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Germination and Fermentation of Soybeans: Two Healthy Steps to Release Angiotensin Converting Enzyme Inhibitory Activity Compounds

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Abstract

Background and objective: Soybean is one of the most important grains with high proteins, good quality edible oils, appreciable amount of minerals and vitamins. Due to some disadvantages soybeans' compounds affecting the flavor, odor and stability, different types and levels of processing are considered to make better products with healthy properties. Hypertension (high blood pressure) is one of the modern world diseases, which increases the risk of serious human health problems. There are several systems in humans' body e.g. angiotensin converting enzyme regulator to blood pressure control. The aim of the present review is to report the effect of germination and fermentation on the concentration of bioactive compounds with angiotensin converting enzyme inhibitory properties.

Results and conclusion: Many scientific research has demonstrated that germination (sprouting, also known as malting) and fermentation are two effective and inexpensive technologies improving soybean quality. During these two processes, anti-nutritional and bioactive compounds affecting human health e.g. anti-hypertension components have been removed and released, respectively. Furthermore, studies have shown effect of soybean isolated compounds to inhibit angiotensin converting enzyme. Therefore, soybean germination and fermentation could affect the concentration of bioactive compounds with angiotensin converting enzyme inhibitory properties.

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1. Introduction

Soybean (*Glycine max* L.) is one of the most cultivated legumes in world and generally recognized as a functional food [1-5]. It is rich in proteins (40-50%), compared to other cereals or legumes (22%-33% protein), lipids (20-30%) and carbohydrates (26-30%) [1-3]. The *β*-conglycinin (7S globulin) and glycinin (11S globulin) are two main soybean protein compounds (nearly 65-85% of the total proteins) and lipoxygenases, lectins, trypsin inhibitors, and α-amylases are other minor proteins/glycoproteins within the soybean [2-6]. Its flour is produced through the milling of whole grains with complete components (protein, fiber, vitamins and minerals) [7]. The high protein content of soybean and its profiles/sequences peptide provide nutrition sources with healthy properties for the consumers [1,3]. Hydrolysis of these proteins releases a group of short-chain peptides with 3-10 amino acids, which show multiple health-promoting functions, including antihypertension, antidiabetes, opioid agonist, immunomodulatory, antioxidant, anxiolytic, anticancer and antimicrobial activities [8,9]. Documented evidence have demonstrated that soybean protein supplementation could significantly decrease serum total cholesterol, low-density lipoprotein cholesterol and tri-

glycerides and increase high-density lipoprotein cholesterol, this supplementation may affect novel cardiovascular disease risk factors and be used against multiple-site cancer cells [10-12].

The most biological soybean active peptides are derived from glycinin by kidney membrane proteases and plasma proteases digestion [2,3,9]. Significant amount of hydrophobic amino acids in green soybean seeds are considered as a source of angiotensin converting enzyme inhibitory (ACE I) peptides [1,6]. An early study showed that four ACE I peptides were isolated from the glycinin proteolysis [13].

Except for proteins, soybeans have high levels of isoflavone, mainly daidzein and genistein [14,15]. These compounds have antioxidant, anti-inflammatory and anti-allergic properties and decrease the risk of cardiovascular diseases and cancer cell growth [15,16].

Because of the presence of anti-nutritional, protease (trypsin) inhibitor factor and allergenic proteins (mainly β-conglycinin, the 30-kD allergen and glycin) in raw soybeans, pretreat of these grains is necessary before consumption. Fermentation and germination are two commercial ways of improving soybean meal properties [17]. These may provide the potential of anti-carcinogen, therapeutic and ACE I compounds, changes in isoflavone forms and reduces trypsin inhibitor and anti-nutritional factors [2,18,19]. In 2015, a study reported that fermentation of soybean and its byproducts increased radical scavenging activity (3.1-24 folds), total antioxidant activity and free amino acid contents (proline, tryptophan, tyrosine, phenyl-alanine and histidine) by protein hydrolysis [20]. In another study demonstrated that the antidiabetic effects of soybeans increased due to its isoflavonoids and peptides during the fermentation process [21]. In addition, a published scientific report has described that soybean products prepared by both germination and fermentation processes might show greater angiotensin converting enzyme (ACE) activity inhibiting effects, compared to those prepared by fermentation or germination separately. They prepared different kinds of doenjang using fermentation regular soybeans (RS), germination soybeans (GS) and germinated black soybeans (GBS) for 90 days. The ACE I enhanced as the fermentation progressed. Doenjang from GBS showed the highest ACE I activity (58.69%) after 75 days of fermentation, compared to that other preparations did [22]. Except for the whole of the health benefits of soybeans mentioned earlier, it has a good effect on hypertension. Hypertension (high blood pressure) is one of the major worldwide diseases causing nearly 12.8% of all the deaths, i.e. around 7.5 million deaths annually and is predicted to affect 1.56 billion people by the year 2025 [3,23]. In fact, there is a positive correlation between high blood pressure and other heart diseases such as coronary heart diseases and ischemic and haemorrhagic strokes [23]. ACE is one of the several hypertension regulators suggested in human body. This enzyme catalyzes the conversion of angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu) into bioactive angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) in renin-angiotensin system and can lead to an elevation of blood pressure [24,25]. Chemical drugs (e.g. thiazide diuretics) are used to inhibit ACE and hence treat hypertension [23]. However, food derived ACE I com-pounds can be appropriate alternatives to synthetic drugs due to lack of after-effects for human body [25]. Many studies have reported that soybeans could generate bioactive compounds with ACE I properties [3,22,24]. Table 1 shows the effects of germination and fermentation on release of ACE I compounds.

