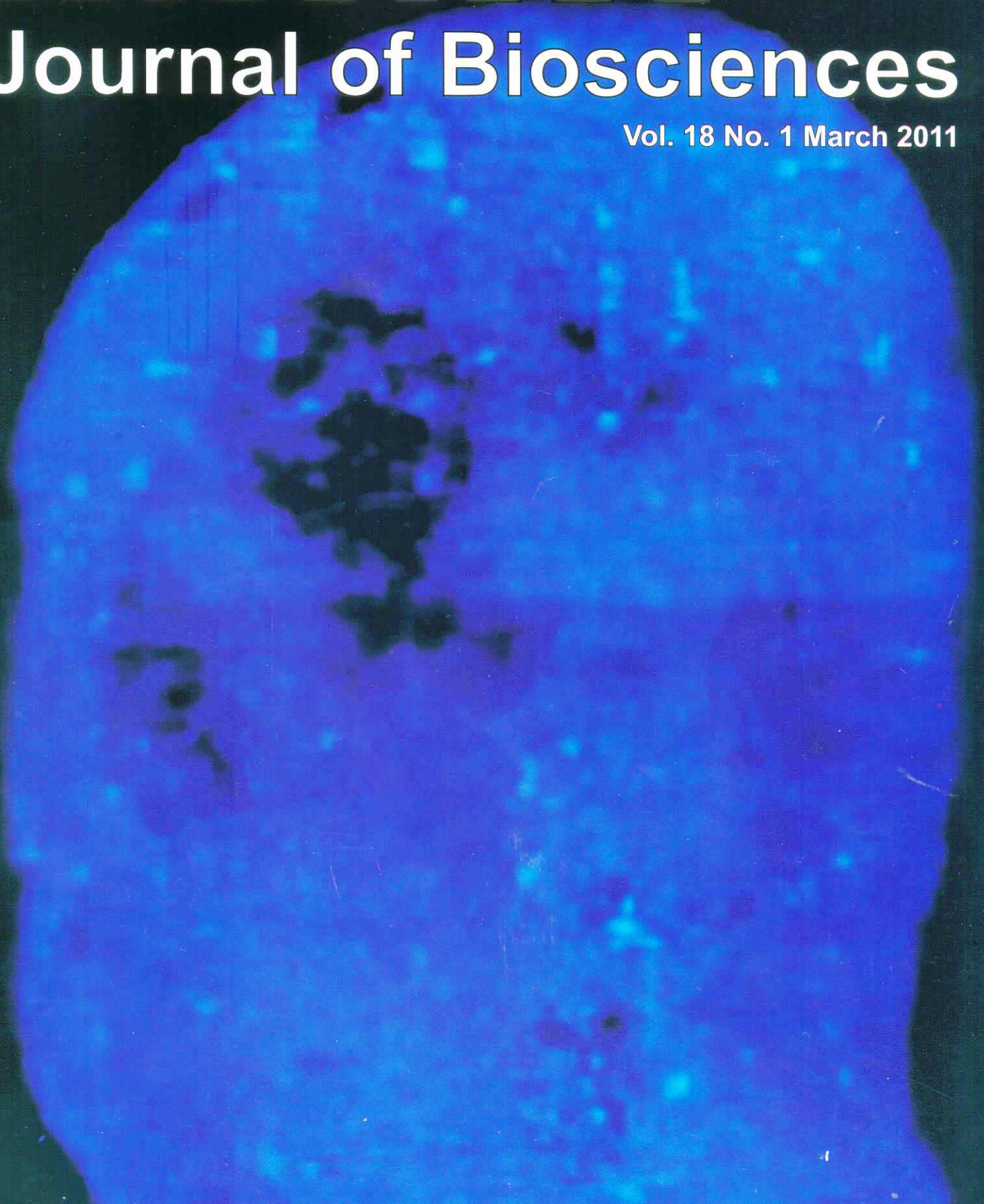


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β -Conglycinin Content Obtained from Two Soybean Varieties Using Different Preparation and Extraction Methods

