Recovery of Natural Folic Acid and It's Identification as Protein Isolate Through Beans Extraction Fermented by *Rhizopus* sp. and *Rhizopus oligosporus* C_1 for Smart Food Formula

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ABSTRACT

Protein isolate produced through fermentation of soy beans (Glycine soja L.), mung beans (Phaseolus radiatus L.) and kidney beans (Phaseolus vulgaris L.) using fungi of Rhizopus sp. (mixture) or Rhizopus oligosporus C_1 (single) are natural source of folic acid for formulation of smart foods. The objective of this experimental activity was to know potential use of fermented beans (tempeh) as protein isolate as a result of pulverizing process and filtration through 100 mesh sieve and characteristic of its monomer, particularly glutamic acid as a part of folic acid via LC-MS. Protein isolate extract was generated via pulverizing mixture between soy tempeh, mung bean tempeh and kidney bean tempeh, and water with ratio of 1 : 1, 1 : 2, 1 : 3, 1 : 4 and 1 : 5, respectively followed by filtration through 100 mesh sieve. The result of experimental activity displayed that interaction of all treatments affected on composition of tempeh extraction result as protein isolate. Identification using LC-MS showed that difference in relative intensity for 3 kinds of tempeh on presence of glutamic acid. Protein isolates from soy tempeh, mung bean tempeh and kidney bean tempeh produced domination of glutamic acid monomer indicated subsequently at chromatogram with T 4.1, T 5.7 and T 7.1; T 4.0 and T 3.9.

Keywords: Tempeh, protein isolate, folic acid, glutamic acid, pulverizing.

ABSTRAK

Isolat protein yang diperoleh melalui fermentasi kacang kedele (Glycine soja L.), kacang hijau (Phaseolus radiatus L.) dan kacang merah (Phaseolus vulgaris L.) menggunakan kapang Rhizopus sp (campuran) atau Rhizopus oligosporus C_1 (tunggal) adalah sumber asam folat alami untuk formulasi pangan pintar. Penelitian ini bertujuan untuk mengetahui potensi kacang-kacangan terfermentasi (tempe) sebagai isolat protein melalui proses pelumatan dan filtrasi lolos 100 mesh serta karateristik monomernya terutama asam glutamat sebagai bagian dari asam folat melalui LCMS. Ekstraksi isolat protein dilakukan melalui pelumatan dan filtrasi lolos 100 mesh pada tempe kedele, tempe kacang hijau dan tempe kacang merah pada rasio tempe dan air 1:1, 1:2, 1:3, 1:4 dan 1:5. Hasil penelitian menunjukkan bahwa interaksi seluruh perlakuan berpengaruh terhadap komposisi hasil ekstraksi tempe sebagai isolat protein. Idensifikasi melalui LCMS memperlihatkan bahwa isolat protein tempe kedele, tempe kacang hijau dan tempe kacang merah menghasilkan dominasi monomer asam glutamat yang ditunjukkan berturut-turut pada kromatogram dengan T4.1, T 5.7 dan T7.1 ; T 4.0 dan T 3.9.

Kata-kata kunci : tempe, isolat protein, asam folat, asam glutamat, pelumatan.

I. INTRODUCTION

Soy bean extract, mung bean extract, and kidney bean extract produced from fermentation processes by using inoculums of *Rhizopus* sp (mixture) and *Rhizopus oligosporus* C_1 (single), respectively under the best condition of fermentation processes, have potential use as protein isolate in order to get folic acid fortified to formulation of smart foods. Folic acid is very important for various human body function starting from synthesis of nucleotide to remetilation of homocysteine, particularly at period of cells fission and growth. Children and adults need folic acid to produce red blood cells and prevent anaemia [1], [2]. Folic acid is sensitive on oxygen, light and high temperature, processes conducted to get protein isolate as concentrate product has get special attention in order to loose folic acid as minimum as possible. Formation of folic acid in tempeh is effected by activity of fungi proteolytic because folic acid is formed by pteridin heterocyclic, para-aminobenzoat acid (PABA) and glutamic acid [3], [4] besides factor of tempeh initial raw material. Beans are the best source of vegetable protein, in which activity of fungi proteolytic will degrade protein into amino acids, particularly glutamic acid as an indicator of its presence of folic acid.

To get tempeh protein isolate, pulverizing method is needed to reduce particles size of tempeh and broad surface tension of particle. Size reduction is aimed to divide a solid material to smaller parts by using mechanical force or pressure [5] and one of the useful unit operation in chemical engineering to generate a high valuable product. Size reduction is divided to two categories, namely solid and liquid [6]. Operation of size reduction on tempeh commodity is proposed to help and facilitate extraction process in order to broad surface of material, which will be processed further or aid mixing process [7]. On tempeh commodity, size reduction is performed by adding water mass at certain ratio of tempeh to water in order to yield suspension of protein extract. Use of blender in pulverizing method is crusher method of a material in scale down [8]. Product of pulverizing is protein isolate suspension, which can be dried as solid and concentrated as thick liquid. This method enables to be extracted folic acid with minimum losses, although use of mechanical force will affect on recovery of folic acid. Through property of water soluble folic acid, a different ratio of tempeh to water enables to extraction faster. In progress, recovery of tempeh paste and tempeh extract as a result of sieving through 100 mesh has both different composition and characteristic of tempeh isolate, especially amino acids or glutamic acids. They can be traced through difference in relative intensity by means of LC-MS. It had been known that glutamic acid is the part of folic acid [9]. which its presence and identification of folic acid is a parameter from its presence of folic acid, in which a ratio of glutamic acid, pteridine heterocyclic and para-aminobenzoat acid (PABA) in folic acid can be known via its difference in molecular weight (MW). Identification of glutamic acid based on MW through LC-MS is performed to know dominant of glutamic acid monomer according to intensity represented as characteristic of protein isolate. Liquid Chromatography (LC-MS) coupled by Mass Spectrometry (LC-MS) is a hybrid between chromatography and mass spectrometry. By using chromatography, it will be separated molecular mixtures according to difference in migration speed and molecule distribution in fixed phase (adsorben) and dynamic phase (eluent), while mass spectrometry will ionize analite based on electro spray ionization (ESI) principle to gas phase (fine aerosol) [10]. Peak of glutamic acid on chromatogram becomes more and more much, it increases intensity and concentration in protein isolate indicating as potential source of folic acid for brain healthy.

