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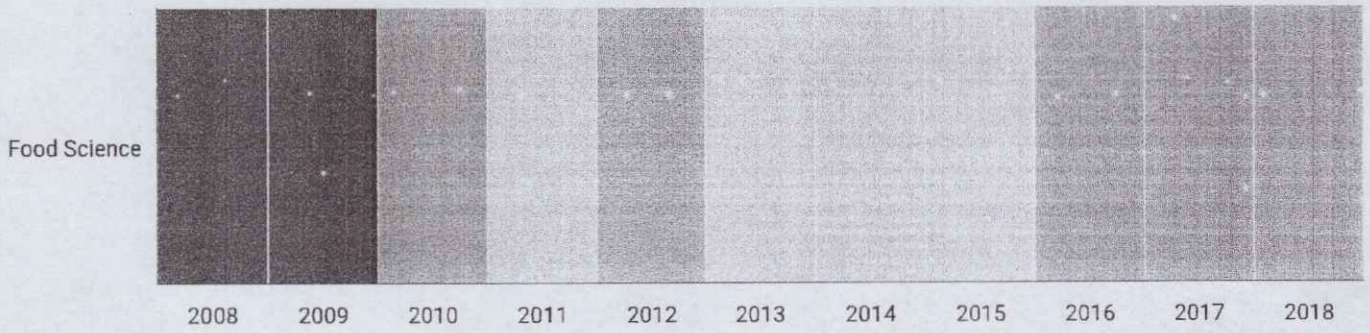

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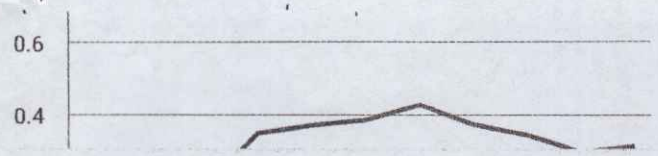

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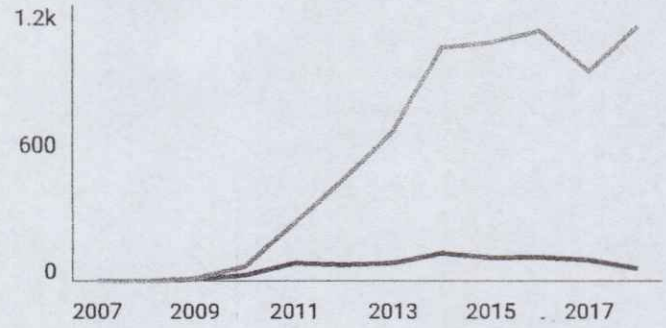

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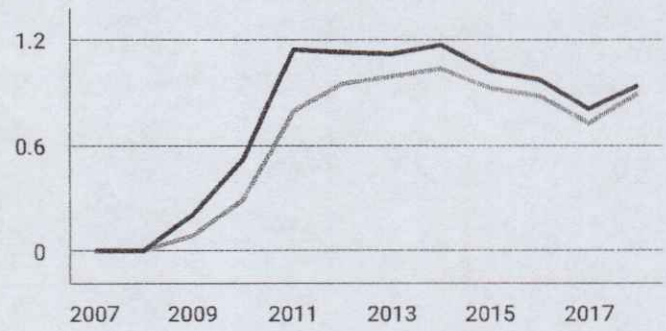

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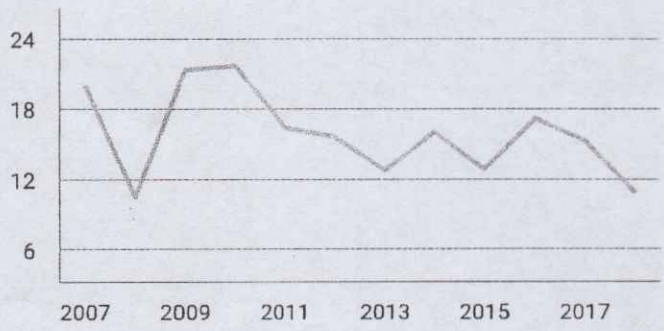