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Soybean is a good source of protein. It has two major fractions, β -conglycinin (7S) and glycinin (11S). β -conglycinin's function was known to suppress food intake, and this effect may be due to stimulating endogenous cholecystokinin (CCK) release. The aims of this study were to determine the highest content of total β -conglycinin and β -conglycinin sub unit- β level obtained from two varieties of soybean i.e. *Wilis* and *Detam 1* varieties using different preparation and extraction methods. These two soybean varieties were prepared into tempeh. Then the seed and tempeh were extracted using Deak and Panthee methods. There were six extracts analysed using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) and coomassie brilliant blue (CBB) staining. The result was shown that *Detam* variety and raw seed contained the highest total β -conglycinin level. And Panthee method was the best method for extraction of total β -conglycinin, while Deak method was the best method for extraction of β -conglycinin subunit- β .

Key words: *Detam 1* soybean, *Wilis* soybean, Deak method, Panthee method, SDS PAGE, CBB staining

INTRODUCTION

Soybean is a source of protein and amino acids for human and animal feeds in the world. Many works have been done on structure, biosynthesis and genetics of the soybean proteins (Nielsen 1984; Clarke & Wiseman 2000).

Soybean storage protein has two major fractions, β -conglycinin (7S) and glycinin (11S), accounting for more than 70% of the total proteins. β -Conglycinin is a trimer with subunits α , α' , and β , with a M.W. (molecular weight) of 180 kDa. Glycinin is a hexamer with a M.W. of 360 kDa, consisting of acidic and basic subunits (Kitamura 1995).

Soybean protein contained 8 essential amino acids. Soybean contained higher amino acids than that of animal products. Protein from legume groups, like soybean, is absorbed slower than protein from livestock (Symolon 2004). Nutritional study showed that soybean protein produced higher thermogenic and satiety effect than that of carbohydrate. This fact made soybean as a good nutrition source for treating obesity (McCarthy 2000). However soy protein is not a perfect protein because of its low level of the sulfur-containing essential amino acids such as methionine and cysteine. Glycinin has three to four times more S-containing amino acids (particularly methionine) than that of β -conglycinin (Kitamura 1995). On the other hand, soybean protein contains β -conglycinin that may be important food components to control lipid accumulation in adipose tissues (Moran 2006).

Based on many studies, β -conglycinin's function was approved to suppress food intake due to releasing β -conglycinin peptone in the lumen stimulating endogenous Cholecystokinin (CCK) with direct acceptance to the intestinal cells (Nishi 2001; Nishi 2003a). The fragment from 51 to 63 of the β subunit (β 51-63) had the strongest binding activity to stimulate releasing CCK and appetite suppression (Nishi 2003b). CCK is an important hormonal regulator of the digestive process. The physiological actions of CCK include stimulation of pancreatic secretion and gallbladder contraction, regulation of gastric emptying, and induction of satiety. Therefore, by its functions which can stimulates CCK made β -conglycinin in soybean as one of the important therapy targets in obesity treatment (Liddle 1997; Little 2005).

There were many varieties of soybean in Indonesia. In this study we focused on 2 varieties of soybean i.e. *Detam 1* and *Wilis* varieties. *Detam 1* variety is a high quality soybean mostly cultivated in Balai Penelitian Kacang dan Umbi-umbian (Balitkabi) plantation in Malang, Indonesia. It contained higher protein level (45.36%) than that of other soybean varieties (MenTan 2008). So it was assumed that it also contained a high level of bioactive compound in the seed. As a comparison we used soybean *Wilis* variety, an Indonesian local soybean which is commonly planted by farmers in Indramayu, Indonesia.

A study focus on the effect of different preparation method of soybean, like tempeh, to produce β -conglycinin was very limited. Fermentation process decreased the protein content of soybean but the amount of protein that can be absorbed was increased due to inactivation of

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antinutrition factors in soybean by heating during fermentation process (Hermana 1999; Buckle 2007). However, its correlation with β -conglycinin production was not clear yet.

