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Original Research Article

Comparative study on protein digestibility, protein patterns, antioxidant activities of raw, cooked and fermented soybeans

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ABSTRACT

Soybean (*Glycine max* L. Merr.) is a versatile food source with protein, polyunsaturated fats, fiber, vitamins, minerals and other essential nutrients for humans. The purpose of this study is to compare the protein digestibility among raw, cooked and fermented soybeans (Natto) inoculated with *Bacillus subtilis* var. natto using simulated *in vitro* gastrointestinal digestion technique (IVD). Amino acid composition and antioxidant activity change during IVD of Natto were also investigated. It was found that trichloroacetic (TCA) soluble peptide yield increased around 46 and 62% in Natto and cooked soybean after IVD, respectively. The soluble protein fractions of cooked soybean and Natto during IVD were digested from large protein fraction into smaller protein sub-fraction observed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After IVD, all essential amino acid especially Arg was found to remarkable increase for 6-fold and 49-fold in cooked soybean and Natto, respectively (P<0.05). Interestingly, antioxidant properties increased when IVD times were increased (P<0.05). The increment of antioxidant activities was 2-4 fold changing between before and after IVD. This can be supported by an increment in antioxidant amino acid composition (Trp, Tyr, Met, Cys, His, Phe and Pro) in IVD digested fractions especially in Natto. The highest antioxidant activity was found in fraction after IVD of Natto. These results indicated that Natto had better digestibility and antioxidant activity compared to raw and cooked soybeans.

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INTRODUCTION

Natto, a traditional Japanese, is one of the well-known products produced from fermentation of soybean with pure culture of *Bacillus subtilis* var. natto. It has been consumed as a traditional food in Japan for thousands of years (Weng, Yao, Sparks, & Wang, 2017). Natto contains isoflavones, dietary fiber, vitamins, linoleic acid and some minerals which are originated from soybeans, in addition, it also contains some functional compounds such as enzymes, bioactive peptides, nattokinase (fibrinolytic agent), gamma-polyglutamic acid (γ-PGA) and so on (Sanjukta & Rai, 2016). During fermentation, storage proteins can be degraded by microbial proteases resulting in

soluble solids, particularly soluble nitrogenous compounds and polypeptides. It can increase its nutritional value, because it can improve protein digestibility, reduce anti-nutritional factors, and hydrolyse oligosaccharides (raffinose and stachyose) (Weng & Chen, 2010, 2011).

Apart from microbial fermentation, the ability of human body in digestion Natto and bioavailability or utilize resulting nutrients are also significant. Gastrointestinal digestion has an important influence on the biological activity of food-derived peptides, allowing the release of new active fragments or on the contrary, giving rise to fragments with less or null activity (González-Montoya, Hernández-Ledesma, Silván, Mora-Escobedo, & Martínez-Villaluenga, 2018). Simulated *in vitro* gastrointestinal digestion (IVD) mimics conditions simulated by the digestive processes occurring in the human gastrointestinal tract through proteolytic enzymes (i.e. pepsin-pancreatin enzyme system), measuring the percentage of proteins which is hydrolyzed by such enzymes (Hur, Lim, Decker, & McClements, 2011). The IVD methods employed in this study are based on the measurement of potential susceptibility of protein peptide bonds to proteolysis by digestive enzymes, which is one of the first characteristics of proteins digestibility and therefore in their nutritional value. Moreover, protein digestibility is an important factor to estimate the protein availability for intestinal absorption after digestion reflecting on the efficiency of protein utilization on diet (Almeida, Monteiro, Costa-Lima, Alvares, & Conte-Junior, 2015). The higher proteins are hydrolyzed the more peptide bonds are broken and the more free amino acids and lower molecular weight of oligopeptides are produced (T. M. Weng & Chen, 2010). Amino acid profile is important in evaluating the protein nutritive quality, the digestion of that protein into free amino acids and small peptides is the primary determinant of the absorption of its amino acids by the human body (Chen, Chiou, & Yu, 2010).

Currently, information on digestibility and change in biochemical characteristics of Natto digested by stimulated human gastrointestinal tract is limited. As well as digestibility and bioavailability has been an important aspect concerning many researchers. Thus, the purpose of this study is to evaluate the protein digestibility of fermented soybeans inoculated with *Bacillus subtilis* var. natto compared to raw and cooked soybean using simulated *in vitro* digestion technique. Digested fractions were investigated for trichloroacetic acid (TCA) soluble peptides, protein digestibility as well as free amino acid content. Moreover, change in totalphenolic content and antioxidant activities of digested fractions among were also investigated.

MATERIALS AND METHODS

Materials and reagents

Soybeans (*Glycine* max, cv. Enrei) were obtained from a local market from Ibaraki Prefecture, Japan. Pepsin from porcine gastric mucosa (EC 3.4.23.1, activity of 800-2500 U/mg protein), pancreatin from porcine pancreas (EC 232-468-9, 8 × USP specifications), 1,1-diphenyl-2-picrylhydrazyl (DPPH), and 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ) were all purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), $FeSO_4$, 3-(2-Pyridyl)-5,6-diphenyl-1,2, 4-triazine-p, p′-disulfonic acid monosodium salt hydrate (Ferrozine), $FeCl_2$ trichloroacetic acid (TCA) and all other chemicals were of analytical grade and purchased from FUJIFILM Wako Pure Chemical Corporation, Tokyo, Japan.

Raw material preparation

Dehulled yellow-seeded soybean (300 g) was washed and soaked in using tap water (ratio beans/water of 1:3, w/v) for 18 h at 20 °C. Raw soybean was collected by discarding soak water. Subsequently the bean was washed again with tap water, cooked in fresh distilled water (DW) at the same ratio in pressure cooker for 90 min, cooled, and superficially dried at room temperature. Cooked soybean (150 g) were transferred into glass beaker and inoculated with 50 mL of diluted culture from commercial Natto product Great Natto S-903® produced by S-903 *Bacillus spp.* Natto (Takonofoods Co., Ltd, Tokyo, Japan). After inoculation, the bean (37.5 g) were packed into paper cup (205 mL volume size), covered the top surface with Polyvinylidene Chloride wrap film and incubated at 40 °C for 18 h.