The aim of this experimental work was to know process condition of pulverizing and separation protein isolate by means of a 100 mesh sieve. Based on the best process condition, concentration of folic acid in both tempeh pulp and extract as protein isolate and its identification as glutamic acid in soy tempeh, mung bean tempeh and kidney bean tempeh had the potential use as a source of folic acid for smart food formula.

II. EXPERIMENTAL SECTION

2.1 Materials

Main materials used in this experimental activity were fermented beans (tempeh) from soy beans (*Glycine soja* L.), mung beans (*Phaseolus radiatus* L.) and kidney beans (*Phaseolus vulgaris* L.) purchased in local market, inoculum of *Rhizopus* sp. (mixture) supplied from P. T. AFI (Bandung), inoculum of *Rhizopus oligosporus* C_1 (Research Center for Chemistry – LIPI), RO water or distilled water and chemicals for preparation and analysis purposes. HCl, Sodium nitrite, sulfamic acid, 3-aminophenol, acetic acid, methanol. All of the chemicals were of reagent grade quality.

2.2 Instrumentations

Main equipments utilized in this experimental activity were glassware, distilling unit (Gesellschaft fur Labortechnik GmbH/GFL, Type 2012,Germany), microbiology equipments, laminar flow system, water bath (Memmert, Germany), incubator (local), fermentation system, blender, sieve of 100 mesh (Retsch, Germany), calibrated pH meter, thermometer, magnetic stirrer (HI 303 N, HANNA Instrument, Japan), pressure gauge of technical nitrogen (Fisher Scientific Company, England), cylindrical tank for technical nitrogen (Local), stop watch (Hanhart Profil 2, Germany), Dead-End Stirred Ultrafiltration Cell (SUFC) (MILLIPORE, Model 8200, U. S. A.) [11], UV-Vis Spectrophotometer (Model RF-550, Shimadzu, Japan) and LC-MS (Mariner Biospectrometry) with LC (Hitachi L 6200) [12].

2.3 Experimental Design and Analysis

Soy bean tempeh, mung bean tempeh and kidney bean, respectively was pulverized by adding water at a 1 part : 1 part ratio of soy bean tempeh, mung bean tempeh and kidney bean to water, followed by 1 : 2, 1 : 3, 1 : 4 and 1 : 5, and filtered through 100 mesh sieve to result tempeh protein isolate as filtrate and residues. Analysis was performed on total solids (Gravimetric method), dissolved protein (Lowry method) [13], N-amino (Copper method) [14], folic acid (UV-Vis Spectrophotometer) [15]. Identification on folic acid was conducted by means of LC-MS (Mariner Biospectrometry) with LC (Hitachi L 6200) [12]. Process and analysis were conducted in duplicate. Data were processed in description based on result of average analysis.

2.4 Procedure

Fermentation Process of beans (tempeh)

A number of soy bean, mung bean and kidney bean, respectively was sorted, washed, blanched for 30 - 45 minutes, cooled to room temperature, and soaked overnight. Soaked beans were measured pH to 5. Soaked beans were subsequently pelled, washed, allowed to cold, and inoculated by tempeh inoculum. Fermentation conditions were as follow : soy beans using inoculum of *Rhizopus* sp. (mixture) 0.2 % (w/w) was incubated at 30 °C for 48 hours; mung beans using inoculum of *Rhizopus oligosporus* C_1 0.2 % (w/w) was incubated at room temperature for 48 hours and Kidney beans using inoculum of *Rhizopus* sp. (mixture) 0.2 % (w/w) at room temperature for 72 hours to produce tempeh with uniform growth of fungi [16].

Pulverizing and separation processes of tempeh protein isolate

A number of tempeh from 3 kinds of beans, respectively was pulverized by adding water at a 1 : 1, 1 : 2, 1 : 3, 1 : 4 and 1 : 5 ratio of soy beans or mung beans or kidney beans to water in order to tempeh suspension. Tempeh suspension was further filtered by 100 mesh sieve to get filtrate as tempeh extract and residue.

III. RESULTS AND DISCUSSION

3.1. Characteristic of Tempeh

Soy beans tempeh (*Glycine soja* L.), mung beans tempeh (*Phaseolus radiatus* L) and kidney mung beans (*Phaseolus vulgaris* L.) have uniform and white micellia growth with specific tempeh aroma and texture according to SNI [17] as shown in Figures 1a, 1b and 1c. Composition of tempeh as raw material in preparation of both protein isolate concentrate and powder as source of folic acid, as indicated in Table 1. This composition tends to differ for each tempeh, particularly pada total solids and folic acid.

3.2. Effect of pulverizing on compositions of tempeh pulp and tempeh extract.
Folic acid (µg/mL)

Pulverizing process of tempeh using blender (crusher system) at a 1 : 1, 1 : 2, 1 : 3, 1 : 4 and 1 : 5 ratio of soy bean tempeh or mung bean tempeh or kidney bean tempeh to water, respectively gave tempeh paste as specific aroma and thick tempeh suspension. Tempeh suspension was filtered through 100 mesh to produce tempeh filtrate with different concentration of folic acid. Ratio of tempeh to water becoming more and more low would increase folic acid concentration on both tempeh paste (pulp) and filtrate from 3 kinds of tempeh, as showed in Figure 2a. Increasing folic acid starts to significant at a 1 : 2 ratio of tempeh to water. In other words, adding water mass becoming more and more much would drop folic acid concentration. The lowest ratio of tempeh to water (1 : 1) resulted optimum concentration of folic acid both in paste (pulp) and filtrate on 3 kinds of tempeh. The whole pulverizing processes and sieving through 100 mesh, a 1 : 1 ratio of tempeh to water gave the highest concentration of folic acid (1,010.8 µg/mL) in soy bean tempeh filtrate