To best of our knowledge, no scientific report has considered the germination and fermentation roles together in releasing bioactive peptides of soybean. So the aim of this paper is to link the effect of soybeans germination and fermentation (fermentation condition, its microbial inoculation, and etc.) to release bioactive peptides with low molecular weight, different sequential and also explains the availability of the important soybean compounds and their possible effects on ACE I (Fig. 1).

2- Effects of germination

In general, soybean germination process improves the nutritional value of seeds, isoflavin [22-27], the digestibility, odor and flavor [27,28], the amount of protein efficiency ratio [18,27], shelf-life due to reduction lipoxygenases activity [28], reduces anti-nutritional factors such as proteolytic inhibitors, phytic acid and TI [1,18,19], and hydrolyzes of oligosaccharides (Table 2). Germination also provides higher levels of methionine, which is the first limiting amino acid in soybean protein [1]. In addition, some of the functional active compounds of soybeans that are originally absent or are present in very low quantities (e.g. γ-aminobutyric acid (GABA) and ACE I) can be concentrated through transformation of the nutrients in soybeans [18,19]. Some researchers produced three types of doenjang by preparing three types of meju using RS, GS and GBS (GS:GBS 7:3 w:w). They assessed ACE I activity by Hip-His-Leu (HHL) method with slight modification and showed that doenjang prepared with greater ACE activity-inhibiting effects, compared to those prepared with RS [22].

Table 1. Effects of fermentation and germination on soybean antihypertension compounds

Compound	Fermentation (inoculated microbes/fermentation condition) or germination	IC_{50} Sequences peptides Amount of phenols	Effect	Ref.
Peptides	Germinating soybean in two temperature (30°C and 40°C)	IC_{50} value of none germinated soybean= 0.174 mg ml^{-1} IC_{50} value of (30°C)= 0.098 mg ml^{-1} IC_{50} value of(40°C)= 0.025 mg ml^{-1} ACE I of none germinated soybean= 33.28 % ACE I of (30°C)= 66.20% ACE I of (40°C)= 83.53%	- Increasing low molecular weight - Increasing ACE I^1 up to 83.5% by Germinating at 40°C - Germination at 40°C was more effective for releasing ACE I.	[21]
Isoflavone and peptides	Inoculating germinated soybeans and germinated black soybeans with 3% (v w^{-1}) spore of *A. oryzae* was for 5 days at 25°C	ACE I%= 58.69%	- The isoflavone contents in GS and GBS-doenjang were slightly higher than in RS-doenjang in the early stage of fermentation. - The ACE I activity increased as the fermentation period progressed. - The ACE I activity were significantly higher in GBS-doenjang (58.69%) than in other preparations after 75 days of fermentation. - Doenjang prepared using GBS (GS: GBS= 7:3) showed greater ACE I activity than RS doenjang.	[25]
Phenols, flavonols, tannins	Investigating effect of germination time (3 and 6 days) and temperature (20 and 30°C) on the phenolic constituents, and bioactive compounds	*NR	Germination at 30°C for 3 days resulted in highest total phenols, flavonols, tannins, saponins, ascorbic acid and tocopherols.	[29]
Isoflavone aglycones (daidzein and genistein) and saponin	Determining the effect of germination time and temperature on producing bioactive compounds	*NR	- Optimal increases in the concentrations of isoflavone aglycones (daidzein and genistein) and saponin glycosides were observed with a 63 h germination time at 30°C. - Both germination time and the temperature had an influence on the composition and concentration of bioactive compounds in germinated soybean flour.	[17]