Determinations

In vitro gastrointestinal digestion (IVD)

The static *in vitro* gastrointestinal digestion model described by Tamura, Singh, Kaur, and Ogawa (2016) was used, with minor adjustment. Briefly, for the preparation of pepsin solution, 0.24 g pepsin from porcine gastric mucosa (activity of 800-2500 U/mg protein; Sigma Aldrich, St. Louis, MO, USA) was dispersed in 50 mL gastric fluid buffer (adjusted to pH 1.20). The mixture was then mixed with a magnetic stirrer (Color Squid White, IKA Works, Wilmington, NC, USA) for 10 min. For the preparation of intestinal enzyme solution, 0.2 g pancreatin from porcine pancreas (Sigma Aldrich), 0.015 g invertase and 4 mL amyloglucosidase (Megazyme, Co. Wicklow, Ireland) were prepared as the same manner as previous step but using 25 mL intestinal fluid buffer (adjusted to pH 6.80). The soybean 15% protein (wet weight) (around 150 g) was blended with gastric buffer for 5 min at the highest speed subsequently homogenized with a homogenizer (The Virtis Company Inc., Gardiner, NY) 10,000×g for 5 min. The homogenous sample solution was poured into a glass reactor before starting the experiment. The reactor was connected to a temperature-controlled water bath (NTT-20S, Eyela, Tokyo, Japan) and liquid sample in the reactor was agitated continuously with a magnetic stirrer. The temperature of the reactor was maintained at 37 °C throughout the experiment, and the pH of the sample was adjusted to 1.20 with 3 M HCl before starting of Gastric phase. The gastric phase was initiated by addition of pepsin solution (19 mL), and the pH was re-adjusted to 1.20 ± 0.01 with 0.5 M HCl. After 120 min of the gastric phase, the pH of the sample was adjusted to 6.80 using 3 N NaOH to inactivate the pepsin. The small intestinal phase was initiated by addition of intestinal enzyme solution (23 mL), and the pH was then readjusted to 6.80 ± 0.01 with 0.5 M NaOH. The sample was maintained at the intestinal condition for 2 h. Homogenous liquid sample (10 mL) was taken from the reactor at intervals before (0 h), gastric (1 h), change from gastric to intestinal (2 h) and intestinal (3 h) and after *in vitro* digestion (4 h) and adjusted pH to 7.0 using 1 M NaOH or 1 M HCl. The digestion was immediately terminated by heating the samples at 95 °C for 10 min using a temperature-controlled water bath (T-25, Thomas

Kagaku, Tokyo, Japan). The centrifuge tubes contained aliquots of digested samples were centrifuged at 4,000 ×***g***, 4 °C for 10 min (Model 2800, Kubota, Tokyo, Japan). The supernatant was separated, collected and lyophilized for use in further analysis. Digestibility experiments were carried out in duplicate.

Trichloroacetic acid (TCA)-soluble peptide and protein digestibility

Protein digestibility was calculated and reported in terms of TCA soluble peptides. The TCA-soluble peptide yield (Ysp) was determined according to the method of Wang, Chi, Cheng, and Zhao (2018) with minor modifications. Each digest sample (100 uL) was added 5% (w/w) TCA 900 uL. Next, the mixture was kept for 1 h at 4 °C and the unsolidified protein was then removed by centrifugation 8,000×***g*** for 10 min. TCA-soluble peptide content in the supernatant was determined by measuring absorbance 280 nm using UV-Visible spectrophotometer (V-630, Jasco, Tokyo, Japan). The content of TCA soluble peptides was calculated as the μmol of tyrosine/g of the samples. Digestibility was calculated as follows:

$$\text{Digestibility (\%)} = (A/B) \times 100\%$$

where A is the TCA soluble peptide content of the supernatant, and B is the total protein content of soybean

Soluble protein fractions and distribution by electrophoretic analysis

The supernatant collected from different stage of IVD was determined for protein content by Biuret method (Gornall, Bardawill, & David, 1949) using bovine serum albumin (BSA) as standard. The samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in order to determine the protein patterns by using NuPAGE® gradient precast gel (4-12% gradient) bis-tris (10 × 10 cm^2) in a Novex Xcell Mini cell (Invitrogen, Thermo Scientific, Rockford, IL, USA). NuPAGE® MES SDS Running Buffer was used as the electrophoresis running buffer. According to the difference of protein content in sample solution, the protein solution was diluted with deionized water (DI) to be the same concentration, and then mixed with NuPAGE® LDS Sample Buffer 12.5 μl and NuPAGE® reducing agent 5 μl. The mixture was heated at 70 °C for 10 min. The samples (all containing 20 μg of protein per well) and the protein standard markers (Thermo Scientific, Rockford, IL, USA) were loaded onto the gel. Electrophoresis was performed at 200 V for 35 min. Proteins were visualized by staining with SimplyBlue™ SafeStain (Thermo Scientific, Rockford, IL, USA) for overnight then de-stained with DW for 1 h. The washing water was changed every h until the background was clear, at which point they were then dried.

Free amino acid analysis

Free amino acid content was analyzed by Chemical research center, Research facility centre for science and technology, University of Tsukuba. In summary, freeze dried powder of digested Natto sample was dissolved with DW (20 mg/mL protein content). After mixing well, the aliquot was pipetted out for 0.2 mL, added into 1.8 mL 5% (w/v) TCA solution, mixed and centrifuged by 10,000×*g* for 10 min at 10°C. The supernatant was diluted to appropriate range and adjust pH 2 to 3 by 0.02 N HCl and filtrated through 0.45 μM filter (Polytetrafluoroethylene (*PTFE*), Advantec® Dismic®-13HP, Toyo Roshi Kaisha, Ltd., Chiba, Japan). Finally, the collected supernatant subjected to amino acid analyzer JLC-500 / V2 Automatic Amino Acid Analyzer equipped with ion exchange column (JEOL Ltd., Tokyo, Japan) and the open type AN-II and Type B (FUJIFILM Wako Pure Chemical, Ltd., Osaka, Japan) were used as reference amino acid standards. Free amino acids were separated in high separation mode (110 min) with lithium citrate buffer system and measured by photometric detection after derivatization with ninhydrin.