There are several kinds of extraction methods to produce β -conglycinin. And in this study we focused on two methods of soybean protein extraction i.e. a Panthee method and a new simplified Deak protein fractionation procedure. The Panthee method is an extraction procedure to get higher glycinin level (Panthee *et al.* 2004; Delwiche 2007). And Deak method is the procedure that is possible to produce protein fractions (> 90% protein) which is rich in either glycinin or β -conglycinin by employing CaCl_2 and NaHSO_3 (Deak 2007). In this study, we consider to find out the best method to extract β -conglycinin especially subunit- β as one of therapy target in obesity treatment in the future.

The aims of this study were to determine the highest content of total β -conglycinin and β -conglycinin sub unit- β level obtained from two varieties of soybean i.e. *Wilis* and *Detam 1* varieties using different preparation and extraction methods analyzed using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) and coomassie brilliant blue (CBB) staining.

MATERIALS AND METHODS

Soybean Variety. Two soybean varieties chosen in this study were *Wilis* and *Detam 1* varieties. The soybean *Detam 1* variety was planted in Balitkabi plantation in Malang, Indonesia. Productivity of *Detam 1* variety was 2.51 ton per acre, seeds were harvested in 84 days. This soybean had a yellow flesh seed covered with hard black skin. It contained 45.36% dry weight protein which was higher than that of other varieties. This variety was approved as a high quality soybean by Minister of Agricultural decree no 240/Kpts/SR.120/3/2008 date March 6th 2008 (MenTan 2008).

Wilis variety is an Indonesian local soybean which is commonly planted by farmers in Indramayu, Indonesia. This soybean has a yellow flesh seed with yellow skin seed. It contains about 39% protein (dry weight).

Sample Preparation. The two soybean varieties were made into tempeh. Then the seed and tempeh from *Detam 1* and *Wilis* varieties were extracted using Deak and Panthee procedures. So that there were 6 extracts of soybean examined in this study i.e. (i) Protein extract of *Detam 1* Soybean seed Deak Method; (ii) Protein extract of *Detam 1* Soybean seed Panthee Method; (iii) Protein extract of *Detam 1* Soybean tempeh Panthee Method; (iv) Protein extract of *Wilis* Soybean seed Panthee Method; (v) Protein extract of *Wilis* Soybean tempeh Panthee Method; (vi) Protein extract of *Detam 1* Soybean skin seed Panthee Method.

Fermentation of Soybean. Procedure steps to make Tempeh was conducted in 8 steps process i.e. boiling, peeling of skin seed, soaking, washing, steaming, inoculating, packaging and stewing. As many as 500 g *Detam 1* and *Wilis* soybean seeds were boiled. Then the

skin seed was peeled and soaked in an acid condition (pH 4.0-5.0). The skin of the seed was also kept to be extracted of their protein. Then the soybean seeds were washed so that they were not sleek. The seeds were steamed until they were soft and wellcooked.

Fungal tempeh inoculum or *laru* was inoculated 1 g for 1 kg of the seeds and they were mixed thoroughly. After the seeds were packed in plastic bags, they were incubated at room temperature (20-37 °C) for 2 days (Hermana 1999; Santoso 2003). It was produced 6,500 g of Tempeh from 500 g of raw seeds.

Protein Extraction Panthee's method. Sample of 10 g was grounded in cool water (20 °C) using Knifetec 1,095 sample mill for 20 s. It produced soybean flour with relatively uniform particle size. Soluble protein was extracted for 1 h at room temperature from 20 to 25 °C (68 to 77 °F). One gram of soybean flour was stirred in 0.2M Tris HCl buffer pH 8.0 with ratio of 1:15 (w/v) contained 0.1 M β -mercaptoethanol. The mixture was centrifuged at 10,000 x g for 10 min at 4 °C. Upon removal of the fat layer, as much as 1 ml of aliquot layer or supernatant was taken from each sample. The proteins in the crude extract sample were dissociated by adding an equal volume of both 5% SDS and 0.1 M β -mercaptoethanol solutions and then warmed at 44.5 instead of 45 °C in a waterbath for 10 min (Panthee *et al.* 2004; Delwiche 2007).