Peptides	NR	Valyl-prolyl-proline (Val-Pro-Pro), isoleucyl-prolyl-proline (Ile-Pro-Pro)and (Tyr-Pro)	Exhibited ACE I activity and blood pressure lowering effect in spontaneously hypertensive rat.	[32]
Peptides	Hydrolyzing by *B. subtilis* Protease	IC_{50}= 26.5 µM Pro-Gly-Thr-Ala-Val-Phe-Lys	Identifying and introducing PGTAVFK sequence with antihypertensive effect	[52]
Peptides	Inoculating soybean with 4 lactobacilli strains: *L. fermentum*, *L. plantarum*, *L. fructosus*, and *L. casei* spp. *pseudoplantarum* at different time period (12, 18, 24, 36 and 48 h) and temperature (25, 37, 40, and 50°C)	1- IC_{50}= 17.2 µg ml^{-1} 2- IC_{50} value of Leu-Ile-Val-Thr-Gln= 0.087 µg ml^{-1}	- The *L. casei* spp. *pseudoplantrum*, LAB strain, exhibited maximum protease activity (37°C/ 36 h). - Purified peptide fractions showed ACE I potency. - Synthesized different peptide analogs LIVTQ, LIVT with ACE I activity	[7]
Peptides	*B. subtilis* protease digestion (40°C, 1 h)	IC_{50} values= 0.1964 µg ml^{-1}	The IC_{50} value of hydrolyzed with *B. subtilis* protease was less than pepsin digestion $IC_{50.}$	[55]
Peptides	Isolating 3 fractions (F535A, F535B, and F535C) from fermented soybean paste	IC_{50} (F535A, F535B, and F535C)= 2.1 to 3.0 µg ml^{-1} His-His-Leu (IC_{50}= 2.2 µg ml^{-1})	- Fraction F53 exerted a strong ACE I activity in vitro tests. - Peptide with His-His-Leu sequences showed lowering activity in vivo tests. - Small decrease in ACE activity accompanied by a strong reduction of blood pressure	[56]
Peptides	Fermenting soybeans by *B. natto* and *B. subtilis* CH-1023	Ala, Phe and His	ACE I activity and antihypertensive effects	[53]
Peptides	Comparing Fermentation douchi qu pure-cultured by *A. Egyptiacus* for 48 h and 72 h with douchi secondary-fermented for 15 d	ACE I for 24h= 7.8% ACE I for 48h= 66.2% ACE I for 72h= 72.4% ACE I for 15d= 86.3%	Improving ACE I activities following the fermentation	[33]
Several compounds	Making Natto by fermenting steamed soybeans with *B. subtilis natto*	IC_{50} = 0.27 mg ml^{-1} total ACE I activity in lyophilized natto (100 g)= 3.7 × 105 units	Reduction the blood pressure of rats	[51]

Peptides	Pre-fermenting Douchi by *A. oryzae*, *Mucor wutungkiao*, *B. subtilis natto* and *B. subtilis* B1	IC_{50} of *A. oryzae*= 0.4991 mg ml^{-1} IC_{50} of *Mucor wutungkiao*= 0.3535 mg ml^{-1} IC_{50} of *B. subtilis* Natto= 0.2294 mg ml^{-1} IC_{50} of *B. subtilis* B1= 0.0901 mg ml^{-1}	- ACE I - The type of starter cultures affected significantly the production of ACE I activity in douchi qu, and that *B. subtilis* B1 was the best starter.	[44]
Peptides	Fermenting Mao-tofu by *Mucor* spp. for 3-9 days		- The extract exhibited the highest ACE I activity in vitro. - Hydrophobic amino acids, especially Pro, are usually found in ACE I peptides.	[50]
Peptides	Obtaining miso paste and natto extracts by using distilled water with the same method as that used for the tofuyo extract, while soybean sauce was used directly	The $IC_{50=}$ 1.77 mg ml^{-1} Ile-Phe-Leu (IC_{50}= 44.8 µM) Trp-Leu (IC_{50}= 29.9 µM) The IC_{50} of Soybean sauce = 3.44 mg ml^{-1} The IC_{50} of Miso paste= 1.27 mg ml^{-1} The IC_{50} of Natto = 0.16 mg ml^{-1} The IC_{50} of Tofuyo= 1.77 mg ml^{-1}	- ACE I activity was observed in a tofuyo. - The ACE I activity of Trp-Leu was completely preserved after treatment with pepsin, chymotrypsin or trypsin. - Ile-Phe-Leu and Trp-Leu isolated from tofuyo were expected to contribute the antihypertensive effect via an in vivo transport system.	[41]
Oligo-peptides	Chungkookjang fermentation	IC_{50} of Lys-Pro= 0.083 mg $(100\ g)^{-1}$	- Chungkookjang had ACE I and antihypertensive effect. - Systolic blood pressure dropped by 15 mmHg and diastolic blood pressure by 8 mmHg 2 h after a single administration of 20 g of fermented soybean.	[40]
Peptides	Fermenting by *B. natto* or *B. subtilis*	Val-Ala-His-Ile-Asn-Val-Gly-Lys or Tyr-Val-Trp-Lys	Releasing several ACE I Peptides	[57]