Total phenolic content

Total phenolic content (TPC) was determined according to the method of Donlao and Ogawa (2018). Briefly, 0.2 mL of the test solution was mixed with 1 mL of 10% (v/v) Folin-Ciocalteu solution and 0.8 mL of 7.5% (w/v) sodium carbonate solution. The mixture was incubated for 1 h at room temperature, and measured for absorbance using a UV-VIS Spectrophotometer at 765 nm. Distilled water (DW) was used as a blank. The TPC was reported as the mg Gallic acid equivalent (GAE) per gram of protein (dry matter).

Antioxidant activity determination
DPPH radical scavenging activity (DPPH)

The DPPH radical scavenging activity of the sample was determined according to the method described in Ketnawa, Benjakul, Martínez-Alvarez, and Rawdkuen (2017). Sample solution (0.5 mL) was added with 0.1 mM DPPH in 95% ethanol (0.5 mL). The mixture was vigorously mixed and allowed to stand for 30 min in the dark at room temperature. The absorbance of the resulting solution was measured at 517 nm. When the DPPH encountered a proton-donating substance such as an antioxidant, the radical would be scavenged, and the absorbance reduced. The DPPH blank is the value of 0.5 mL of 95% ethanol mixed with 0.5 mL of 0.1 mM DPPH in 95% ethanol. The DPPH radical scavenging activity was calculated by a Trolox standard curve (0-1,000 μmol/L) and expressed as μmol Trolox equivalents (TE)/g protein (dry matter).

ABTS radical scavenging activity (ABTS)

ABTS radical scavenging activity was determined according to the method of Ketnawa et al. (2017) with slight modification. ABTS radicals ($ABTS^+$) produced in a reaction of 7 mM ABTS was dissolved in 2.45 mM potassium persulfate allowing the mixture to react in the dark at room temperature for 12 h before use. The $ABTS^+$ solution was diluted with distilled water to obtain an absorbance of 0.7xx at 734 nm. To initiate the reaction, 0.02 mL of the sample was mixed with 0.98 mL of diluted $ABTS^+$ solution. The extent to which the $ABTS^{\bullet+}$ was quenched was measured at 734 nm after 10 min incubation at 30 °C in the dark. Ascorbic acid standard curve (0-0.1 mg/mL) was prepared. Distilled water was used instead of the sample and prepared in the same manner to obtain the control. $ABTS^+$ radical scavenging activity was expressed as mg Ascorbic acid equivalent (AC) per gram of protein (dry matter).

Ferric reducing antioxidant power activity (FRAP)

The capacity of Natto digested fractions to reduce the ferric-tripyridyltriazine complex was evaluated by the FRAP assay as described by Ketnawa et al. (2017) with a slight modification. Freshly prepared FRAP reagent (10 mM TPTZ solution in 40 mM HCl plus 20 mM $FeCl_3 \cdot 6H_2O$ solution and 300 mM acetate buffer, pH 3.6 in the ratio of 1:1:10 (v/v/v)) (1.30 mL) was incubated at 37 °C before being mixed with 0.2 mL of the sample. The mixture was incubated at the same temperature for 30 min in the dark. Absorbance at 595 nm was recorded after 30 min of reaction. The control was prepared in the same manner previously described with DW instead of the sample. The FRAP was calculated from the $FeSO_4$ standard curve (0-100 μmol/L) and expressed as μmol $FeSO_4$ equilavents/g protein (dry matter).

Metal chelating activity (MIC)

The chelating activity on $Fe^{2+}$was measured using the method of Ketnawa et al. (2017) with a slight modification. Diluted sample (0.8 mL) was mixed with 2 mM $FeSO_4$ (0.01 mL) and 5 mM Ferrozine (0.02 mL). The reaction mixture was allowed to stand for 10 min at room temperature. The absorbance was then measured at 562 nm. The blank was conducted in the same manner but DW was used instead of the sample. A standard curve of EDTA (0-10 mmol/L) was prepared and used as the positive control. The control was prepared in the same manner as before with DW instead of the sample. Ferrous chelating activity was expressed as μmol EDTA equivalents/g protein (dry matter).

Statistical Analysis

IVD of Natto was done in duplicate. The other experiments were carried out in triplicate. Data were subjected to the analysis of variance, and mean comparisons were performed using Duncan's multiple range test (Steel and Torrie 1980). Statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS for Windows, SPSS, Inc., Chicago, IL). The differences were considered to significant at $P<0.05$.

RESULTS AND DISCUSSION

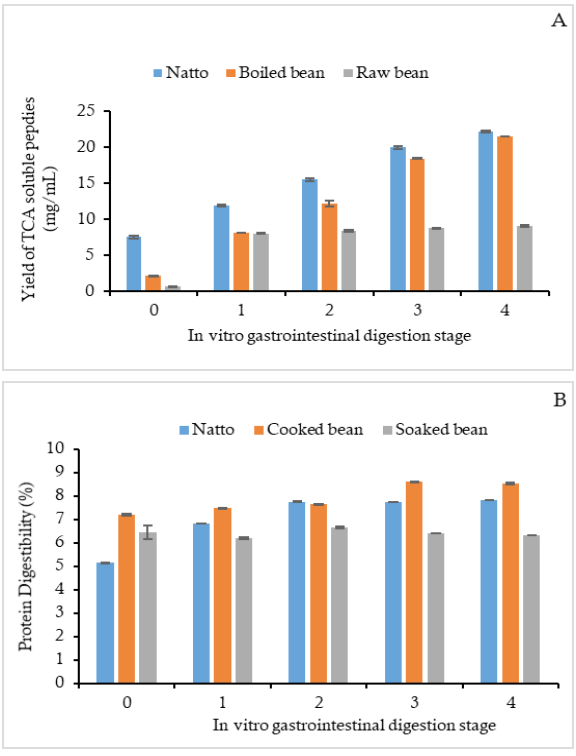
***In vitro* digestibility parameters**

Threes parameters have been often used to evaluate the protein digestibility of Natto, cooked bean and raw bean during a two-stage *in vitro* gastrointestinal digestion (IVD). They are trichloroacetic acid (TCA) soluble peptides, SDS-PAGE profile, and free amino acid composition. Figure 1. shows the variability on TCA soluble peptide and *in vitro* protein digestibility (expressed by the percentage of TCA soluble peptides comparted to total protein content in raw soaked bean). The progress of hydrolysis also confirmed by the determination of TCA soluble peptides content and showed in Figure 1A. Besides, it has been reported that peptides could be more rapidly utilized than amino acids and proteins which might also contribute to the anti-oxidative activity and metal-chelating activity (Kodera, Hara, Nishimori, & Nio, 2006)