followed by concentration of folic acid in mung bean tempeh filtrate (629.8 µg/mL) and concentration of folic acid in kidney bean tempeh filtrate (752.6 µg/mL). While, concentration of folic acid in mung bean tempeh paste (602.78 µg/mL) was higher than that in soy bean tempeh paste (502.56 µg/mL) and in kidney bean tempeh paste (400.3 µg/mL). Tempeh texture is possibility relating to maturity of tempeh resulting different particles of tempeh at pulverizing runs. Texture of kidney bean tempeh is better than soy bean tempeh and mung bean tempeh, so that they generate smaller particles sizes at pulverizing process and same ratio of tempeh to water. Its presence of folic acid in 3 kinds of tempeh pastes fluctuate for each pulverizing treatment. This matter is caused by crusher system using disc at blender followed by suspension mixing using resiprocating mechanical power. This resiprocating mechanical power causes smaller particles size will mix with other particles in order to re-crush particles to uniform size, as a consequence of fluctuated folic acid concentration.

Figure 1. (a) Soy bean tempeh, (b), mung bean tempeh and (c) kidney bean tempeh from the best process condition, respectively.

Table 1. Compositions of soy bean (*Glycine soja* L.) tempeh, mung bean (*Phaseolus radiatus* L) tempeh and kidney bean (*Phaseolus vulgaris* L.) tempeh.

Kinds of Tempeh	Component			
	Total solids (%)	Dissolved protein (mg/mL)	N-Amino (mg/mL)	Folic acid (µg/mL)
Soy beans	42.92	0.33	1.68	219.8
Mung beans	43.59	0.31	1.12	381.5
Kidney beans	38.01	0.34	1.12	251.1

At adding water mass with different volume causes different system due to same mechanical power (rotation speed of knife and blender) and different loads at each ratio of tempeh to water. Adding water mass on same tempeh weight becoming more and more large will weigh rotation load and lower folic acid concentration extracted. It had been known that particles size of folic acid is proporsional with its molecular weight (MW) (441 Da.) or range of 0.008 – 0.1 µm [18] so that they will pass more much in tempeh filtrate when compared to they retained on the surface of sieve (100 mesh). Folic acid is very sensitive on mechanical treatment, air and light so that interaction of each treatment affected on kinds and initial material composition. Optimum pulverizing and filtration processes at a 1 : 1 ratio of tempeh to water increased subsequently concentrations of folic acid in soy bean tempeh paste, mung bean tempeh paste and kidney bean tempeh paste of 219.8 µg/mL to 502.55 µg/mL (128.64 %, 1.28 folds), 381.5 µg/mL to 629.85 µg/mL (0.651 %, 0.65 folds), and 251.1 µg/mL to 400 µg/mL (0.593 %, 59.3 folds), whereas raising folic acid in filtrate passing through 100 mesh sieve were 219.8 µg/mL to 1,010.85 µg/mL (360 %, 3.6 folds), 381.5 µg/mL to 629.85 µg/mL (0.65 %, 0.65 folds).

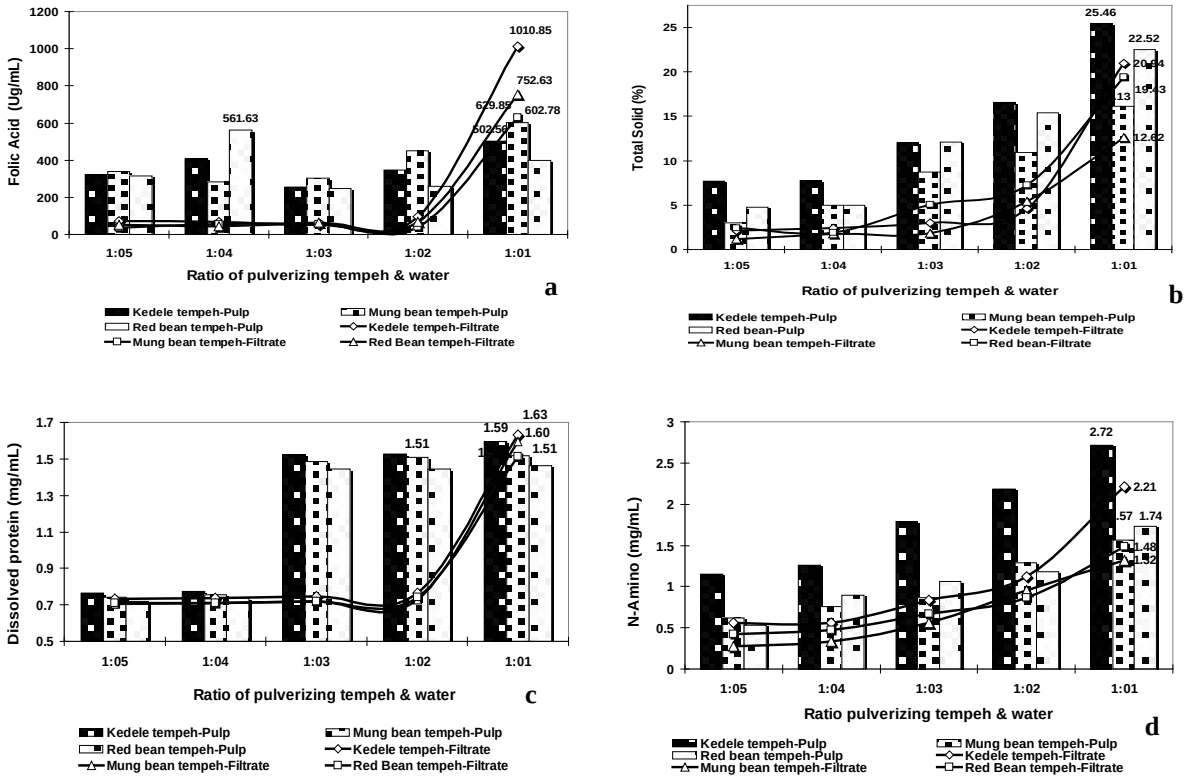


Figure 2. Relationship between kinds of tempeh and ratio of pulverized tempeh to water on (a) folic acid; (b) total solid; (c) dissolved protein and (d) N-Amino in both tempeh pulp and filtrate.