Deak's Method. Defatted soy flour of 100 g was extracted using deionized water with ratio of 1:15 (w/v) at pH 8.5 adjusted with 2 N NaOH. The slurry was stirred for 1 h and centrifuged at 14,000 g and 15 °C for 30 min. The protein extract (first protein extract) was decanted. The extract was then added with sufficient NaHSO_3 and CaCl_2 to obtain concentration of either SO_2 and Ca_2^+ to 5 mM and the pH was adjusted to 6.4 with 2 N HCl. The slurry was stored at 4 °C for 12-16 h. The slurry was centrifuged at 14,000 g for 30 min at 4 °C. A glycinin-rich fraction was obtained as the precipitated curd, which was neutralized and treated as described on Wu procedure.

The supernatant (second protein extract) was adjusted to pH 4.8 with HCl. Then it was stirred for 1 h and centrifuged at 14,000 g and 4 °C for 30 min. A β -conglycinin-rich fraction was obtained as the precipitated curd and treated as described previously. The amount of supernatant (whey) was determined and sampled.

Freeze-drying steps were modified to evaporation process in cycling evaporator at 30 °C until the solution extracts become thick liquid. Samples were placed in sealed containers and stored at 4 °C up to analysis (Figure 1).

There are two steps in Deak's procedure i.e. D4C (in 4°C) and DRT (in 25 °C). The Deak β -conglycinin-rich fraction D4C (the methods which performed in this study) comprised 23.1% of the solids, 37.1% of the protein and 37.5% of the isoflavones in the starting soy flour. Protein purity was > 80%. Their D4C method produced 85.6% β -conglycinin and 14.4% glycinin. The β -conglycinin subunit consisted of 27.3% subunit α' 38.0% subunit α , 34.7% subunit α (Deak 2008).