Peptides	Preparing different fermented soybean products from various locations Chinese	IC_{50} value of tempeh = 0.51 mg ml^{-1} IC_{50} value of tofuyo = 0.66 and 1.77 mg ml^{-1} IC_{50} value of miso = 2.38-1.27mg ml^{-1} IC_{50} value of natto= 0.16-0.44 mg ml^{-1} IC_{50} value of soybeanpast= 0.012 mg ml^{-1}	The lowest ACEI belonged to fermented soybean past.	[42]
Peptides	Modified soybean past fermentation, termed Fermented Soybean Seasoning (FSS),	IC_{50} value of fermented soybean seasoning= 454 µg ml^{-1} IC_{50} regular soybean sauce= 1620 µg ml^{-1} The ACE I peptides isolated from FSS were Alae-Trp IC_{50} =10 µg ml^{-1} Alae-Tyr IC_{50} =48 µg ml^{-1} Glye-Trp IC_{50}= 30 µg ml^{-1} Sere-Tyr IC_{50}=67 µg ml^{-1} Glye-Tyr IC_{50}=97 µg ml^{-1} Vale-Pro IC_{50}=480 µg ml^{-1} Alae-Phe IC_{50}=190 µg ml^{-1} Alae-Ile IC_{50}=690 µg ml^{-1} Vale-Gly IC_{50}=1100 µg ml^{-1}	- Fermented soybean seasoning showed low IC_{50} compared to regular soybean sauce. - Several dipeptides with ACE I potency were re-cognized.	[46]
Peptides	Hydrolyzing soybean and the soybean-fermented foods were dephosphorylated, deglycosylated and digested with a variety of endoproteases (pronase, trypsin, Glu C protease, plasma proteases, and kidney membrane proteases) to generate oligopeptides	IC_{50}= 0.1, 0.3, 0.5 and 0.7 mM	- Peptide with ACE I potency was produced by a tryptic-like cleavage with a C-terminal arginine. - The proteases of lower specificity produced showed more oligopeptides and a higher percentage of bioactive peptides than the proteases of higher specificity, namely trypsin, and Glu C.	[5]
Isoflavonoids and peptides	Fermenting Meju for 20d or 60 d), without the use of salt	Amount of two kinds iso flovine after 60 d fermentation: Daidzein: 152 Genistein :170	- Longer fermentation period (60 d) enhanced the antidiabetic effect of soybeans. - After 60 d fermentation:15 kd (96.9%)	[24]

*NR, not reported
GS=germinated soybeans, GBS=germinated black soybeans, RS=regular soybeans, ACE I= angiotensin converting enzyme inhibitory
PGTAVFK =Proline (P)-Glycine (G)-Threonine (T)-Alanine (A)-Valine (V)-Phenylalanine (F)-Lysine (K), LIVTQ = Leucine (L)-Isoleucine (I)-Valine (V) -Threonine (T)- Glutamine (Q), LIVT=
Leucine-Isoleucine-Valine-Threonine
Aspergillus= A, *B. subtilis=Bacillus subtilis, L. plantarum=Lactobacillus plantarum, L. fructosus= Lactobacillus fructosus, L. casei= Lactobacillus casei*
LAB=Lactic Acid Bacteria