Total solids (%)

Similar patron with folic acid seems at total solids from tempeh paste and tempeh filtrate as a result of filtration through 100 mesh sieve, in which low ratio of tempeh to water will increase total solids both in tempeh paste and tempeh filtrate from 3 kinds of tempeh, as demonstrated in Figure 2b. Pulverizing soy bean tempeh, mung bean tempeh and kidney bean tempeh at a 1 : 1 ratio of tempeh to water result subsequently tempeh paste with the highest total solids of 25.46, 16.12 and 22.52 %, while filtration of pulverized soy bean tempeh or mung bean tempeh or kidney bean tempeh through 100 mesh sieve pass total solids in filtrates of soy bean tempeh, mung bean tempeh and kidney bean tempeh of 20.94, 12.62 and 19.42 %, respectively. In other words, total solids in paste and filtrate of soy bean tempeh was lower than that in kidney bean tempeh and mung bean tempeh. Increasing total solids in tempeh paste was caused by decreasing water mass so that tempeh suspension formed become more and more thick. Besides, difference in particles size from each tempeh relating with kinds of beans, such as roundness), sphericity will affect on surface square of beans seed mass [19] and its total solids content. Spreading inoculum micellia on tempeh is also as contributor of total solids, in which fungi growth on beans surface will bind or cement seeds to tough and dense mass so that adding water mass with large volume to tempeh materials will form suspension with different viscosity affecting on total solids. Whereas, raising total solids in tempeh filtrate is caused by its accumulation of material mass passing through 100 mesh sieve both dissolved and indissolved from adding water mass with less water volume. However, when compared with initial tempeh materials prior to pulverizing and filtration processes at a 1 : 1 optimum ratio of tempeh to water drops total solids in pastes of soy bean tempeh, mung bean tempeh and kidney bean tempeh subsequently 42.92 % to 25.46 %, 43.59 % to 16,12 % and 38.01 % to 22.52 % or it takes place a decrease 68.57 % (0.68 folds), 170.4 % (1.7 folds) and 68.78 % (0.68 folds). While, pulverizing and filtration processes at a 1 : 1 optimum ratio of tempeh to water decreases total solids in filtrates of soy bean tempeh, mung bean tempeh and kidney bean tempeh of 42.92 % to 20.94 %, 43.59 % to 12.62 % and 38.01 % to 19.42 % or it occured a drops 104.96 % (1.04 folds),

254.4 % (2.45 folds) and 95 % (0.95 folds). These declines of total solids are caused by adding water mass at various ratios.

Dissolved Protein (mg/mL)

Similar profile from folic acid and total solids on dissolved seemed at both tempeh paste and filtrate as a result of filtration through 100 mesh sieve, in which low ratio of tempeh to water would increase dissolved proteins in tempeh paste and tempeh filtrate for 3 kinds of tempeh, as demonstrated in Figure 2c. Increasing total protein start to seem subsequently by adding water mass at a 1 : 3 ratio of tempeh to water in tempeh paste, and by adding water mass at a 1 : 2 ratio of tempeh to water in tempeh filtrate. Optimization of dissolved protein was reached at the highest concentration of soy bean tempeh filtrate (1.63 mg/mL) when compared to all others treatments. Dissolved protein is all amino acids, peptides and protein derivatives linked to other components as a result of enzymatic activity during fermentation, particularly fungi activities of proteolytic, amylolytic and lipolytic. At a 1 : 5 ratio of tempeh to water and 1 : 4 ratio of tempeh to water, its presence of dissolved protein in paste and filtrate have similar pattern. However, by adding less water mass seems a difference in sufficient high dissolved protein to the lowest ratio of tempeh to water (1 : 1). This matter showed that processes relating to recover tempeh protein isolate affects on the whole compositions. Pulverizing process using crusher system (blender) and accurate volume of water mass (1 : 3, 1 : 2 and 1 : 1) in tempeh paste is able possibility to extract protein, whereas its extract filtered through 100 mesh sieve is reached at lower ratio of tempeh to water (1 : 2). This matter is not only caused by process condition, but also by water dissolved tempeh protein property and concentration dissolved protein in initial tempeh material. Pulverizing process and filtration under optimal ratio tempeh to water (1 : 1) increased subsequently dissolved proteins in soy bean tempeh paste, mung bean tempeh paste and kidney bean tempeh pastes from 0.33 mg/mL to 1.6 mg/mL, 0.31 mg/mL to 1.51 mg/mL, and 0.34 mg/mL to 1.46 mg/mL or it occurred an increase 385 % (3.85 folds), 364 % (3.64 folds), and 329 % (329 folds). While, increases of dissolved protein contained in soy bean tempeh filtrate, mung bean tempeh filtrate and kidney bean tempeh filtrate as a result of sieving through 100 mesh are subsequently from 0.33 mg/mL to 1.63 mg/mL, 0.31 mg/mL to 1.6 mg/mL, and 0.34 mg/mL to 1.51 mg/mL or it takes place increasing 393 % (3.93 folds), 416 % (4.16 folds) and 344 % (3.44 folds), respectively.