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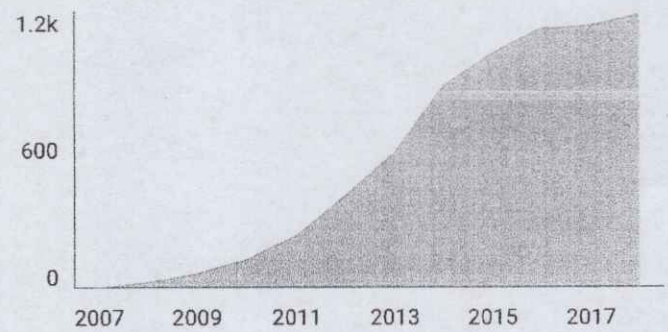

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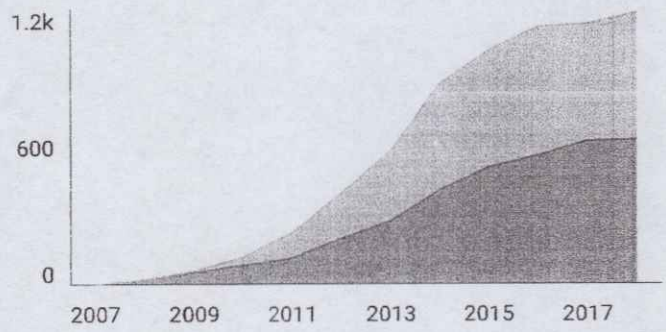

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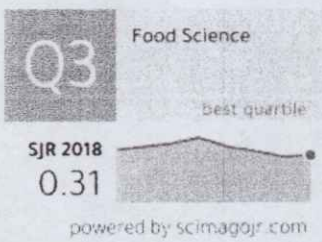

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Amino acid profile of soybean *(Glicine max)* sprout protein for determining insulin stimulation amino acids

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Abstract

Seed germination has been known increased free amino acids, and changed the amount and kind of amino acids. Soybean protein has popularly known as functional food for diabetes, it is due to its specific amino acids that are known as insulin stimulation for reducing blood glucose. This research was conducted to know the contain of specific amino acids for stimulation of insulin secretion, including Leucine(Leu), Arginine(Arg), Alanine (Ala), Phenylalanine (Phe), Isoleucine (Ile), Lysine (Lys) and Metionine (Met), based on the profile of total and free amino acids of soybean sprout in various of protein isolation methods. Proteins of soybean sprout and seed as control were isolated by 2 methods that were distinguished by pH extraction and pH precipitation. On the 1st and 2nd method, protein was extracted at pH4 and pH9 respectively, then precipitated at pH3 and pH4 respectively. The protein precipitates from soybean sprout and seed were analyzed moisture content, protein, free amino acids, the profile of total and free amino acids. The result of this research showed that germination of soybean seed for 36 hr increased the specific free amino acids, particularly Ala, Leu, Lys, and Phe that was known as stimulation of insulin secretion, which were 8.5; 3,5; 2.8; and 1.7 times respectively compared to the specific free amino acids of the original seeds.

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Introduction

The fact show that 90% of diabetes patients are identified as type 2 diabetes mellitus. There are many causes of type 2 diabetes mellitus, among them is impaired insulin secretion that is due to the lack of energy for insulin secretion, hence the secretion of insulin from pancreatic β-cell is inadequate to normalize blood glucose. The patient must control their blood glucose by oral hypoglycemic drugs (OHG), but they get harmful effects for the body. Therefore, functional foods have to be invented and for the future they must be part of diabetes patient consumption in order to stop using of OHG. Diabetes in Indonesia is rank 5 in the world (Shashank and Aravind, 2017). Kanetro *et al.* (2008) invented that protein isolate of soybean sprout could stimulate insulin secretion through in vitro bioassay that was higher than protein isolate of soybean seed. Therefore soybean sprout may be used for reducing diabetes prevalence in Indonesia.