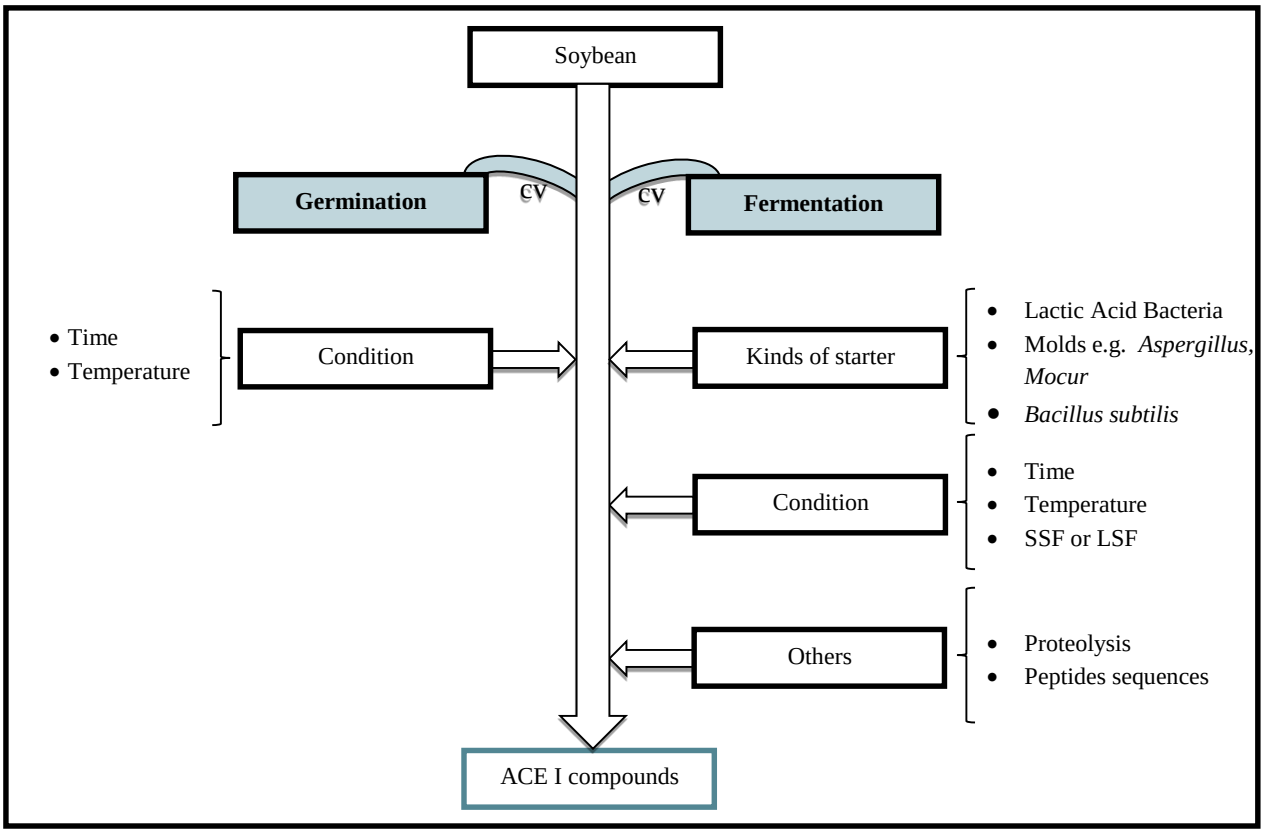

Figure 1. Proposed review-diagram for the characterization of germination and fermentation to release ACE inhibitory from soybean
SSF =solid-state fermentation, ACE= angiotensin converting enzyme

Table 2. Various soybean fermented foods and their processing

Name	Definition and Production	Ref.
Doenjang	Doenjang is a representative traditional Korean fermented food that has played an important role in providing. protein in typically graincentered, protein-scarce diets. It is prepared by fermented soybean paste.	[58]
Sufu	Sufu is a traditional and highly flavored fermented tofu. Its preparation consists of a former fermentation by inoculation of tofu with *Actinomucor elegans* and incubation for 48 h to produce pehtze (pizi). Fermentation is usually performed between 20°C and 35°C.	[18]
Miso	Miso is commonly produced from koji. Soybean koji can be prepared by soaking soybeans in water and mixing with the conidia of *A. oryzae* or *A. sojae* and incubated at room temperature.	[18]
Natto	Natto is a *B. subtilis* fermented soybean. Its preparation consists of splitting, soaking, and boiling of soybeans, followed by fermentation with *B. subtilis* at 37°C for 48 h.	[18] [34]
Tempeh	Tempeh is produced by soaking soybeans at room temperature for 10-12 h and dehulling them by hand. They are then heated up to the boiling point and boiled for 20 min. After cooling to 35-40°C, an inoculum of *R. oligosporus* for incubation in the dark at 37°C for 22 h.	[18]
Douchi	Douchi is prepared by soaking soybeans in water for 8 h at room temperature; after draining, soybeans are cooked (>100°C) and then inoculated either with Mucor, Bacteria, or Aspergillus strains at 30-35°C (pre-fermentation).	[18]
Tofuyo	Tofuyo is a traditional fermented tofu from Okinawa in Japan.	[41]
Chunggugjang	Chunggugjang is a traditional fermented Korean soybean product.	[49]

Aspergillus= A, *B. subtilis=Bacillus subtilis*, *R. oligosporus = Rhizopus oligosporus*

Both germination time and temperature influence on the composition and concentration of bioactive compounds in GS flour. Germination time and temperature could affect the amount and kinds of compounds that are released during the process for instance; germination at 30°C for 3-6 days increased contents of total phenols, flavonols (genistein and daidzein), ascorbic acids, saponins and tannins [14,26-29], or germination at 40°C enhanced the concentrations of LMW proteins (< 17 kDa) [18,27] and germination at 37°C increased legume protease activity up to maximum [30]. After five days of germination, the amount of phytic acid content reduced [27], and during 72h of the germination process, the lipoxygenases activity reduced [28], and after two days of germination, the quantity of inhibitors reduce (25 to 32%) and the lowest inhibitory concentration was seen on the 6th day of germination [19].A study was carried out in 2013 found that the GABA content in the sprouts of soybean was increased by 27 times after germination for 24 h at the room temperature [31]. In addition, another study reported that the extracts of germinated soybean at 40°C exhibited the IC_{50} value of 25 µg ml^{-1} and increased ACE I up to 83.5% [18]. Based on earlier studies, the bioactivity of compounds released during germination depends on the type of legume and the germination temperature (the higher compounds archives in germination at 35-40°C [14,18,26-30].

3- Effects of fermentation

Soybean is consumed in both unfermented (e.g. roasted and fried soybeans, soybean powder, soybean butter, soybean oil, and etc.) [1,3] and fermented (e.g. soybean sauce, Tempeh, notto, soybean milk, etc.) forms [34-37].

During fermentation, complex organic compounds are broken down into smaller molecules by microorganisms [9]. Silva et al. promoted the transformation of isoflavone glycoside into aglycones [7,13]. Slavin et al. observed that fermented soybeans contained more aglycones (e.g. genistein and daidzein) than glycosides and also the bioavailability of the isoflavones increased in In vivo experiments [38]. In addition, the proteins are partly hydrolyzed then released bioactive peptides and amino acids such as glutamic acid (Glu) and aspartic acid (Asp) [17,32,39]. These peptides have been studied for various therapeutic properties such as antioxidant, antihypertensive, antitumor, antidiabetic, negligible human IgE immunore and ACE I activity properties [2,6,32].