TCA-soluble protein means the amount of digestible soybean protein that dissolves in TCA; it measures the portion of protein soluble in TCA which contains polypeptides of fewer than 10 amino acids and free amino acids whereas nondegraded proteins are precipitated by TCA (Chen et al., 2010). It was shown that TCA soluble peptides increased by increasing IVD time. Natto digested fraction showed the highest TCA soluble peptides (7.51-22.13 mg/mL) followed by that in cooked bean (2.09-21.47 mg/mL) and raw bean (0.64-9.07 mg/mL). The higher TCA soluble peptides (7 mg/mL) in Natto before digestion (0) is due to the large amount of soluble peptide initially present in Natto generated by *Bacillus subtilis* fermentation (Kiers, Van laeken, Rombouts, & Nout, 2000). During digestion, TCA soluble peptides was generated more because of the reaction of digestive enzymes. Obviously, TCA soluble protein contents for all kind of bean after IVD (4) was significantly higher than that before IVD (0) ($P< 0.05$). After gastric digestion, the increment of TCA in Natto, cooked bean, raw bean was 2.06-, 5.81- and 13.06-fold whereas total increment after IVD was 2.95-, 10.27-, 14.17-fold, respectively. From these numbers, the huge change was observed in cooked bean digested fraction. There results corresponded with free amino acid content and the increment of free amino acids by grouping reported (data not shown). Increment of TCA soluble peptides from the reaction of gastric enzyme in Natto 2.06-fold and cooked bean 5.82-fold whilst that were 1.43-fold (Natto) and 1.77-fold (cooked bean) from the reaction of digestive enzyme at intestinal stage. These can be inferred that protein digestion happen more in stomach stage more than intestinal stage. Slightly change between before and after digestion in raw bean can be understood by protease inhibitors present especially trypsin inhibitor (TI) in raw bean (Chi & Cho, 2016). that trypsin and α-chymotrypsin inhibitors were 46±6 and 2.7±1.6 IU/10 g Natto. Besides, Natto and cooked bean trypsin inhibitors can be found in slighter amount. TI is a protein-based anti-nutrient substance which inhibits pancreatic protease, proteolysis, and the absorption of dietary proteins (Chi & Cho, 2016). The soybean meal containing TI 4.77 mg/g and decreased to 1.3 mg/g after heat (by steaming at 100 °C for 30 min in an autoclave) (Chi & Cho, 2016).

Change in TCA soluble peptides between before and after digestion showed that cooked bean and Natto protein were digested and significantly improved protein utilization. Previous research also reports an increase in TCA soluble peptides in fermented soybean compared to raw material or protein flour (Weng et al., 2010). The increase in the digestibility values improved the nutritional quality the bean proteins by favoring hydrolysis and the absorption of amino acids and short chain peptides, which is essential to human metabolism as can be seen from amino acid compositions.

Figure 1. Change in Trichoroacetic acid (TCA) soluble peptides (A) and protein digestibility (B) of Natto during stimulated *in vitro* gastrointestinal digestion. Bars represent the standard deviation from triplicate determinations. D.B.: Dry basis



Protein digestibility was calculated from TCA soluble peptides and shown in Figure 1B. Protein digestibility increase as digestion time increased. Cooked bean showed the highest protein digestibility (7.20-8.53 %) followed by Natto (5.14-7.82%) and raw bean (6.19-6.43%). As aforementioned results about the ratio in increment of TCA soluble peptides, cooked bean showed than better increment than others so better digestibility outcome. Raw soaked bean showed stable protein digestibility. Protein digestibility of Natto was higher ratio about 1.48- and 1.66-fold than cooked bean 1.06- and 1.18-fold after gastric and intestinal digestion, respectively. This can be assumed

that during IVD, Natto protein was digested better than cooked bean. As aid from bacteria fermentation, protein ready and easily to digest. The protein digestibility that increased showed the ability of digestive enzymes in gastric digestion stage to digest protein in Natto. A half of the dietary protein leaving stomach is usually in the peptides form with a large proportion being soluble in TCA, i.e. peptides having ten or fewer amino acids (Kodera et al., 2006).

Soluble protein fractions and distribution by SDS-PAGE

Soluble protein distribution profile different stages of IVD in raw soaked bean (S), cooked bean (B) and Natto (N) presents by Bis-Tris-SDS-PAGE analysis and shown in Figure 2. The SDS-PAGE technique has originally been used to study soybean protein subfractions and to evaluate degradation rate of individual IVD digested fraction. The protein subfractions were divided into three parts according to the MW. The protein bands ranged of >55 kDa were group as large size fractions, 17-55 kDa as medium-size fractions, and smaller than 17 kDa as small-size fractions. The major protein subunits can be clearly identified in the raw soybean (lane 2, S0).

Soybeans contain two main storage proteins, β-conglycinin and glycinin, which are composed of several subunits. As been shown in lane 2, S0, 7S globulin consists of three subunits α' (ca 71 kDa), α (ca 67 kDa) and β (ca 55 kDa). 11S globulin is a hexamer, and is made up of five different subunits, each of which consists of an acidic subunit A (acidic pI) with a molecular mass about 35 kDa and basic subunit B (basic pI) of molecular mass about 20 kDa (Nishinari, Fang, Guo, & Phillips, 2014). The protein band at 20.46 kDa, corresponding to Kunitz inhibitor. The BowmanBirk (BB) inhibitor is capable of inhibiting both trypsin and chymotrypsin at independent reactive sites and has a molecular weight of 7.8 kDa (Chen et al., 2013). The major allergen proteins are β-conglycinin (α, α' subunit, β subunit), the 30-kDa allergen (Gly m Bd 30), and glycinin (Chi & Cho, 2016). Extensively degraded when heat-denatured as can be seen in cooked soybean when heating by pressure cooker for 90 min (Figure 2., B0, lane 7). This could be due to heat denaturation of proteins and consequent dissociation of both 7S and 11S globulins (Chi & Cho, 2016). However, the protein with molecular weight < 28.0 kDa was fairly heat stable and persisted in all forms of heat treatment (B0, lane7). These results based on the basis of previous studies raw soybean protein profile are in agreement with those of previously reported (González-Montoya et al., 2018; Nishinari et al., 2014).