Soybean protein has popularly known as functional food for diabetes, it is due to its trypsin inhibtior (Kanetro *et al*, 2005; Kanetro *et al*, 2007) and its specific amino acids that are known as insulin stimulation for reducing blood glucose. Specific amino acids, such as Leu, Ile (Sans *et al*., 2006; Yang *et al*., 2006), Arg (Kim *et al*., 2004; Yang *et al*., 2006), Ala, Phe, Lys, Met (Vanloon *et al*., 2000; Calbeat and Maclean, 2002; Vanloon *et al*., 2003) were known as stimulation of insulin secretion. Amino acids may influence insulin secretion via a number of possible mechanisms, including generation of metabolic coupling factor, depolarization of the plasma membrane, or enhancement of mitochondrial function (Newsholme *et al*., 2006). In the pancreatic β-cell, mitochondria serve to couple nutrient metabolism to the exocytosis of insulin, a process that can be stimulated by glucose or by amino acids. The specifics amino acids that are known as insulin stimulation can activate mitochondrial metabolism in pancreatic β-cell via the tricarboxylic acid (TCA) cycle, resulting in the formation of ATP. The rise in ATP levels leads to closure of ATP-dependent K^+ channels, which in turn depolarizes the cell membrane, thus opening of voltage-dependent Ca^{2+} channels and increasing intracellular Ca^{2+} concentration, which triggers insulin exocytosis and hence facilitating insulin secretion from pancreatic β-cell (Argmann and Auwerx, 2006; Newsholme *et al*., 2007).

The mobilization of protein reserves in germination is started by increasing of protease activity, then proteins are hydrolysed by proteases,

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resulting several small peptides and free amino acids. The free amino acids may be used for protein synthesis, or to provide energy by oxidation of the carbon skeleton after deamination, and converted into other amino acid. Protein and amino acids metabolism during seed germination changed molecular weight of protein (Ahmed *et al.*, 1995). Seed germination increased free amino acids (Khalil *et al.*, 2006; Martinez *et al.*, 2006), changed the amount and kind of amino acids (Chiou *et al.*, 1997).

The investigation about germinated seed for solution the problems of diabetic has been conducted by Usuki *et al* (2007). This research showed that using diet germinated brown rice could decrease blood glucose significantly of streptozotocin-induced diabetic rats compared to brown rice and white rice diet. Germination for 48 hr of brown rice reduced glycaymic index from 60.1 to 37.76 (Cornejo *et al.*, 2015). Pathak (2005) also has been explored that germinated soybeans for 24 hr could control blood glucose and were more effective than the utilization of OHG. The development of the anti-diabetes property in soybean seed during germination was as the result of the synthesis of phosphatydylinositol 3 kinase, and it was an important component of insulin receptor/tyrosin kinase (Pathak, 2005). Soybean sprout protein also showed the role as insulin-like protein (Pathak dan Martirosyan, 2011). Germination Phaseolus vulgaris produced bioactive peptides with the ability to inhibit DPP-IV, resulting in increased insulin released from pancreatic cells (Rocha *et al.*, 2015). This research was conducted to know that the protein of soybean sprouts contain the specific amino acids for stimulation of insulin secretion, such as Leu, Arg, Ala, Phe, Ile, Lys and Met.

Materials and Methods

The variety of soybean, Sinabung, was obtained from Research Center of Legumes, Malang, Indonesia. Chemical agents such as amino acids standard, OPA (ortho phentaldialdehyde) etc were purchased from Sigma, Fluka and Merck Chemical Co. The sequences of experimental activities were performed as follows:

Selection of germination time

Soybean seeds were soaked for 8 hr, and then germinated for 12, 24, 36, 48, 60, or 72 hr, and ungerminated soybean as a control. Germination for 36hr was selected for producing soybean sprout based on previous research for increashing protein during germination. This research showed that germination for 36hr was the highest protein content especially trypsin inhibitor (Kanetro *et al.*, 2007). The selected soybean sprouts were used for material of protein isolation.

Isolation of protein from soybean sprout and seed

Proteins of soybean sprout and seed as a control were isolated by 2 methods that were distinguished by pH extraction and pH precipitation (Kanetro *et al.*, 2008). The 1st method, the proteins were extracted at pH4 and then precipitated at pH3. While the 2nd method, the protein were extracted at pH9 and then precipitated at pH4. The 2nd method procedure based on the solubility of soybean seed protein in pH9 solution was the highest, and the isoelectric point of soybean seed protein was at pH4 (Chau *et al.*, 1997). The 1st method procedure as control for indicating the mechanism of free amino acids in the precipitates of protein was trapped in the macromolecule of denatured protein, and the amount of protein precipitate of the 2nd method was more than 1st the method.

These results of this experimental activity were the four various samples of protein precipitate, which were seed protein precipitate from 1st method, sprout protein precipitate from 1st method, seed protein precipitate from 2nd method, and sprout protein precipitate from 2nd method. The precipitates of protein samples were dried by freeze dryer before stored in cool room at 4°C and analyzed

Chemical analysis: moisture, protein, free amino acid, and amino acid profile.