N-Amino (mg/mL)

N-Amino is perception of protein as amino acids produced from fermentation process of soy beans, mung beans and kidney beans. Proteolytic activity of fungi will degrade protein in beans to amino acids, flavor, taste, aroma and appearance from the whole tempeh. There is a real correlation between the whole tempeh appearance and organoleptic quality based on difference in N-amino for each kind of tempeh. It had been known that tempeh with “maturity” apperance will be dark brown, thin micellia and more wet. N-amino is generally higher than that fresh tempeh appearance to be consumed. This matter showed that kind and concentration of inoculum, temperature and time of fermentation and fermentation environmental condition (humidity) affects on N-Amino. Pulverizing process on soy bean tempeh, mung bean tempeh, and kidney bean tempeh at a low ratio of tempeh to water increase N-Amino concentration in paste and filtrate as a result of sieving through 100 mesh, in which higher concentration of N-Amino in tempeh paste than that concentration of N-Amino in filtrate, as represented in Figure 2d. At 1 : 1 ratio of tempeh to water yielded the highest concentrations of N-Amino in tempeh paste and tempeh filtrate as a result of filtration through 100 mesh sieve when compared to all treatments of ratio tempeh to water. Process optimization was reached by soy bean tempeh paste with N-Amino concentration of 2.72 mg/mL, which is higher than mung bean tempeh paste (1.57 mg/mL) and kidney bean tempeh paste (1.74 mg/mL mesh sieve at a 1 : 1 ratio of tempeh to water from 3 kinds of tempeh increased N-Amino concentrations in soy bean tempeh paste, mung bean tempeh paste and kidney bean tempeh paste subsequently from 1.68 mg/mL to 2.72 mg/mL, 1.12 mg/mL to 1.57 mg/mL and 1.12 mg/mL to 1.74 mg/mL or it takes place a raise 62 % (0.62 folds), 40 % (0.4 folds) and 55 % (0.55 folds). While, raising N-Amino concentrations in soy bean tempeh filtrate, mung bean tempeh filtrate and kidney bean tempeh filtrate subsequently from 1.68 mg/mL to 2.21 mg/mL, 1.12 mg/mL to 1.32 mg/mL and 1.12 mg/mL to 1.48 mg/mL or 31 % (0.31 folds), 17 % (0.17 folds) and 24 % (0.24 folds).

From results of feasibility for all pulverizing process and filtration through 100 mesh sieve on soy bean tempeh and mung bean tempeh had been known that 1 : 1 ratio of tempeh to water increased concentrations

of folic acid, dissolved protein and N-Amino, but decreased total solids in tempeh pulp and tempeh filtrate as a result of filtration of tempeh suspension through 100 mesh sieve. Optimization for all pulverizing process and filtration through 100 mesh sieve was reached by kind of soy bean tempeh. Figures 3a, 3b and 3c showed soy bean tempeh pulp, mung bean tempeh pulp and kidney bean tempeh pulp as a result of pulverizing at a 1 : 1 ratio of soy bean or mung bean or kidney bean to water, respectively.

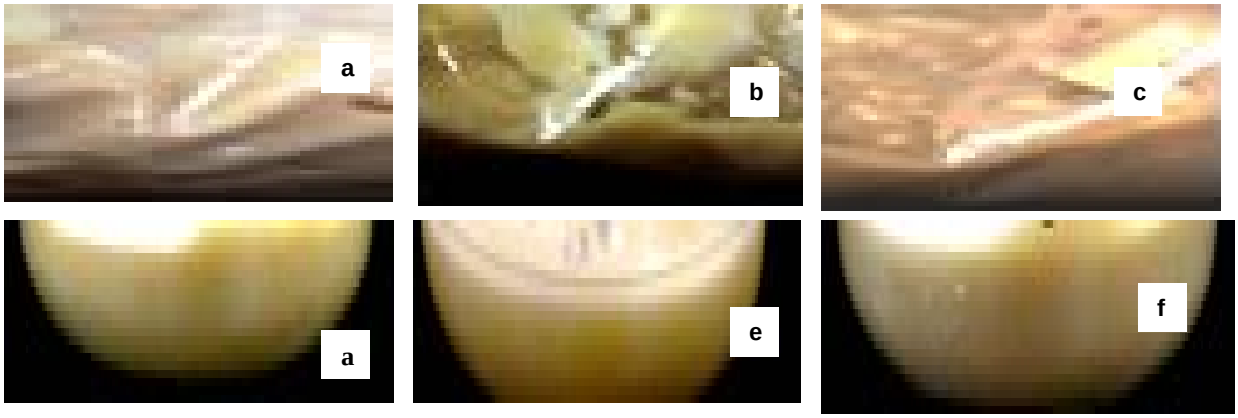


Figure 3. (a) Soy bean tempeh pulp, (b) mung bean tempeh pulp , (c) kidney bean tempeh pulp , (d) soy bean tempeh filtrate, (e) mung bean tempeh filtrate and (f) kidney bean tempeh filtrate .

Identification of tempeh isolate monomer

Identification of folic acid monomer on tempeh isolate was performed by referring its presence as glutamic acid with assumption that glutamic acid is a part of folic acid. The result of identification on standard glutamic was reached 1 peak (T 3.0) at retention time 0 – 10 minutes with relative intensity 100 %, in which at mass spectra 111 – 784 mz from T 3.0 showed compound domination with molecular weight (MW) 148.1479 Da. (100 %), as shown in Figures 4a and 4b. On standard folic acid was get 1 peak (T 3.1) at retention time 0 – 10 minutes with relative intensity 100 %, in which at mass spectra 69 – 1200 mz from T 3.1 indicated compound domination with MW 267.2922 Da. (100 %), which is considered as folic acid as a result of degradation by LC-MS system. Folic acid indicated MW 441 Da. [9] is represented by relative intensity 77 %, as showed in Figures 4c and 4d. All data of monomer as glutamic acid (MW of 148 Da.) or folic acid (MW of 441 Da.) in this analysis as indicated in Table 2.