Comparing S0, B0, and N0, because of the proteolysis that occurred during fermentation with *B. spp.* Var Natto, the protein-based antinutrient factor for example trypsin inhibitor and allergens were almost completely breakdown and hydrolyzed into small molecular-weight peptides. Besides, the protein larger than 28 kDa was mostly eliminated resulting in an accumulation of low molecular weight compounds as shown in Figure 2. (N0, lane 12). Apart from the action of microbial enzyme by fermentation, Figure 2 showed a change in the distribution of proteins during IVD. In B0-4 and N0-4, the large-molecular weight proteins were obviously decreased, and the small proteins accumulation were increased by increasing digestion time. Our result showed that the ratio of small protein fraction in stage 4 is the highest. During pepsin digestion (1-2), the intensities of the protein bands corresponding to 7S and 11S fractions were decreased and many peptide bands appeared at <36 kDa indicating protein hydrolysis (B1-B4). IVD of Natto caused a complete degradation of polypeptides >20 kDa and increased the abundance of oligopeptides with molecular weight <10 kDa (N4, lane 16, Figure 3). After IVD for 3 h (N3, lane 15), all protein subunits are degraded to a large extent, and after 4 h of IVD virtually all big molecular size proteins have disappeared (lane 16). The disappearance of those allergens disappeared in N4 whereas the presences in B4. due to the degradation of allergens into peptides. The peptides can be easily absorbed by an animal and transported within an organism (Gilbert, Wong, & Webb, 2008). These results show that some peptides with high molecular weight were degraded during IVD. This result suggests that digestive enzyme containing active protease that is able to decompose the larger proteins. This also confirmed the stronger hydrolyzing ability of pancreatin, which contains a mixture of digestive enzymes (including proteases) to hydrolyze the proteins.

In the case of pepsin hydrolysis, the subunits of Natto protein were partially digested within 1-2 h, while in the intestinal digestion, the most disappearing of larger molecule was found after 2 h. Generally, basic subunits were less easily digested than acidic subunits. This may be attributed to the different location of subunits in molecule structure. The interior subunits are usually more inaccessible to the catalytic sites of the enzymes. Therefore, Natto improve digestibility of the presence of antinutritional factors such as protease inhibitors (trypsin-/chymotrypsin inhibitors), tannins and lectins. The phenomenon suggests that this subunit may be present in the exterior of the 7S-form complex, thus easily accessible to protease hydrolysis. Furthermore, it is possible that the small-molecular weight proteins have anti-oxidative activity and metal ion-chelating activities, which can inhibit lipid oxidation and utilization of minerals in the functional foods.

Total phenolic content

Change in total phenolic content (TPC) of different stage digestion of Natto, cooked bean and raw bean was shown in Figure 3A. Overall, there were increasing in TPC by increasing of digestion time. Excepted in raw bean, significance increment was found through IVD digested

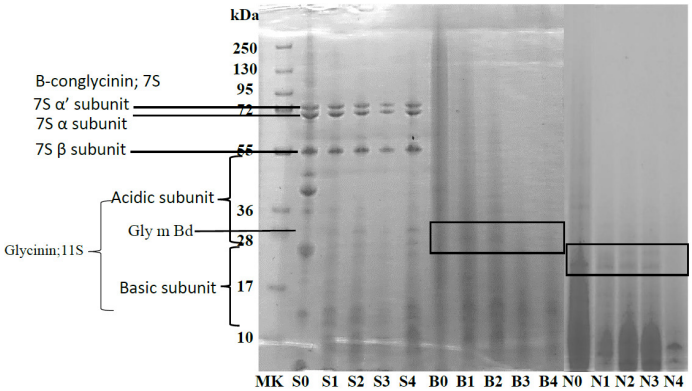


Figure 2. Change of SDS-PAGE electrophoretogram in Natto, cooked bean and raw bean protein at different digestion stages. Lane 1 represents standard molecular marker (MK), lane 2-6 represent raw soybean before digestion (S0), gastric digestion 1 h (S1), gastric digestion 2 h (S2), intestinal digestion 3 h (S3) and intestinal digestion 4 h (S4), respectively; lane 7-11 represent cooked bean in the same *in vitro* gastrointestinal digestion (B0-B4); lane 7-16 represent Natto in the same *in vitro* gastrointestinal digestion (N0-N4).

fractions of Natto and cooked bean. The releasing of TPC in to digested solution of cooked bean (15.85-25.17 as mg gallic acid per g protein) was greater than Natto (10.41-25.65 as mg gallic acid per g protein) whereas pretty stable in raw bean (10.58-14.00 as mg gallic acid per g protein). This may be due to the cell can be disrupted by heat, thus cell break and soluble phenolic release outside. In addition, the digestive enzymes playing a role in exposing water-soluble polyphenols from the structure because more water-soluble compounds were free. Increments of 1.68- and 1.39-fold were found in Natto and cooked bean gastric fractions, respectively. Besides, 1.47- and 1.13-fold of increment were found between gastric and intestinal digestion for Natto and cooked bean, respectively. Event high amount of TPC in digested cooked bean, higher increment of TPC in Natto was observed. TPC in cooked bean and Natto were comparable in IVD stage 3 and 4. This may be corresponding with releasing of TPC easier in fermented beam than cook bean. The structure between protein and polyphenolic was broke by hydrolysis.