The protein precipitates samples from soybean sprout and seed were subsequently analyzed their moisture content by oven method, protein content by micro-kjeldahl method, and free amino acids content in samples were prepared by precipitation in TCA solution, and then determined by micro-kjeldahl method, total and free amino acids profile by OPA (ortho phentaldialdehyde) method (Antonie *et al.*, 1999 with modification) using HPLC (High Performance Liquid Chromatography). Total amino acids are amino acids that combine each other to make long chain (protein or peptide) + free amino acids, while free amino acids are amino acids that do not combine with each other in the form protein macromolecule or liberated-amino acids from protein or peptide. The amino acids that have been known as insulin stimulation (Leu, Arg, Ala, Phe, Ile, Lys and Met) could be determined based on the amino acid profile.

Preparation of sample for HPLC analysis of total amino acid

Take 0.37 g of protein isolate, and then wasadded 10 ml 6 N HCl, and hydrolyzed by autoclafe at 110°C, 12 hr. After that, the samples were cooled and neutralized by adding 6 N NaOH. Add 2.5 ml 40% Pb-acetate and 1ml 15% oxalate acid and then was

fixed 50 ml with aquabides. Take 3 ml and filtered by millex 0.45 μm, and then mixed with OPA solution for 3 minutes. The 30 μL sample solution was injected to HPLC.

Preparation of sample for HPLC analysis of free amino acid

Take 0.15 g of protein isolate, and then was added 25 ml aquades, and mixed by vortex for 10 minutes. After that the solution of protein was filtered by millex 0.45 μm, and then mixed with OPA solution for 3 minutes. The 30μL sample solution was injected to HPLC.

Preparation of amino acids standar solution for HPLC analysis

Take 50 μL 2.5 ppm amino acid standard solution and then was mixed with 950 μL OPA solution for 3minute. The 30 μL standar solution was injected to HPLC. The conditions of HPLC of the analysis were Column: Eurospere 100-5 C18, 250 x 4.6 mm with precolumn P/N: 1115Y535; Eluen: A = 0.01 M Acetic buffer pH5.9; Eluen B = MeOH: 0.01 M Acetic buffer pH5.9:THF 80:15:5; λ Fluorescence: Ext = 340 nm; Em = 450 nm.

Experimental design and data analysis

The experimental design of this research was randomized complete design with 1 factor, which was the four various protein precipitate, i.e seed protein precipitate from 1st method, sprout protein precipitate from 1st method, seed protein precipitate from 2nd method, and sprout protein precipitate from 2nd method. Statistical analysis by Analysis of Varian and Duncan Multiple Range Test.

Results and Discussion

As shown in Table 1 and 2, there were free amino acid in the precipitates of protein from soybean sprout and seed that were isolated by 1st and 2nd methods respectively. The analysis of amino acids profile were preceded by determination of protein and free amino acids in protein samples. The amount of free amino acids which could be trapped in the precipitate of protein macromolecule from 2nd method was higher than 1st method. The mechanism of free amino acid was explained in the following discussion.

Table 1 showed that the amount of free amino acids from 1st method were no significant different between soybean sprout and seed, and a lot of free amino acids of protein precipitate were no detected. Table 2 showed that the protein of sprout and seed did not showed significant differences. Free amino acid

Table 1. The total and free amino acid profile of soybean sprout (seed as a control) protein that were isolated by 1st method

Group of amino acids	Amino acids	Total amino acid profile (%db of seed)		Free amino acid profile (%db of seed)	
		Seed	Sprout	Seed	Sprout
Stimulation of Insulin	Leu	0.02	0.02 *	0.00	0.00 *
	Arg	0.03	0.03 *	0.00	0.00 *
	Ala	0.09	0.10 *	0.00	0.03 *
	Phe	0.00	0.01 *	0.00	0.00 *
	Ile	0.02	0.03 *	0.01	0.01 *
	Lys	0.02	0.02 *	0.00	0.00 *
	Met	0.01	0.02 *	0.00	0.00 *
	Sub-total	0.18	0.24 *	0.02	0.06 *
Non-Stimulation of Insulin	Asp	0.05	0.08 *	0.00	0.01 *
	Glu	0.07	0.09 *	0.00	0.01 *
	Ser	0.02	0.02 *	0.00	0.00 *
	His	0.01	0.02 *	0.00	0.00 *
	Gly	0.00	0.01 *	0.00	0.00 *
	Thr	0.02	0.02 *	0.00	0.00 *
	Tyr	0.00	0.00 *	0.00	0.00 *
	Trp	0.02	0.01 *	0.01	0.01 *
	Val	0.01	0.02 *	0.01	0.00 *
	Sub-total	0.20	0.27	0.03	0.03
Total		0.39	0.50 *	0.05	0.10 *