SDS-PAGE. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according

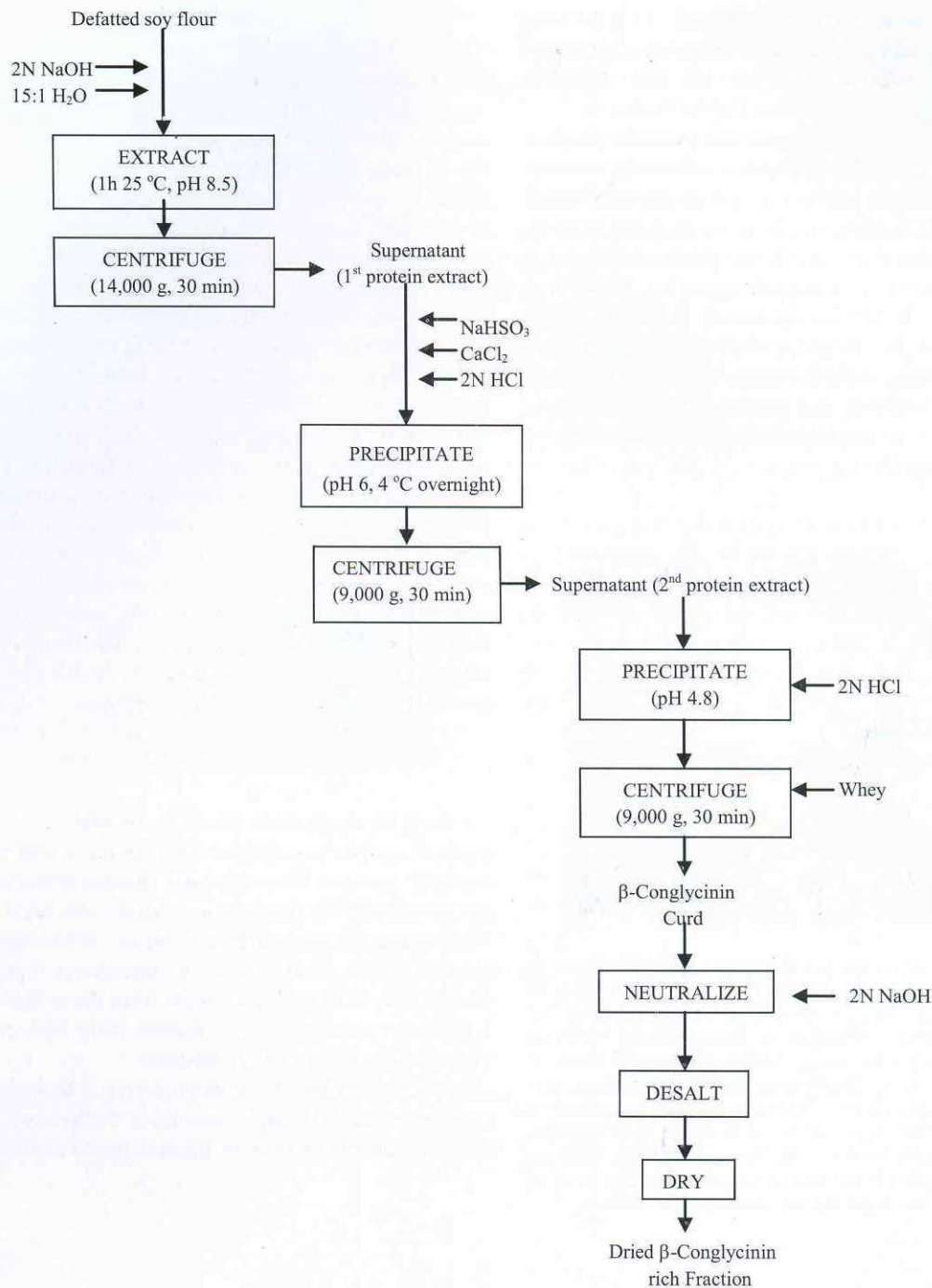


Figure 1. Step in new simplified Deak soy protein fractionation procedure (Deak 2007; Panthee 2007).

to the method of Laemmli (1970). Protein concentrations were firstly measured using a BCA protein assay kit (Pierce, Rockford, IL) with BSA as a standard. The SDS-PAGE was carried out in 80- (high) \times 100- (width) \times 1-mm (thick) vertical slab gel consisting of stacking gel of 5% acrylamide, and main running gel of 20% acrylamide in pH 8.8 of 0.375M Tris-HCl (5-20% ReadyGel Bio-Rad, Tokyo). Twenty micrograms of protein was run on an electrophoresis at 100 v and 400 mA of constant electric current for 100 min. Visualization of protein bands was done by staining with 0.05% Coomassie brilliant blue R-250 (Bio-Rad, Tokyo) (Moriyama 2004; Yoshino 2006; Vasconzelos 2006; Imoto 2008; Motoyama 2009).

Protein markers employed were myosin (206 kilo Dalton; kDa), β -galactosidase (118 kDa), bovine serum albumin (97 kDa), ovalbumin (54 kDa), carbonic anhydrase (37 kDa), soybean trypsin inhibitor (29 kDa), lysozyme (17 kDa) and aprotinin (6 kDa). Determination of the protein levels of each molecule was conducted with pan-actin (Cell Signaling, MA) as a loading control. Comparison of these levels among each sample was performed by using acquisition into the Photoshop software (Adobe) and analyzed by the Quantity one (Bio-Rad). Determination of the protein based on the size as reported by Thanh (1977) and Nielsen (1985). β -conglycinin (7S globulin of soybean) was a trimeric protein composed of various

combination of three subunits (namely, α' - ~72, α - ~68, and β - ~52 kDa) and glycinin (11S globulin of soybean) was acidic and basic subunits of ~35 and ~20 kDa, respectively (Thanh 1977; Nielsen 1985) (Figure 2).

β -conglycinin detected using an anti pan actin (Sigma) served as control was checked and calculated the amount of protein loaded into gel well to get an accurate equal amount for each sample using computerized system. Horizontal scanning densitometry was performed by using acquisition into Adobe Photoshop (Apple, Inc., Cupertino, CA) and analysis by the Quantity One (BioRad). Comparison of the densitometer result of CBB staining of *Detam* seed Panthee method extract with *Detam* tempeh Panthee method extract and *Detam* skin seed Panthee method extract was conducted to determine the best preparation (Figure 3).

