market, furthermore assesment of fried slices tempe Malang was conducted.The result indicated that fried slices tempe Malang had zero activities of enzyme β-glucosidase. There are correlationship between inoculum with enzyme activities, the content of enzyme in tempe product made with mixed culture inoculum in laboratory and fermented for 48 h or two days was lower, compared with commercial inoculum used for tempe Malang. The different combination of inoculum produces various isoflavone compounds with different content of daidzein and genistein. This is due to the difference in capability of each combination of fungi and bacteria in producing enzyme. The analysis of isoflavonoids compound in soybean as well as in tempe was carried out by using HPLC. Further, chromatographic analysis of standard isoflavone aglycone is showed in figure 2.

The identification of isoflavone present on the chromatogram was carried out by comparing their retention times with the standars run under the same conditions.The solution of standard isoflavone mixture was run several time until the difference between each retention time was less than 1%. The average data of these retention times were then use as reference such as factor 2 (32.82), daidzein (35.03), glycitein (36.16) and genistein (37.87). Several peaks appeared on this chromatogram smaller retention times have been identified as isoflavone glucosides namely daidzin, acetyl daidzin, genistin, acetyl genistin and glycitin. The application of the HPLC procedure for the determination of isoflavones from tempe and soybean seed is known to contain daidzein (7,4-dihydroxyisoflavone) as well as glycosides of daidzin and genistein (5,7,4-tryhydroxyisoflavone).

The chromatogram is shown in figure 3, 4 and 5, illustrates that HPLC system can also be used to separate closely related compounds. As presented in figure 3, genistein content in tempe Malang which was made traditionally 15.57 mg/100 g deffated tempe (peak 4) and daidzein content that was 10.01 mg/100 g deffated tempe (peak 2). As demonstrated in figure 4, genistein content in Laboratory made tempe 5.45 mg per 100 g deffated tempe (peak 4) and daidzein content 2.33 mg per 100 g deffated tempe (peak 2). As shown in figure 5, genistein content in fried slices Malang that was 11.45 mg per 100 g deffated tempe (peak 4) and daidzein content 5.38 mg per 100 g deffated tempe (peak 2).

According to data above, total amount of isoflavone compounds in tempe changed significantly during frying.The amount of each isoflavone aglycone

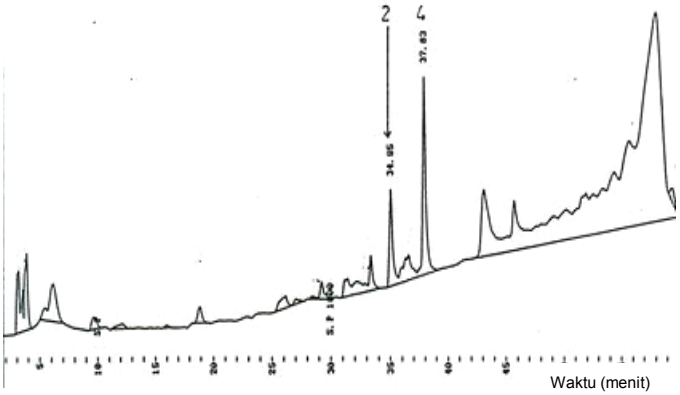

Figure 4. HPLC profile of tempe laboratory.