Soy Tempeh

The result of identification showed that soy bean tempeh isolate is yielded 3 peaks, T 4.1, T 5.7 and T 7.1 at retention time 0 – 10 minutes with relative intensity 100, 43 and 44 %, as indicated in Figure 4e. Mass spectra of 117 – 196 mz from T 4.1 showed compound domination of MW of 132.1727, 166.1808, 148.1423 and 176.2013 Da. with relative intensities 100, 95, 55 and 45 %. However, there is monomer enabling as glutamic acid with MW of 146.0373, 147.188 and 148.1422 Da. and relative intensities 0.14, 20.6 and 52.07 % (Table 3). In other words, monomer with MW of 132.1727 and 166.1808 Da., and relative intensities 100 % and 95.55 % is a result of degradation of folic acid, as demonstrated in Figure 4f. Via LC-MS method had been known that a compound indicated difference in MW, in which its possibility is as M^+, M^+ Na^+, $2M^{++}$ or $2M^+$, Na^+. This matter is caused by its presence of ionization as a consequence of sensitivity of LC-MS instrument relating to eluent used. Operation condition of LC-MS is injection volume of 20 µL, flow rate 1 mL/minutes with eluent mixture of metanol and water (containing 0.3 % acetic acid) at a ratio of 90 : 10 [12] Mass spectra at 59 – 1200 m/z from T 5.7 showed domination of monomer with MW of 219.1393 Da. and relative intensity 100 %. However, monomer get is considered as glutamic acid with MW of 147.8167 and 142.63037 Da. and relative intensities 1.42 and 0.19 % (Table 2). as displayed in Figure 4g, while at T 7.1 is dominated by monomer with MW of 219.2028 Da. and 247.2463 and relative intensities 100 dan 45 %. On the other hand, monomer reached is considered as glutamic acid with MW of 146.4572, 147.2790 and 147.8044 Da. and relative intensities of 1.13, 1.51 and 0.15 % (Table 2) as

demonstrated in Figure 4h. The whole soy bean tempeh filtrate (extract) as a result of pulverizing and filtration via 100 mesh sieve fermented by *Rhizopus* sp at 30 °C for 48 hours resulted 3 peaks of mass spectra T 4.1, T 5.7 and T 7.1 dominated by monomer of glutamic acid with MW of 148.14226, 147.8167 and 147.2790 and relative intensities of 52.07, 1.42 and 1.51 %. Monomer of folic acid (MW of 441 Da.) is not found caused possibility by its occurrence of degradation by LC-MS system.

Table 2. Mononer dominant on mass spectra of folic acid standad, glutamic acid standar , soy bean tempeh and kidney bean tempeh.

Kinds of Mass spectra / Figures	Peak/Index	Centroid Mass	Relative Intensity (%)	Area
Standar of folic acid / T 3.0/ 4b	1	148.157631	11.37	95.19
	2	148.546075	1.34	15.39
	26	442.496215	50.55	631.03
	27	443.166533	8.35	24.35
	28	443.509427	14.98	60.46
	29	444.479853	2.44	78.59
	30	464.491583	1.51	32
Standard of glutamic acid/ T 3.0 /4d	4	148.147897	100.00	2542.26
	5	148.466240	8.51	2542.26
	6	149.145287	7.06	165.60
Soy bean tempeh/ T 4.1	8	146.037322	0.14	31.08
	9	147.188089	20.6	191.48
	10	148.142257	52.07	470.99
Soy bean tempeh/ T 5.68	3	142.630377	0.19	14.97
	4	147.816734	1.42	140.68
Soy bean tempeh/ T 7.1	3	146.45727	1.13	38.65
	4	147.27903	1.51	13.32
	5	147.80446	0.15	82.56
Kidney bean tempeh T 4.0	8	144.187065	4	47.68
	9	147.199931	68.81	696.35
	10	147.575906	5.49	51.03
	11	148.155758	59.44	620.51
	12	148.532616	4.85	52.36
	13	149.162417	3.53	39.42
Kidney bean tempeh T 4.2	7	140.16516	20.39	94.55
	8	144.99348	0.47	172.75
	9	147.19942	18.02	99.91
	10	148.16398	39.43	196.62