* Not significant different between seed and sprout in the same of amino acid profile.
** Significant different between seed and sprout in the same of amino acid profile

of protein precipitate from 2nd method were detected and showed significant different between seed and sprout, indicating that the germination for 36 h did not change N total of seed. Therefore the following discussion for comparing amino acid profile between soybeen seed and sprout were based on 2nd method protein isolation, which was seen at Table 2.

Mechanism of free amino acid in the protein precipitatesbased on 1st and 2nd methods of protein isolation

Based on 1st method and 2nd method of protein isolation could be known that the mechanism of free amino acids in the precipitates of protein was trapped in the macromolecule of denatured protein and then free amino acid could be precipitated. These mechanisms could be proved through this experiment that was indicated by the amount of free amino acid from 2nd method was higher than 1st method. It was due to the protein total and the yield

Table 2. The total and free amino acid profile of soybean sprout (seed as a control) protein that were isolated by 2nd method

Group of amino acids	Amino acids	Total amino acid profile (%db of seed)		Free amino acid profile (%db of seed)	
		Seed	Sprout	Seed	Sprout
Stimulation of Insulin	Leu	1.19	1.08 **	0.02	0.07 **
	Arg	1.05	0.94 *	0.08	0.03 **
	Ala	2.28	2.13 *	0.06	0.51 **
	Phe	0.44	0.49 **	0.15	0.26 **
	Ile	1.14	1.12 *	0.04	0.04 *
	Lys	1.65	1.64 *	0.04	0.11 **
	Met	0.83	0.88 *	0.15	0.07 **
	Sub-total	8.60	7.80 **	0.52	1.08 **
Non-Stimulation of Insulin	Asp	2.81	2.92 *	0.10	0.13 *
	Glu	5.24	5.43 *	0.06	0.30 **
	Ser	0.84	0.75 **	0.03	0.27 **
	His	0.66	1.92 **	0.02	0.19 **
	Gly	0.22	0.03 **	0.00	0.02 **
	Thr	1.37	1.37 *	0.03	0.26 **
	Tyr	0.44	0.39 *	0.05	0.06 *
	Trp	0.37	0.36 *	0.30	0.06 **
	Val	1.29	1.06 **	0.06	0.06 *
	Sub-total	13.24	14.23	0.65	1.35
Total		21.83	22.01 *	1.13	2.39 **

* Not significant different between seed and sprout in the same of amino acid profile.
** Significant different between seed and sprout in the same of amino acid profile

of protein precipitate from 2nd method was higher than 1st method, hence the amount of free amino acids which were trapped in the macromolecules of protein as much as the amount of protein which was precipitated. In 2nd method, protein was extracted at pH9 and precipitated at pH4. The solubility of soybean seed protein in pH9 solution was the highest, and the isoelectric point of soybean seed protein was at pH4 (Chau *et al.*, 1997). On the other hand, The adjustment of pH (extraction at pH9 and followed precipitation at pH4) has been applied in commercial and laboratory scale of soybean protein isolate production. While in 1st method, a lot of free amino acids of protein precipitate was no detected, and the amount of free amino acids were no significant different between soybean sprout and seed.

This experiment indicated that free amino acid could be trapped in protein precipitate, although every amino acid has a different isoelectric point. Fifteen of the 20 amino acids, those with non polar neutral side chain have isoelectric points in the range of 4.8-6.3. The three basic amino acids have higher isoelectric points, and the two acidic amino acids have lower ones (Murry, 2010). Based on the discussion of this section could be concluded that free amino acids could be got through 2nd method protein isolation, due to the amount of free amino acids which could be trapped in the precipitate of protein macromolecule from 2nd method was higher than 1st method. Therefore, the following discussion to know the effect of soybean seed germination on amino acid profile was based on the result of amino acids profile analysis of protein precipitate from 2nd method. It is due to the amount of free amino acids of protein precipitate from 1st method were no significant different between soybean sprout and seed,that was seen at Table 1. While, the amino acid profile of protein precipitate from 2nd method changed significantly between soybean sprout and seed that was seen at Table 2.