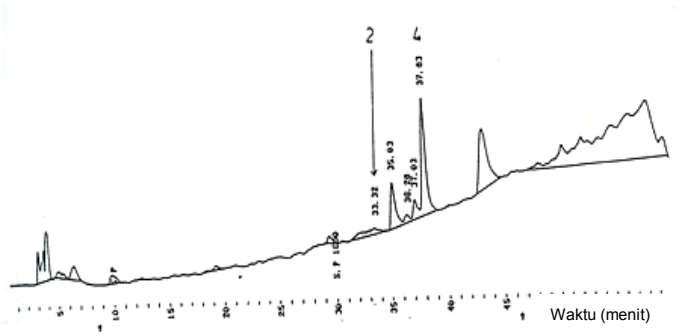

Figure 5. HPLC profile of tempe fried slices

Table 1. Daidzein and genistein of various tempe

Sample	Water Content (%)	Fat Content (%)	Daidzein (mg)	Genistein (mg)
Tempe M1	62.32	22.70	6.97	15.38
Tempe M2	61.92	21.61	7.29	16.82
Tempe M3	63.25	21.73	6.82	13.61
Tempe M4	63.64	23.35	6.82	16.07
Tempe M5	63.50	22.65	6.66	15.97
Lab tempe 1	68.85	14.18	2.26	5.72
Lab tempe 2	66.17	13.76	2.40	5.17
Lab tempe 3	65.85	16.62	2.52	6.23
Lab tempe 4	63.21	13.80	2.30	4.12
Lab tempe 5	60.65	13.19	2.20	6.03
Fried slices M1	1.91	38.67	6.16	14.14
Fried slices M2	1.49	48.66	5.33	10.74
Fried slices M3	1.73	46.44	5.18	10.33
Fried silces M4	1.40	34.69	4.92	8.94
Fried slices M5	2.22	35.22	5.31	13.10

Notice :* 100 g tempe
** 30 g powder deffated

Table 2. Isoflavone aglicone content of soybean extraction

Soybean Sample	Daidzein (mg)	Genistein (mg)
Variety of Willis	1.73	4,57
Variety of Galunggung	6.46	8,21
Variety of Import Soy Bean	1.66	2,67

of tempe Malang have the highest genistein content compared with others. As shown in Table 1, revealed that the averages of genistein content in tempe Malang were about 15.57 mg per 100 g defatted tempe greater than those found in tempe fermented in Laboratory scale which used mixed *R. oligosporus* C and *R. oryzae* L 16 e.g 5.45 mg per 100 g deffated tempe. However, after changing tempe Malang into fried slices tempe, genistein total decreased 28% so that genistein content occured 11.03 mg per 100 g deffated tempe. Decreasing genistein content in fried slices tempe caused by heating process of fried oil, where the temperature can be reached 180 °C. Soybean fermentation on an industrial manufacture scale may involve *Rhizopus* species other than *R. oligosporus* C or *R. oryzae* L16. Numerous bacteria and yeast exist in tempe to cause greater hydrolysis of glycosides than laboratory scale. Or after cooking, the beans are usually cross contaminated through handling, equipment,etc. Apart from the uncertain significance of using mixed cultures for tempe preparation, this type fermentation in industrial tempe manufacturing processes may also lead to the accumulation of undesirable isoflavonoids such as the isoflavan equol. The averages of daidzein content in tempe produced from laboratory that was 2.33 mg per 100 g defatted tempe lower than tempe Malang (*Mendoan*) with average 6.91 mg per 100 g defatted and fried slices tempe 5.38 mg per 100 g deffated tempe. Decreasing daidzein content caused by frying tempe when was changing into fried crispy slices tempe that was 22%. By comparing the data presented in table 1, it may be concluded that tempe Malang (M1, M2, M3, M4 and M5) contained higher genistein than similar product prepared with tempe laboratory. The inoculum tempe Malang mainly consist of mixed culture and may naturally contains other microorganism. Meanwhile tempe prepared in laboratory mainly *R. oligosporus and R. oryzae.* Daidzein and genistein content in tempe Malang e.g 10.01 and 15.57 per 100 g defatted tempe with plastic wraped 7.38 mg and 13.58 mg per 100 g defatted tempe, banana leaves wraped 9.57 and 18.19 mg per 100 g deffated tempe respectively. The data indicated that tempe with plastic wrapped purchased in Bandung traditional market had isoflavone content lower than purchased in Malang. The composition of microbe and *Rhizopus* species of inoculum which was used in fermentation proccess can caused differences of isoflavone content. Generally, tempe purchased in the traditional market by using mixed culture microbes consist of *Rhizopus* sp, however yeast and also bacteria can be detected.