Furthermore, peptides isolated from fermented soybean products and foods had been associated with the potential of ACE I activity [32-37]. The most commonly fermented soybean foods in Asian countries include natto, miso, tofuyo (Japan), douchi, sufu, doubanjiang (China), soybean sauce, cheonggukjang, doenjang, kanjang, meju (Korea), tempeh (Indonesia), thua nao (Thailand), kinema, hawaijar and tungrymbai (India) [6,15,17]. Soybean fermentation products and their processing have been shown in Fig. 2. In 2004, a study was carried out to examined antihypertensive activity in chunggugjang in 4 volunteers. They reported that the systolic and diastolic of blood pressure dropped (by 15 and 8 mm Hg, respectively) 2 h after a single administration of 20 g of fermented soybeans. Also, the purified sequence with Lyse-Pro showed ACE I activity (0.083 mg/100 g sample), with the IC_{50} value of 32.1 µM [40]. Kuba et al. isolated two ACE I peptides with the IC_{50} value of 44.8 µM and 29.9 µM and observed ACE I activity with the IC_{50} value of 1.77 mg ml^{-1} from tofuyo extract [41]. Natto and Monascus exhibited ACE I activity and could be considered as an important source of ACE I peptides [35]. Another study reported that soybean past showed higher ACE I (the IC_{50} value of 0.012 µg ml^{-1}) between other fermented food includes: tempeh (the IC_{50} value of 0.51 µg ml^{-1}), tofuyo (the IC_{50} value of 0.66 and 1.77 µg ml^{-1}), miso (the IC_{50} value of 2.38-1.27 µg ml^{-1}), and natto (the IC_{50} value of 0.16-0.44 µg ml^{-1}) [42]. The effect of soybean fermentation on releasing ACE I compounds have been shown in table 3.

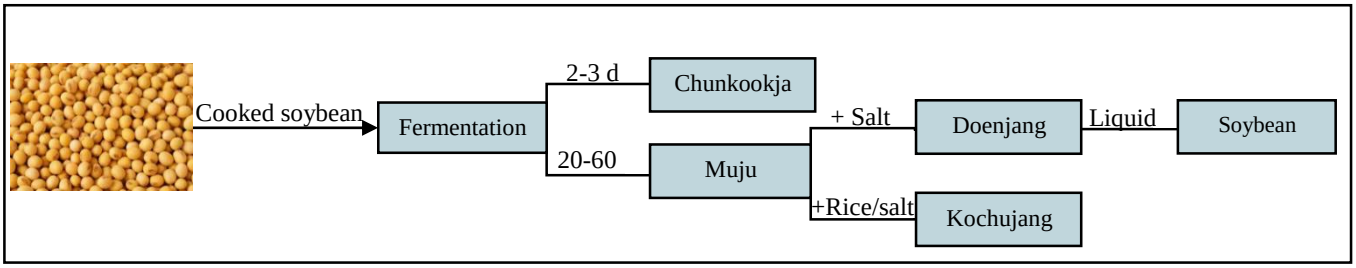

Figure 2. Schematic of soybean food production

d= Day

Table 3. Time and temperature used to achieve the major components during germination

Effect	Time and temperature	Ref.
Isoflavones	After 24 h days of germination	[25]
ACE I activity	24 h germination/ 75d fermentation	[25]
ACE I activity	40°C	[21]
The lowest quantity of inhibitors	After 6 days of germination	[22]
Maximum activity of legume proteases	37°C	[30]
GABA	After 24 h days of germination	[31]
The highest daidzein, glycitein, and genistein content	After 3, 1, and 3-day germination	[26]
Reduction lipoxygenases activity	Short periods of germination (72 h)	[28]
Genistein, daidzein	6 days germination	[27]
Reduction phytic acid	5 days germination	[27]
9.4-10.9 kDa MW peptides	40°C	[27]
highest total phenols, flavonols, tannins, saponins, ascorbic acid	30°C for 3 days	[29]
Optimal increases in the concentrations of isoflavone aglycones and saponin glycosides	After 63 h germination time at 30°C	[17]

GABA= γ-aminobutyric acid, MW= molecular weight, ACE I= angiotensin converting enzyme inhibitory

3-1- Effects of fermentation starter

Kinds of microorganism determine sensory properties, nutritional changes and favor compositions developing during the soybean fermentation [6,43]. Actually, different starter especially proteolytic strains and their enzymes improved biofunctional compounds such as isoflavones and peptides with antioxidant, ACE I and etc., properties and the amount of proteins proteolysis and as a result [6,35]. *Bacillus* (*B.*) *subtilis* is the most favor starter for soybean fermentation [35,38,45].