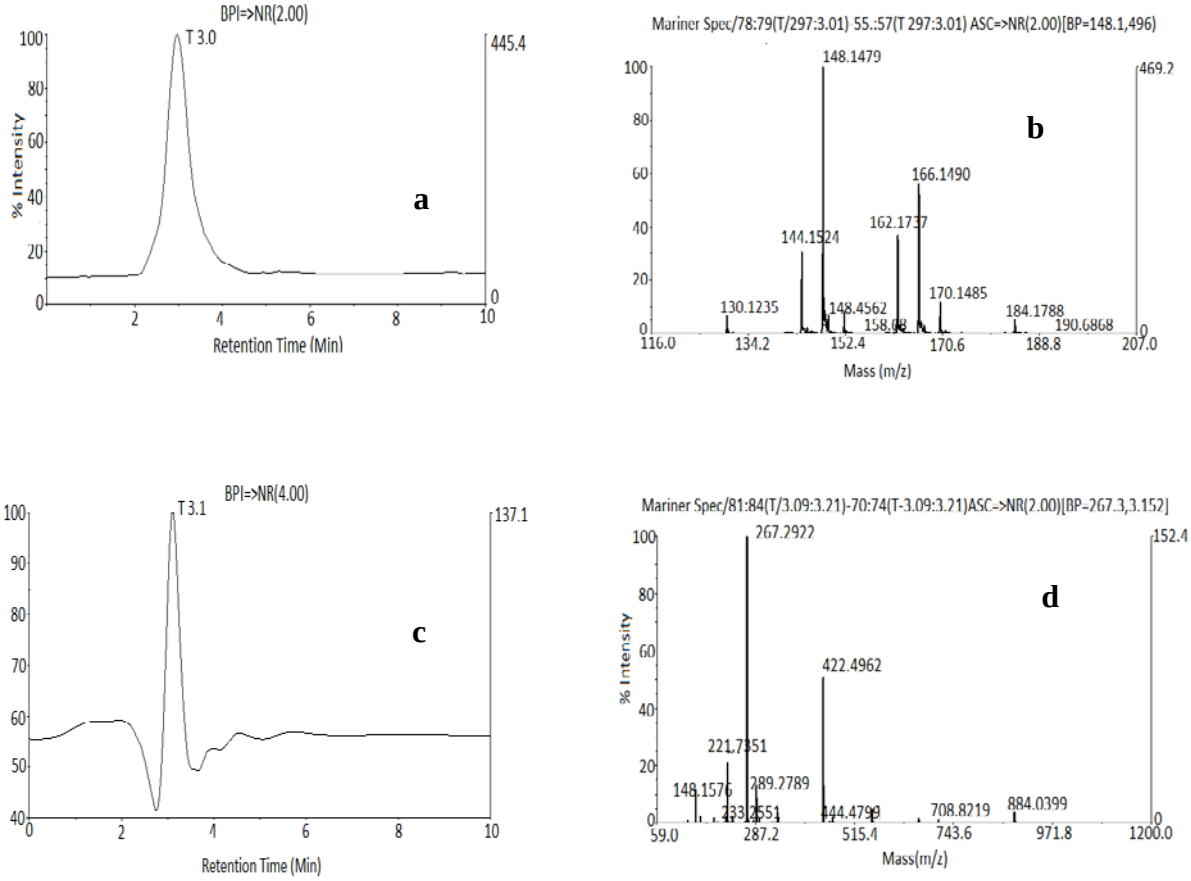
Mung bean tempeh

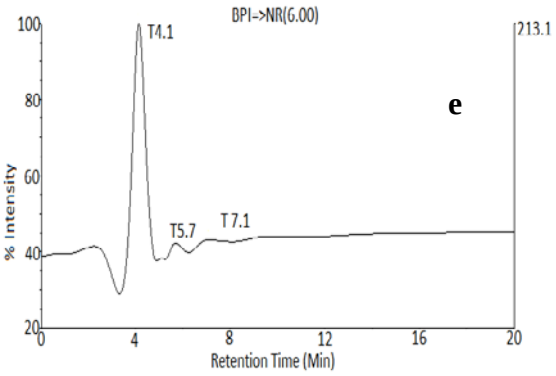
Identification of folic acid in mung bean tempeh filtrate (extract) as a result of fermentation using *Rhizopus oligosporus* C_1 at room temperature for 48 hours displayed 1 (one) peak, namely T 4.0 at retention time 0 – 10 minutes with relative intensity 100 %, as shown in Figure 4i. Mass spectra on T 4.0 at 180 – 1200 m/z showed monomer dominated by MW of 156.1712, 147.1712, 148.1558 and 132.1845 Da. with relative intensities 100, 68.81, 59.44 and 52 %. On same peak is also identified monomer as glutamic acid

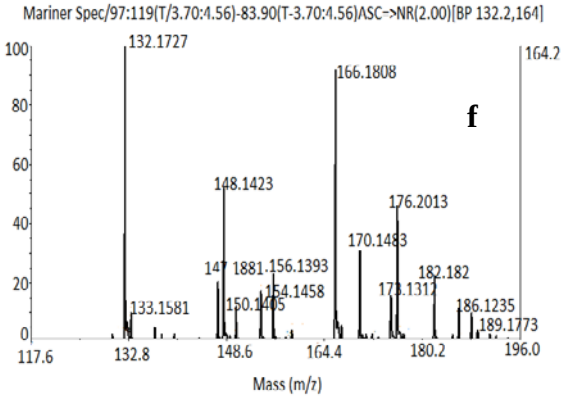
with MWs of 144.1870, 147.5759, 148.1557, 148.5326 and 149.1624 Da. (Table 2). and relative intensities of 4, 5.49, 59.44 and 85 %, as shown in Figure 4j. This matter demonstrated that mung bean tempeh filtrate (extract) has potency as source of folic acid due to high relative intensity of glutamic acid. It had been known that glutamic acid is a part of folic acid, besides para-aminobenzoat acid (PABA).

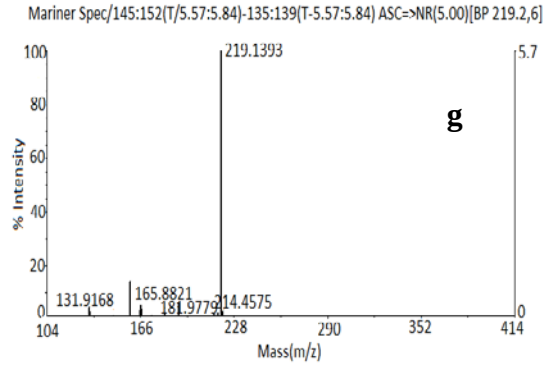
Kidney bean tempeh

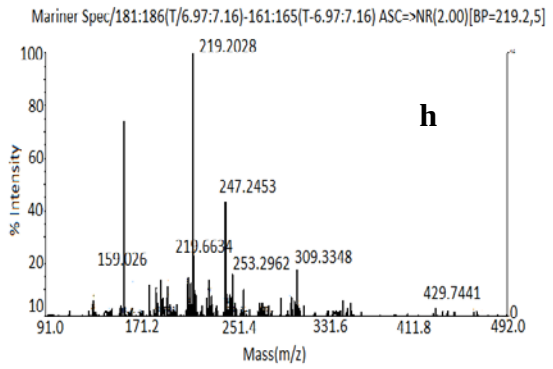
Identification on folic acid in kidney bean tempeh filtrate (extract) as a result of fermentation by using *Rhizopus* sp (mixture) at room temperature for 72 hours gave 1 (one) peak (T 4.2) at retention time 0 – 20 minutes with relative intensity 100 %, as showed in Figure 4k. Dominant monomer at mass spectra T 4.2 showed compounds with MWs of 156.1561, 166.2033, 132.1921 and 148.164 Da. and relative intesities of 100, 79, 59 and 40 %, respectively. Monomer as glutamic acid is considered as MWs of 140.1652, 144.9935, 147.1994 and 148.164 Da with relative intensities of 20.39, 0.47, 18.02 and 39.43 % (Table 2), respectively, as represented in Figure 4l.