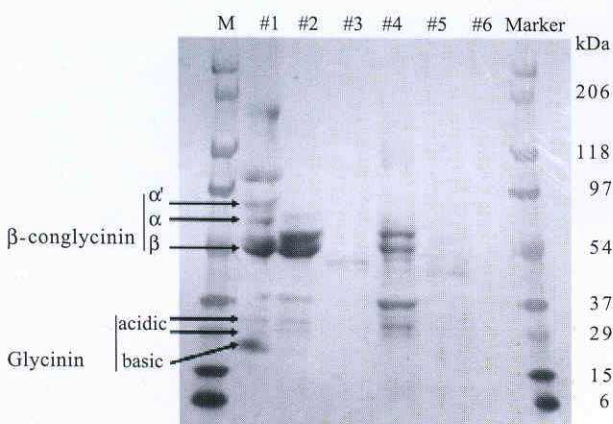


Figure 2. Polyacrylamide gel electrophoresis (SDS-PAGE) of β -conglycinin and glycinin from Indonesian soybean determined by pan-actin (Cell Signaling, MA). M: Molecular mass standards; #1: Seed of *Detam 1* Soybean using Deak modification method; #2: Seed of *Detam 1* Soybean using Panthee modification method; #3: Tempeh of *Detam 1* Soybean Panthee modification method; #4: Seed of *Wilis* Soybean with Panthee modification method; #5: Tempeh of *Wilis* Soybean with Panthee modification method; #6: skin seed of *Detam 1* Soybean Panthee modification method.

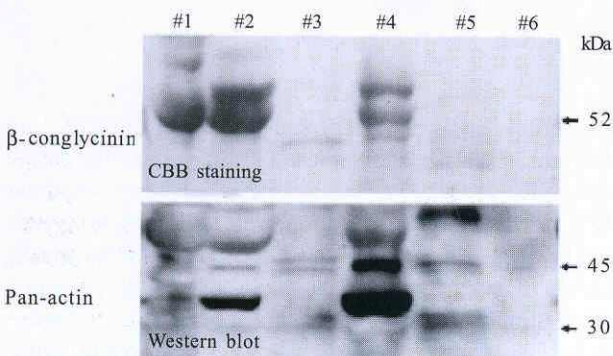


Figure 3. Coomassie brilliant blue (CBB) staining and Western blot analysis of β -conglycinin and its unit calculated as the arbitrary densitometric of bands after normalization of each expression level with pan-actin.

RESULTS

The bands of pan actin as internal control were not equal probably due to the differences of either the purity or quality the extracts. However there was able to determine the expression of β -conglycinin from the extracts by dividing the densitometry value with the pan actin value as internal control. Protein extract from seed of *Detam 1* Soybean using Panthee modification method (Figure 4; line 2) had the highest level of total β -conglycinin (51.7 densitometric unit, DU), alpha aksen subunit (7.9 DU) and alpha subunit (20.8 DU) also. On the other hand, protein extract from seed of *Detam 1* Soybean using Deak modification method (Figure 4; line 1) which contained total β -conglycinin 37.1 DU, had the highest level of β -conglycinin beta subunit (25 DU). Protein extract from *Wilis*-seed of soybean with Panthee method (line 4) and from *Wilis*-tempeh of Soybean with Panthee modification method (line 5) showed low concentrations of total β -conglycinin, 6.4 and 1.2 DU, respectively (Figure 4; line 4 and 5). However, protein extract from tempeh of *Detam 1* Soybean Panthee method (Figure 4; line 3) and from skin seed of *Detam 1* Soybean Panthee method (Figure 4; line 6) showed no expression of β -conglycinin.

DISCUSSION

Total β -conglycinin level in *Detam* seed Panthee method extract was higher than in *Wilis* seed Panthee method extract. However total β -conglycinin level in *Detam* tempeh Panthee method extract was lower than in *Wilis* tempeh Panthee method extract. The average total β -conglycinin level of *Detam* variety was higher than that of *Wilis* variety. Protein level from the seed of *Detam 1* Soybean was higher than protein from *Wilis* soybean variety or its fermentation products.