In gastric stage, TPC more leaching than intestinal stages. Pepsin could play a free TPC from bound TPC in soy structure. The effectiveness of polyphenols was related to their antioxidant potential. In addition, increase in free phenolic acids in fermented soybean is probably due to β-glucosidase production during fermentation (Bhanja, Kumari, & Banerjee, 2009). An increase in free phenolics is expected to improve their bioavailability in the intestine, which will further improve the beneficial effects associated with them. Since we don't use special solution to extract phenolic out, the number of total phenolic shown only one that dissociate in supernatant derived from each digested fraction. From the reported of Juan and Chou (2010) extracted phenolic yield depends on the solvent that used for extraction as well. The total phenolic content ranging from 6.04 to 26.60 and 12.44-40.42 mg gallic acid equivalent/g extract, respectively, was reported with the extracts of non-fermented- and fermented black soybeans, respectively (Juan & Chou, 2010). Authors found that the total phenolic content was the highest with the acetone extract. On the other hand, the total phenolic content observed on the water extract was the lowest. 6.04-12.44 mg gallic acid equivalent/g extract, for non-fermented and fermented black soybean.

Antioxidant activities

Many researchers have suggested that increase in antioxidant activity is due to the increase in free polyphenols. Sanjukta, Rai, Muhammed, Jeyaram, and Talukdar (2015) suggested that Soybean fermented with potential proteolytic *Bacillus subtilis* var. natto strains enhanced antioxidant activities due to increase in peptides as well as polyphenols. Besides, antioxidant activity in the water soluble fraction of fermented Kinema may be due to the amino acids and peptides formed during fermentation. However, not only phenolic compound that play a role in antioxidant activities but also regarding to the generated oligopeptides from the action of digestive enzymes. These active peptides are either naturally occurring or hidden in a latent state within the precursor protein sequence but can be released by enzymatic proteolysis during gastrointestinal digestion, or during processing by enzymatic hydrolysis or fermentation (Möller, Scholz-Ahrens, Roos, & Schrezenmeir, 2008; Sánchez & Vázquez, 2017).

From Figure 3. it could be stated that there was a correlation between peptide content and antioxidant activities more than that between the phenolic compounds content and antioxidant, which means that the antioxidant activities of peptides are higher than phenolic compounds. All antioxidant activities have improved between before and after digestion. Natto IVD digests showed better activity in DPPH, FRAP and MIC than those of cooked bean except ABTS. Raw soaked soybean IVD digested fraction showed higher DPPH than cooked bean however it was stable even IVD time increased. Besides, the antioxidant activities of raw bean were stable over IVD time increased. It may be because raw bean did not contain oligopeptides like Natto and cooked bean therefore only phenolic compound reaction play a role in antioxidant activities.

For DPPH, Natto digested fraction showed the highest DPPH (24.12-68.00 as μmol TROLOX per g protein) followed by raw bean (40.03-51.52 as μmol TROLOX per g protein) and cooked bean (4.19-22.12 as μmol TROLOX per g protein) (Figure 3A). The DPPH activity increased with increasing time in IVD except stable in raw bean. Increment during gastric digestion was 2.42- and 1.96-fold whereas the increment after intestinal stage was 1.17- and 2.69-fold in Natto and cooked bean, respectively. Even the DPPH activity in Natto digest fractions was higher than that of cooked bean, the greatest change in increment was found in cooked bean. The same trend like DPPH was observed in ABTS that the activity increase by increasing IVD time. ABTS ranged from 0.84 to 3.62, 2.07 to 2.99 and 0.97 to1.08 mg L-Ascorbic acid per g protein in Natto, cooked bean and raw bean respectively and showed in Figure 3B. The biggest change found in Natto, gastric (2-h), ABTS increased ($P < 0.05$) around 2.05-fold gastric stage (1.72 mg L-Ascorbic acid/g protein), following intestinal stage increase around 4.31-fold (3.62 mg L-Ascorbic acid/g protein) compared to undigested stage (0) (0.84 mg L-Ascorbic acid/g protein). No big differences in ABTS were observed during IVD for cooked bean and raw bean. In Natto digested fractions, the increment of DPPH, ABTS, FRAP, MIC compared to before IVD was reported as 2.85, 4.32, 1.93 and 2.21-fold, respectively. The highest change (4.32-fold) was observed in ABTS.

Furthermore, reducing power (FRAP) of Natto digested fraction continuously increased during IVD up to 1.72 for gastric stage (18.80 μmol $FeSO_4$)/g protein) and 1.93-fold for Intestinal stage (21.10 μmol $FeSO_4$/g protein) ($P < 0.05$) whereas slightly increased for cooked bean (9.96 to18.95 μmol $FeSO_4$/g protein) and stable for raw bean (7.13 to 9.22 μmol $FeSO_4$/g protein) (Figure 3D). The increase in the reducing power of IVD-digests shows that Natto protein can be more effective hydrogen or electron donors after *in vitro* digestion. Since, transition metals such as Fe^{2+} and Cu^{2+} are well-known stimuli of lipid peroxidation and their chelation helps to retard the peroxidation and subsequently prevent food rancidity, the changed of MIC activity in the IVD of Natto, cooked bean and raw bean also investigated and was shown in Figure 3E. For Natto digested fractions, the MIC value was drastically increased approximately 1.98-fold for gastric stage (5.47μmol EDTA)/g protein) and 1.15-fold for intestinal stage (6.10 μmol EDTA)/g protein) ($P < 0.05$). Surprisingly, the same increment in MIC was also found in raw bean (0.92 to 4.28 μmol EDTA)/g protein) whereas stable in cooked bean (1.73 to 2.18 μmol EDTA)/g protein) even higher phenolic compound was higher. At gastric stage increase 2.13-fold and intestinal stage 2.00-fold of increment were observed in raw bean.