In addition, lactic acid bacteria (LAB) via possessing galactosidase and protease enzyme can hydrolyze soybean oligosaccharides and proteins during fermentation, reduce its beany flavor and released ACE I peptides [4,36]. Hydrolysis of the soybean proteins by LAB and other bacteria varies between different strains of the same species [6]. Sanjukta et al. showed that *B. subtilis* MTCC5480 showed a higher degree of protein hydrolysis and free amino acids during fermentation of soybeans, compared to that *B. subtilis* MTCC 1747 did [20]. *Lactobacillus* (*L.*) *casei* spp. pseudoplantrum strain exhibited the maximum protease activity during soybean fermentation, compared to that *L. fermentum*, *L. plantarum* and *L. fructosus* did. This strain released two peptides that inhibited ACE with the IC_{50} value of 17 and 30 µg ml^{-1} [4]. However, fewer studies used LAB for the hydrolysis of soybean proteins, compared to studies used *Bacillus* spp. [24]. This might occur because *Bacillus* spp. result in alkaline fermentation while LAB result in acidic fermentation, producing lactic acids [6].

Apart from the bacterial starters, filamentous fungi are used in fermented soybean products to provide aroma, color, and biological activity [6,33]. Silva et al. reported that fermentation of whole autoclaved soybean flour using *Aspergillus* (*A.*) *oryzae* resulted in hydrolysis of soybean proteins and formation of LMW peptides [7]. Moreover, ACE I activity of douchi qu pure cultured by *A. Egyptiacus* was improved during the fermentation [33].

The type of the starter cultures significantly affects production of ACE I in foods. A study investigated effects of different types of cultures (Aspergillus-type, Mucor-type, and Bacteria-type) on ACE I activities of douchi qu, Pre-fermented douchi. The IC_{50} value of douchi qu fermented by *A. oryzae*, *Mucor wutungkiao*, *B. subtilis natto* and *B. subtilis* B1 were 0.4991, 0.3535, 0.2294 and 0.0901 mg ml^{-1}, respectively. They indicated that *B. subtilis* B1 could be a candidate as an inoculant to prepare fermented soybeans with strong ACE I activity [44]. In addition, *B. subtilis* enhanced more antioxidant activity than *A. oryzae* (five-folds/ three folds) [17]. Also, fermentation with *B. subtilis* had higher in vitro digestibility of crude protein than fermentation with *A. oryzae*. While *B. subtilis* showed more proteolytic activity than *A. oryzae* (8.37% to 0.34%) [1].

Based on scientific studies, *B. subtilis*, *Rhizopus* (*R.*) *oligosporus*, *A. oryzae* and *A. sojae* are the main microorganisms involved in production of fermented soybean foods [45]. However, complex starters are typically used for fermented soybean foods; of which, Bacilli species ferment soybeans during the early stages of fermentation while Aspergillus sp. play their roles after several days of fermentation [21]. It appears that the various proteases in Bacillus and Rhizopus strains break down main soybean proteins into large peptides, subsequently, large peptides are degraded to oligopeptides by peptidase activity during the soybean preparation process [2].

3-2- Fermentation conditions

Suitable fermentation of soybean can remove TI, allergen and release more ACE I activity ingredient [45]. Modification of the soybean fermentation process can enhance ACE I. Published reports have demonstrated that ACE activity of fermented soybean seasoning (FSS), was approximately three times lower (the IC_{50} value of 454 µg ml^{-1}) than RS sauce (the IC_{50} value of 1620 µg ml^{-1}). Furthermore, FSS has shown antihypertensive effects on spontaneously hypertensive rat in long-term continuous

feeding [46]. Two-step fermentation also can decrease molecular weight over 20 kDa after 24 h of fermentation [39].

3-2-1- Effects of time and temperature

Conversions of β-glucosides to corresponding aglycones, proteins to peptides and polyphenols to genisteins and daidzeins can be changed by the fermentation time [6,18,47,48]. Short-term fermentation of soybean foods such as natto, chungkookjang and meju increased quantities of genistein, daidzein and smaller peptides by enzyme activity [6,21,49]. While Dae Kwon et al. demonstrated that the highest protein degradation rate in meju water extracts occurred after 20 days of fermentation and proteins were much smaller after 60 days of fermentation. They also mentioned that longer fermentation times of soybeans by *B. subtillus* and *A. oryzae* improved the insulin-sensitizing mechanism [21]. Zhang et al. revealed that the ACE I activity increased with fermentation time, too. They compared the ACE I activity of fermented douchi qu pure cultures using *A. Egyptiacus* (for 48 and 72 h) with douchi secondary-fermented (for 15 days) [33]. In another study, Mao-tofu was fermented by Mucor spp. for 3-9 days. The higher extraction yield or degree of hydrolysis of protein fractions determined in fermentation with longer times. In addition, five days fermentation of Mao-tofu and extraction by the ethanol/water solvent exhibited the highest ACE I activity [50].

The optimal growth temperature varies for different kinds of used culture. A study in 2003 reported that the optimal growth temperature for *A. elegans* was 25-30°C [15]. Incubation temperature for *B. subtilis* and *A. oryzae* were 37 and 28°C, respectively. The 9°C difference of fermentation temperature was a good reason for the large proteolysis gap between the two cultures [45]. Effects of time and temperature has been shown in Table 4.