Seed (raw) of soybean without preparation contained the highest total β -conglycinin level. Fermented process may decreased the protein content in the soybean. For

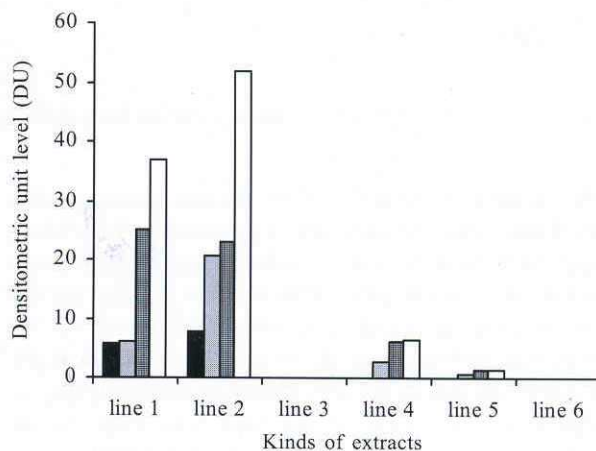


Figure 4. Results of horizontal scanning densitometry performed by acquisition into Adobe Photoshop (Apple, Inc., Cupertino, CA) and analysis by the Quantity One (BioRad). ■: alpha aksen, □: alpha, ▒: beta, □: total.

illustration, dry raw Detam 1 soybean seed contained 41.82% of protein, but pure soybean tempeh only contained 25.35% (Hidayat 2010). There was no β -conglycinin level in tempeh and skin seed of *Detam 1* as well as *Wilis* tempeh. So it is not a good sources for β -conglycinin.

Total β -conglycinin level in *Detam* seed Panthee method extract (line 2) was higher (51.7 DU) than in *Detam* seed Deak method extract (line 4) (37.1 DU). Comparison of β -conglycinin subunit showed that *Detam* seed Panthee method extract (line 2) contained alpha aksen (7.9 DU) and alpha subunit (20.8 DU) higher than that of *Detam* seed Deak method extract which contained alpha aksen (5.7 DU) and alpha subunit (6.2 DU). However *Detam* seed Deak method extract contained beta subunit (25 DU) slightly higher than *Detam* seed Panthee method extract (23 DU).

Comparison of percentage proportion of subunits from total β -conglycinin with Deak and Panthee's data, it was shown that α subunit' in our study (Deak method 15.5% and Panthee method 15.3%) was lower than that of the references (Deak reference 27.3% and Panthee reference 22.8%) (Deak 2007; Panthee 2007). Our study showed that the the β -conglycinin α subunit in Deak method (16.8%) was lower than that of Panthee method (40.2%) and both references (Deak reference 38.0% and Panthee reference 40.3%). Our result for β -conglycinin α subunit Panthee method was almost the same as references (Deak 2007; Panthee 2007).

On the contrary, the level of β -conglycinin β subunit in Deak method (67.7%) was higher than that of Panthee method (44.5%) and both references (Deak reference 34.7% and Panthee reference 36.9%). Liu (1997) noted that the different methods used in isolation of different protein fractions reported by different researchers on the characterization of soy protein fractions were sometimes controversial. This appears to be an issue in this study as well. The difference of the result might be caused by variety difference due to each variety had each specific content. Deak study used white flakes soybean variety IA2020 which contained 57.3% protein, while Panthee study used soybean variety 101 F6-derived recombinant inbred lines (RIL) developed from a cross of N87-984-16 x TN 93-99 which contained 38.89% protein (Deak 2007; Panthee 2007). Therefore Deak method is desirable to be used for extraction the fragment from 51 to 63 of the β -conglycinin β subunit. Because it had the strongest binding activity to stimulate CCK release and appetite suppression (Nishi 2003).

As conclusion, *Detam* variety and raw seed contained the highest total β -conglycinin level. and Panthee method was the best extraction method for producing total β -conglycinin, while Deak method was the best method for producing β -conglycinin subunit- β .

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