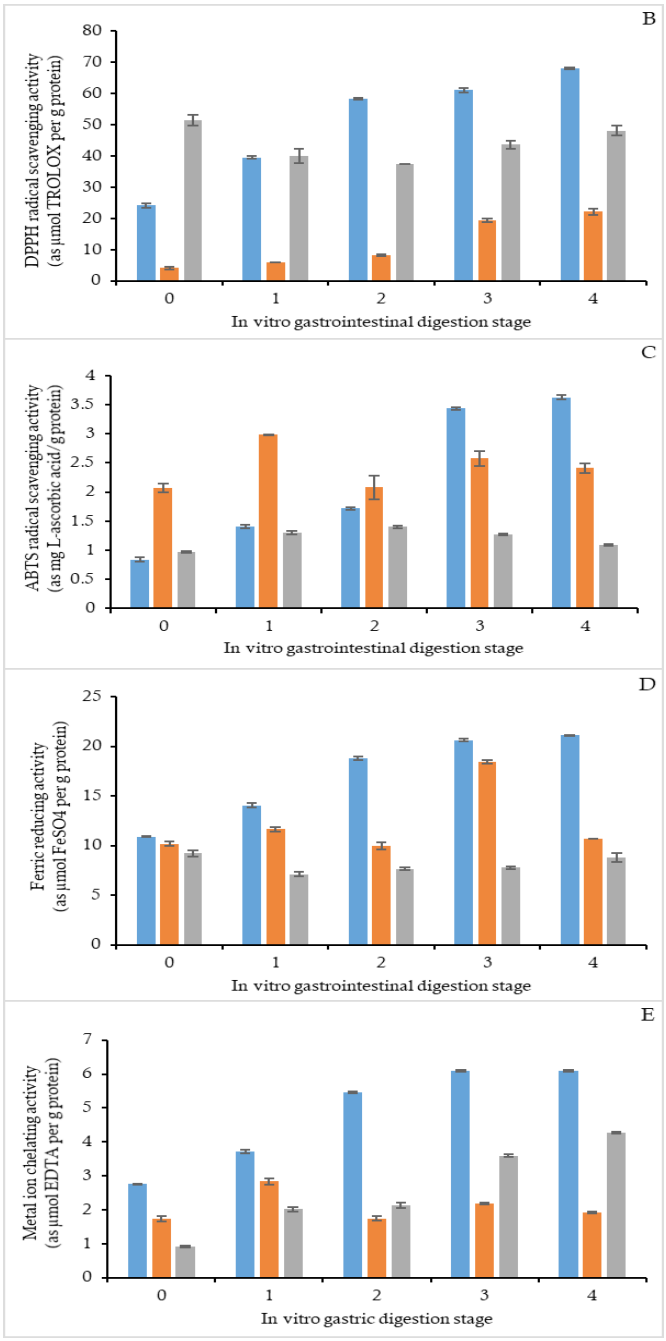


Figure 3. Total phenolic content (TPC) expressed as mg of gallic acid equivalents/g protein (A), DPPH radical scavenging capacity (DPPH) expressed as as µmol TROLOX equivalents/g protein (B), ABTS radical scavenging capacity (ABTS) expressed as mg of ascorbic acid equivalents/g protein (C), ferric reducing ability (FRAP) expressed as mmol $FeSO_4 \cdot 7H_2O$ equivalents/g protein (D) and metal ion chelating activity (MIC) expressed as µmol EDTA equivalents/g protein (E) of Natto at different digestion stages. Bars represent the standard deviation from triplicate determinations.

Phenolic compounds have been reported to be chelators of free metal ions. In a complex system like food, organic acids, amino acids and sugars can be sequester of transition metal ions. Raw beans can present significant values of chlorogenic, gallic and protocatechuic acid. On the other hand, identified higher concentrations of kaempferol, catechin, vanillic, gallic and sinapic acid in beans after cooking (Silva, Brigide, Toledo, & Canniatti-Brazaca, 2018). Furthermore, metal chelating potency of phenolic compounds are dependent upon properly oriented functional groups and their unique phenolic structure and the number and location of the hydroxyl groups as well as the presence of ortho-dihydroxy polyphenols (Andjelković et al., 2006). When a phenolic group is conjugated with a carbohydrate moiety, as in naturally occurring phenolic glycoside, it can no longer bind metals. Therefore, a sample high in polyphenols might not chelate metal if the polyphenols present did not have suitable groups that could chelate cations (Moktan, Saha, & Sarkar, 2008).

This study showed that antioxidant activity increased (in Natto) or stable (cooked bean) by IVD time increased. However, Sanjukta et al. (2015) reported soybean fermented with *B. subtilis* MTCC 5480 showed a decrease in antioxidant activity on pepsin digestion followed by an increase in activity on pancreatin digestion. The decrease after pepsin digestion may be due to the degradation of some original peptides and increase in activity is due to further formation of peptides with higher antioxidant activity. In case of soybean fermented with *B. subtilis*, MTCC 1747 antioxidant activity increased on pepsin digestion which further reduced on pancreatin digestion. Besides, the significant increment (11–35%) in antioxidant activity was documented with different concentrations of trypsin, pepsin and pancretin using IVD of soy milk fermented by *Lactobacillus plantarum* strain C2 (Singh & Vij, 2018). Hence, the antioxidant of fermented bean depends on not only type of microbial used but also how digestive enzyme react and generate antioxidant peptides. Moreover, in this study proved that Natto showed an improvement in antioxidant activity through increasing digestion time and those generating antioxidant peptides tolerate with digestive enzymes. The antioxidant activity of Natto came from both phenolic compounds and bioactive peptides. From preliminary test for the digestion of raw bean and cooked bean, not only total phenolic content plays a part in antioxidant activities but released peptides during the digestion also improved antioxidant activities. In addition, Sanjukta et al. (2015) suggested that antioxidant activity in the water soluble fraction of ***B. subtilis*** MTCC5480 fermented soybean be due to the amino acids and peptides formed during fermentation as well as antioxidant peptides that are released from gastrointestinal digestion.

The results clearly indicated that the Natto at the last stage of IVD showed the highest activity. This may be due to resulting short chain peptides showed activity based on rather than parental protein, contained peptides, which are electron donors and could react with free radicals to convert them to more stable products and terminate the radical chain reaction (Aluko, 2015). From the results, Natto protein should be resistant to gastrointestinal digestion to reach the target tissue in an active form. Furthermore, the size of peptides is known to be a significant factor in the overall antioxidant activity of hydrolyzed proteins. The antioxidant activities of these hydrolysates are shown to be largely dependent on enzyme specificity, molecular weight distribution, amino acid composition and the specific sequences of the released peptides (Sarmadi & Ismail, 2010). Another point, the size of peptides is known to be a significant factor in the overall antioxidant activity of hydrolyzed proteins. Previous studies have reported that peptides with lower MW (5-16 amino acids) could have a higher probability to cross the intestinal epithelia and exert better biological activities (Ketnawa, Martínez-Alvarez, Benjakul, & Rawdkuen, 2016). From Figure 2. The smaller size found in SDS-PAGE may supported the antioxidant activity. In the present study, the final digest of Natto (N4), which exhibited the highest overall antioxidant potential, larger molecular weight was disappeared.