The isoflavone aglycone of dries soybean, can be shown in Table 2. Soybeans contain two isoflavone glycosides, daidzin and genistin and their respective aglycones such as genistein and daidzein.

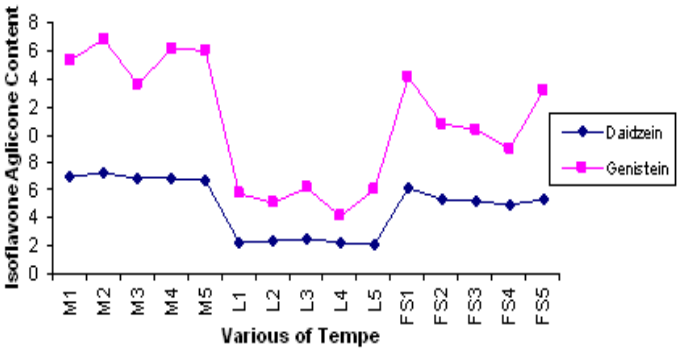

Figure 6. Isoflavone aglicone content of tempe Malang (M), fried slices Malang (FS) and tempe laboratory (L).

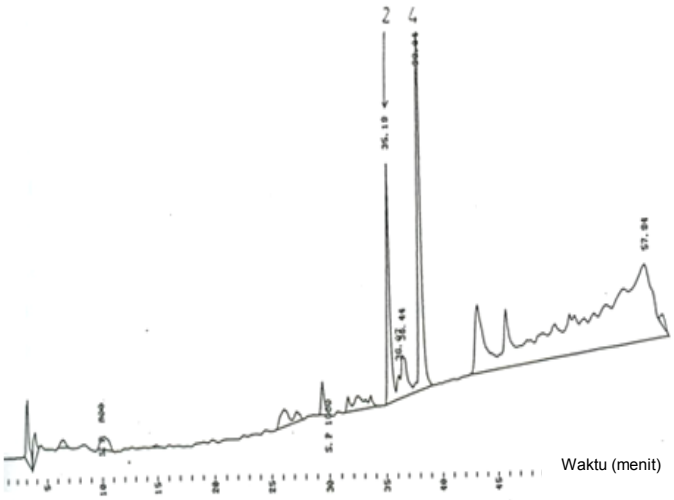

Figure 7. HPLC profile of tempe plastic wrapped.

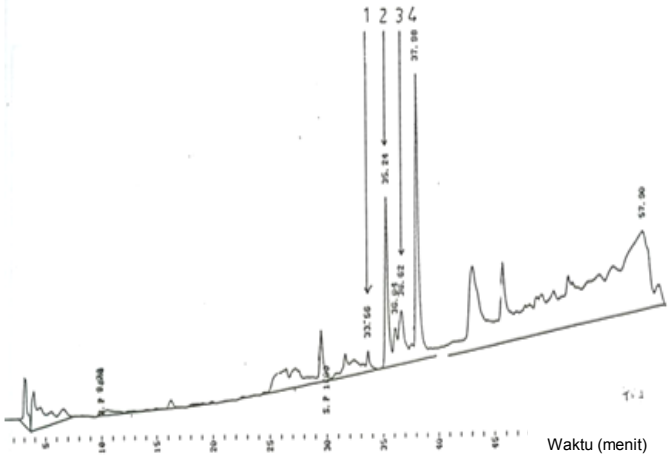

Figure 8. HPLC profile of tempe banana leaves wrapped.