Comparison of total and free amino acid profile of soybean sprout and seed for determining insulin stimulation amino acids.

Germination of soybean seed for 36 hr increased free amino acids which were indicated by the free amino acid of protein precipitate from soybean sprout was higher than soybean seed, that was seen at Table 2. The result also showed that the free amino acids of sprout protein was about 2 times compared to seed protein. The seed germination for 36hr increased free amino acids, it was possible due to increasing proteases activity during germination that hydrolyzed protein and liberated amino acids. Some of the high molecular weight proteins disappeared after germintaion of *Lupinus angustifolius* L. (Rumiyati *et al.*, 2012). Martinez *et al.*, (2006) explained that soybean seed germination for 96hr increased free amino acids. The amino acid such as glutamic acid content of eighteen varieties of Malaysian brown rice increased significantly after pre-germination (Roohinejad *et al.*, 2011). The free amino acids in raw soybeans were not detected but determined in germinated soybeans (Huang *et al.*, 2017)

The analysis of total amino acids profile showed that there were no significant different between sprout and seed protein, except a few kind of total amino acids (6 kind of total amino acids) that changed significantly. Seed germination for 36 hr decreased Leu, Ser, Gly, and Val but increased Phe, and His. While the analysis of free amino acids profile

showed that many kind of free amino acids (12 kind of free amino acids) changed significantly between sprout and seed protein. Seed germination for 36 hr increased Leu, Ala, Phe, Lys, Glu, Ser, His, Gly, and Thr but decreased Arg, Met, and Trp. The changes of some kind of amino acids may be caused by protein synthesis and generation of energy through oxidation of the carbon skeleton from amino acids during seed germination. The research was conducted by King and Puwastien (1987) showed that germination of winged bean changed many kind of amino acid. Chiou *et al.* (1997) also invented that germination of peanut changed the amount and kind of amino acids. The essential amino acids clearly increased after germination in peanut seed (Li *et al.*, 2014). The leucine to iso-leucine ratio decreased from 1.05 to 0.98 whereas % Essensial amino acid index increased from 80.92 to 90.50% after germination of *Cicer arietinum* (Sibian *et al.*, 2016).

The analysis of amino acids profile also indicated that the total and free amino acids as insulin stimulation of soybean sprout and seed showed significant differences. The free amino acids as insulin stimulation of soybean sprout increased significantly, but the total amino acids as insulin stimulation decreased significantly. Soybean seed germination for 36 hr decreased Arg and Met but increased Ala, Leu, Phe, and Lys, which were 8.5; 3.5; 2.8; and 1.7 times respectively compared to the original seed. These result showed soybean sprout contained higher amino acid for stimulation of insulin secretion than soybean seed. Therefore protein isolate of soybean sprout that was extracted at pH9 and then precipitated at pH4 might be potential as functional food for diabetic.

Kanetro *et al* (2008) had showed that the protein isolate of soybean sprout that was extracted at pH9 and then precipitated at pH4 could increased stimulation of insulin secretion by in vitro bioassay of pancrease islet of normal and diabetic rats, compared to protein isolate of the original seed. The amino acids in soybean seed naturally combined each other to make long chains in the form of protein macromolecule complex. Germination liberated and increased free amino acid, so the acceleration of free amino acids in protein soybean sprout to be utilized by pancreatic β-cell for stimulation of insulin secretion may be faster than amino acids in the form of macromolecule. The specific free amino acids could increase stimulation of insulin secretion (Newsholme *et al.*, 2007). The development of the anti-diabetes property in soybean seed during germination was as the result of the synthesis of phosphatydylinositol 3 kinase, and it was an important component of insulin receptor/tyrosin kinase (Pathak, 2005). Soybean sprout protein also showed the role as insulin-like protein (Pathak dan Martirosyan, 2011).

Conclusion

Based on the research could be concluded that germination of soybean seed for 36 hr increased the specific free amino acids as stimulation of insulin secretion, particularly Ala, Leu, Lys, and Phe of protein soybean sprout which were 8.5; 3,5; 2.8; and 1.7 times respectively compared to the specific free amino acids of the original seeds. The protein isolate of soybean sprout that was extracted at pH9 and then precipitated at pH4 might be potential as functional food for type 2 diabetes mellitus especially due to the lack of energy for insulin secretion.

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