Free amino acid composition

Pepsin plays an important role in primary digestion of food proteins, resulting in long chain peptides followed by secondary digestion by intestinal enzymes, resulting in the formation of short chain peptides (Goodman, 2010). The nutritional quality of a protein is primarily related to its amino acid composition. In addition, amino acid availability is also a key factor of protein quality. Availability depends on the process of digestion, which may be affected by many factors such as the characteristics of the protein itself. Raw bean, cooked bean and Natto and their digested fractions were analyzed for free amino acid profile responsible for nutritional aspect and bioactive activities divided by group of amino acids. The change of free amino acid profile was reported in term of increment between before (0) and after IVD (4) (data not shown). When compare to raw bean before IVD (time 0), total increment of free amino acids in raw bean, cooked bean and Natto after IVD (time 4) was 2.15-, 6.77- and 21.10-fold, respectively. The distinctive amino acids found in the bean were Ile, Glu, Val, Leu, Tyr, Phe, Lys and Asg (>20 nmol/mL). All other amino acid content also increased, especially the highest of increment was observed in cooked bean and Natto after IVD (4). When compare between Natto before (N0) and after IVD (N4), total increment was 3.54-fold. Most represented amino acids were increased significantly around 1.5 to 3.5-fold. The change in free amino acid content by different group showed that the maximum change was found in Arg (49.43-fold) whilst Pro was found to slightly decrease (0.95-fold) in N4. For cooked bean, the big difference in some amino acids between before and after digestion. There was drastically increased in Leu, Tyr, Phe, Lys and Arg around 5 to 28-fold found in B4.

Consideration of amino acid divided by group we found the maximum change of 9.52-fold in B4 and 4.19-fold in N4 in essential amino acid group (EAA: Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Try, Val). As aforementioned details, Arg was increase the most while His was the least. This can infer that IVD improved the generate of EAA. Apart of EAA, we found an remarkable increment also in different groups of amino acid, for example, hydrophobic amino acids (HAA: Ala, Val, Ile, Leu, Tyr, Phe, Trp, Pro, Met and Cys); aromatic amino acids (AAA: Phe, Trp, Tyr and His), antioxidant amino acids (AXA: Trp, Tyr, Met, Cys, His, Phe and Pro) for around 5.29 to13.04-fold in cooked bean and 2.99 to 4.13-fold in Natto. Pepsin is most effective at cleaving peptide bonds between hydrophobic and preferably aromatic amino acids such as Phe, Trp and Try. Pancreatin exhibited the activities of trypsin (cleavage of peptide bonds at Arg and Lys sites), chymotrypsin (cleavage of peptide bonds at Phe, Trp, Tyr and Leu sites), and elastase (cleavage of peptide bonds at Ala and other aliphatic amino acids) (Udenigwe & Aluko, 2012). Beyond nutritional quality, the increase in free amino acids content is responsible for the remarkable increase in antioxidant activities that shown in Figure3. The result of amino acid contents showed the same trend like that of the fermented soybean meal with ***B. subtilis*** reported by Song, Frias, Martinez-Villaluenga, Vidal-Valdeverde, and de Mejia (2008). By the increase of amino acids in this study we inferred that Natto and cooked bean digested fraction by IVD could increase the nutrition values and physio functions for human.

The nutritional quality of a protein is primarily related to its amino acid composition. In addition, amino acid availability is also a key factor of protein quality. The antioxidant activity of a peptide is widely recognized to be based on the free amino acid composition formed up on IVD (Ajibola, Fashakin, Fagbemi, & Aluko, 2011; Sánchez & Vázquez, 2017). It has been reported that peptides rich in HHA and AAA show an scavenging effect of free radicals through direct electron transfer, and inhibit the propagation of oxidized lipid by-products (Wiriyaphan, Xiao, Decker, & Yongsawatdigul, 2015). In addition, the presence of hydrophobic amino acids (HAA) residues in the digested Natto offers structural activities that can enhance interactions with lipids in foods and enhance antioxidant entry into target organs through hydrophobic interactions with membrane lipid bilayers (Wu et al., 2015). Besides, it was suggested that aromatic amino acids and His residues contributed to the radical scavenging activity because of their capacity to donate proton easily to electron deficient radicals and maintain its stability (Sanjukta & Rai, 2016). The increase in free amino acids such as Leu, Met, Tyr, His and Try have also been attributed to strong reducing power (Ajibola et al., 2011). The side chain of amino acids, for example, His (imidazole group), Trp (indolic group) and Tyr (phenolic group), contributes to the strong radical scavenging activities of peptides (Guo, Kouzuma, & Yonekura, 2009). However, the antioxidant activity of peptides depends not only on their amino acid composition, but also on other factors such as molecular weight, amino acid sequence structure and configuration of peptides (Zhang et al., 2018).

CONCLUSIONS

Cooked bean showed greater digestibility in each stage of gastrointestinal digestion than that of Natto. Natto digested through *in vitro* digestion showed an improved in protein utilization. Due to leaching out of phenolic compounds and bioactive peptides/amino acids during digestion, digested soybean fractions can act as a hydrogen donor, a water-soluble radical quencher, and a transitional metal ion sequester. These activities were retained or improved following stepwise enzyme digestion simulating the human digestive tract. The peptide molecular weight size, the concentration of peptides/free amino acids, the high amount soluble phenolic compounds and the high percentages of antioxidative amino acid residues present contribute to the strong bioactivities of the protein digests. Therefore, the benefits of soybean and fermented soybean like Natto could be extended beyond its role as staple food but food with a potential antioxidant activities because when ingested, it may help promote human health beyond basic nutrients.

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