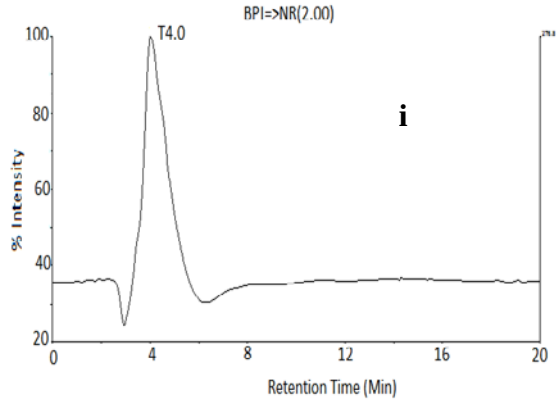


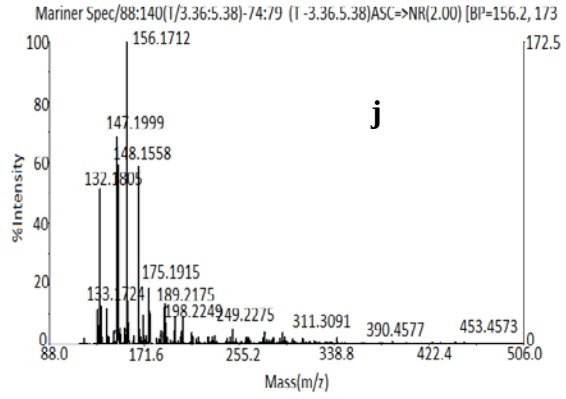

BPI->NR(6.00)
T4.1
T5.7
T7.1
213.1
% Intensity
Retention Time (Min)
e


Mariner Spec/97:119(T/3.70:4.56)-83.90(T-3.70:4.56)ASC->NR(2.00)[BP 132.2,164]
132.1727
166.1808
148.1423
176.2013
170.1483
182.182
133.1581
150.1405
186.1235
189.1773
164.2
Mass (m/z)
f


Mariner Spec/145:152(T/5.57:5.84)-135:139(T-5.57:5.84) ASC=>NR(5.00)[BP 219.2,6]
219.1393
5.7
131.9168
165.8821
214.4575
% Intensity
Mass(m/z)
g


Mariner Spec/181:186(T/6.97:7.16)-161:165(T-6.97:7.16) ASC=>NR(2.00)[BP=219.2,5]
219.2028
247.2453
159.1026
219.6634
253.2962
309.3348
429.7441
% Intensity
Mass(m/z)
h


BPI=>NR(2.00)
T4.0
% Intensity
Retention Time (Min)
i


Mariner Spec/88:140(T/3.36:5.38)-74:79 (T-3.36:5.38)ASC=>NR(2.00) [BP=156.2, 173
156.1712
172.5
147.1999
148.1558
132.1805
175.1915
133.1724
189.2175
198.2249
249.2275
311.3091
390.4577
453.4573
%Intensity
Mass(m/z)
j

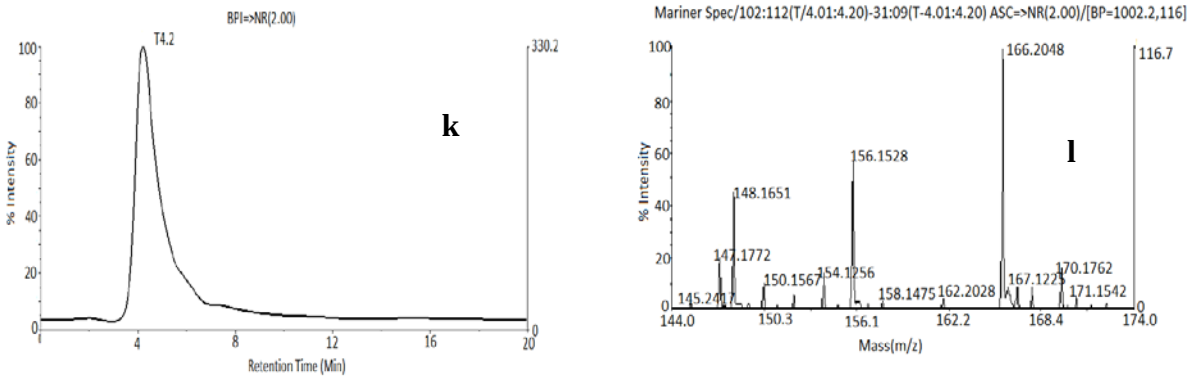


Figure 4. (a) Chromatogram of standard glutamic acid; (b) mass spectra T 3.0 of folic acid standar ; (c) chromatogram of standard folic acid; (d) mass spectra T 3.1 of glutamic acid standar; (e) chromatogram of soy tempeh extract , (f) mass spectra T 4.1 extract of soy tempeh, (g) mass spectra T T 5.7 soy tempeh extract, (h)) mass spectra T 7.1 extract of soy tempeh ; (i) chromatogram of mung bean tempeh extract and (j) mass spectra from T 4.0 extract of mung bean tempeh, (k) Chromatogram of kidney bean tempeh extract and (l) mass spectra from T 4.2 at kidney bean tempeh extract.

IV. CONCLUSIONS

Tempeh and water ratio becoming more and more low will increse total solids, folic acid, dissolved protein, and N-Amino in both soy tempeh pulp and filtrate, mung bean tempeh pulp and filtrate, and kidney bean tempeh pulp and filtrate. Based on composition, optimal pulverization and filtration processes were achieved at a 1 : 1 ratio of tempeh to water. Under this ratio, it can occur in increasing folic acid, dissolved protein and N-Amino, but decreases total solids both in tempeh pulp and filtrate when compared to initial material composition. Increases of folic acid, dissolved protein and N-Amino in soy tempeh pulp, mung bean tempeh pulp and kidney bean tempeh pulp when compared to initial materials were subsequently 1.29, 0.57 and 0.59 folds; 3.85, 3.64 and 3.29 folds; and 0.62, 0.4 and 0.55 folds. While, increases of folic acid, dissolved protein and N-Amino in soy tempeh filtrate, mung bean tempeh filtrate and kidney bean tempeh filtrate when compared to initial materials were subsequently 3.6, 0.65, and 2 times; 3.93, 4.16 and 3.44 times; and 0.31, 0.17 and 0.24 times. Identification on monomer from protein isolates of soy tempeh extract, mung bean tempeh extract and kidney bean tempeh extract displayed subsequently compound domination as glutamic acid with molecular weight (MW) 148.1422 Da., 148.1557 Da., and 148.164 Da., and relative intensity 52.07, 59.44 and 39.43 %, this matter showed that mung bean tempeh extract produced from fermentation by *Rhizopus oligosporus* C_1 at room temperature for 48 hours had a potency use as source of folic acid.

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