It has been reported that these isoflavones have certain physiological and antifungal activities. Two varieties of soybean such as Willis and Galunggung has been used to measure effectivities isoflavone extraction. Fortunately, both of local soybean contain daidzein that were 1.73 and 6.46 mg per 100 g deffated soybean, greater than those found in import soybean which were used for making tempe industry scale that was 1.66 mg per 100 g deffated soybean. The other hands, Willis and Galunggung varietas also had genistein content that was 4.57 and 8.21 mg per 100 g deffated soybean compared to imported soybean only reached 2.67 mg per 100 g deffated soybean. Although, isoflavone aglycone content in local soybean greater than import soybean, but, according to tempe producer, they prefer to used import soybean than local soybean because the bean was bigger and easy to get in the traditional market place. As shown in table 2, the amount of isoflavone aglycone in soybean extract obtained from 100 g material was very small only 1.66 mg daidzein and 2.67 mg genistein. This concentrations increased after the soybean was fermented for 36 h with *R.oligosporus* C and *R.oryzae* L16 as inoculum. The results showed that, the amount of both aglycones in tempe were much higher (2.33 mg for daidzein and 5.45 mg for genistein). This increase presumably reflects fungal hydrolysis of the various glycosides during the fermentation process.

As demonstrated in figure 6, genistein content in tempe Malang and fried slices Malang indicated higher concentration content than daidzein. This effect has already been demonstrated by tempe Malang and tempe laboratory and fried slices tempe. Interestingly, the amount of genistein in those materials was always higher than daidzein. This result is preferred, since genistein posses a stronger activity than daidzein.

As shown in Figure 7, the genistein content particularly in tempe plastic wrapped was 13.58 mg/100 g deffated tempe (peak 4) and daidzein content was 7.38 mg/100 g deffated tempe (peak 2). In Figure 8, tempe which was wrapped with banana leaves revealed that genistein content was 18.19 mg/100 g deffated tempe (peak 4) and daidzein content was 9.57 mg/100 g deffated tempe (peak 2). There was factor-2 at retention time 33.56 (peak 1) and glycitein at retention time 30.02 (peak 3).

Preparing functional food from fermented soybean sources, should be based upon the data resulted, which had highest productivity of isoflavone content of concentrate. Isoflavone compounds in tempe are very prospective compounds to be used in the field of pharmaceutical and medicine among other things as antioxidant, antihemolysis, anticholesterol and anticancer (3). Furthermore, the data can establish the optimal fermentation conditions for maximal results.

CONCLUSION

It can be concluded that soybean tempe have attracted much attention because they are unique as nutritionally significant dietary sources of isoflavones. Tempe typically contain more genistein than daidzein,

although this ratio varies among the different soybean product. The study revealed that genistein content in conventional tempe Malang was 15.57 mg per 100 g deffated tempe higher than tempe which was made in laboratory using mixed fungal *R. oligosporus* C and *R. oryzae* L16 (1:1) that was 5.45 mg per 100 g defatted tempe.

Daidzein and genistein content in tempe Malang e.g 10.01 and 15.57 per 100 g defatted tempe with plastic wraped 7.38 mg and 13.58 mg per 100 g defatted tempe, banana leaves wraped 9.57 mg and 18..19 mg per 100 g deffated tempe respectively. The data indicated that tempe with plastic wrapped and banana leaves wrapped had isoflavone content lower than purchased in Malang, however tempe banana leaves wrapped had a litlle bit of factor-2 and glycitein. The composition microbe and *Rhizopus* species of inoculum which was used in fermentation proccess can caused differences of isoflavone content.

The β-glucosidase specific activity of tempe Malang indicated 2486 U/g protein at day two, tempe plastic wrapped that was 1844 U/g protein, tempe banana wrapped 2053 U/g protein and tempe laboratory 1693 U/g protein. There are correlationship between inoculum with enzyme activities, the content of enzyme in tempe product made with inoculum in laboratory and fermented for 48 h or two days was lower, compared with commercial inoculum used for tempe Malang. Meanwhile, fried slices tempe had zero activities of enzyme β-glucosidase.

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