Actually, in vitro and in vivo studies have shown that various starter types need different fermentation time or/and temperature that these can affect the number of bioactive components during preparation different soybean products [7,21,51]. For example, meju fermented by Bacillus and Aspergillus for 20-60 days, contained many more isoflavonoid aglycones than that chungkookjang fermented by *B. subtilus* for 2-3 days did [21]. The best fermentation conditions producing the fermented autoclaved whole soybean flour was at 30°C for 48 h [7]. Sachie Ibe et al. reported that fermentation at 39℃ for 20 h was the optimal conditions for the production of natto (inoculation by 10^4 CFU g^{-1} of *B. subtilis natto* O9516) with the highest ACE I activity (the $\underline{IC_{50}}$ value of 0.27 mg ml^{-1} and total inhibitory activity of 3.7×10^5 U) [51]. Fermentation of fermented autoclaved whole soybean flour by whole autoclaved soybean flour using *A. oryzae* at 35°C and for 36 h resulted in hydrolysis of soybean proteins and formation of LMW peptides [1]. A study was carried out in 2013 reported the best fermentation time and temperature for maximum protease activity by *L. casei* spp. *pseudoplantrum* included at 37°C for 36 h [4].

Totally, based on earlier studies, type of the starter is important for the determination of soybean fermentation time and temperature.

3-2-2- Effects of solid-state fermentation

The most common method for soybean fermentation is solid-state fermentation (SSF) [17,45]. Yang et al. reported that the nutritional quality of soybean meal was improved via SSF using *A. oryzae* or *B. subtilis* in conical flasks [17].

Table 4. Time and temperature used to produce soy fermented foods

Product	Starter	Time and temperature	Ref.
Fermented whole soybean flour	*A. oryzae* CCT 4359	30°C for 48 h	[10]
Whole autoclaved soybean flour	*A. oryzae*	35°C and 36 h	[4]
Fermented soybean	*A. oryzae*	28°C and 72 h	[45]
Fermented whole soybean flour for best protease activity	*L. casei spp. pseudoplantrum*	37°C and 36 h	[7]
Fermentation soybean bean	*B. subtilis*	37°C and 72 h	[45]
Fermentation soybean bean	*B. subtilis*	42°C and 24 h	[23]
Natto	*B. subtilis natto*	39°C for 20 h	[51]
Tofu Mao	*Mucor* spp.	20°C and 5 d	[50]
Chungkookjang	*B. lichemiformis*	40°C and 42 h	[49]
Meju	*Bacilli* spp.	Short-term fermentation Environment temperature	[24]
	A. oryzae	20-60 d	
Douchy	*A. Egyptiacus*	30°C and 48 h	[33]

A. oryzae=Aspergillus oryzae, L. casei=Lactobacillus casei, B.subtilis=Bacillus subtilis, B. lichemiformis= Bacillus lichemiformis, A. Egyptiacus= Aspergillus Egyptiacus

3-3- Released peptide sequences during fermentation and their effects on ACE I activity

Based on studies, ACE I potency is associated with the presence of hydrophobic amino acids such as proline, alanine, phenyl alanine, valine, glycin, isoleucine and

leucine [1,52]. Peptides with amino acid sequences of Ile-Phe-Leu and Trpe-Leu, found in fermented soybeans showed good ACE I activity [41]. Bioactive peptides such as Val-Pro-Pro, Ile-Pro-Pro and Tyr-Pro isolated from fermented soybeans exhibited ACE I activity and blood pressure-lowering effect in spontaneously hypertensive rat [32]. A study reported that the most and the less effective peptides with ACE I properties isolated from FSS included Alae-Trp and Vale-Gly with the IC_{50} value of 10 and 1100 µg ml^{-1}, respectively [46]. In another study, Ala, Phe and His (ACE I peptides) have been isolated from soybeans fermented by *B. natto* and *B. subtilis* [32,53]. When comparing different peptide sequences, peptides with C-terminal Ala or Pro sequence had the most ACE I potency [32,46,53]. In 2014, a study on amino acid compositions and that extracts with more total hydrophobic amino acids or proline showed higher ACE I activity [50]. Table 5 shows the identified peptide sequences from research and their ACE I potency.

Table 5. Identified peptide sequences from the study research and their ACE I potency

Sequence	IC_{50}	Ref.
Leu-Ile-Val-Thr-Gln	0.087 µM	[7]
(LIVTQ)	0.110 µM	
Leu-Ile-Val-Thr (LIVT)	not shown ACE I	
Leu-Ile-Val (LIV)	activity	
Leu-Ile-Val (LI)	not shown ACE I	
Val-Leu-Ile-Val-Pro	activity	
	Not reported*	
Ile-Phe-Leu	44.8 µg ml^{-1}	[41]
Trp-Leu	29.9 µg ml^{-1}	
Phe-Phe-Tyr-Tyr	1.9 µM	[34]
Trp-His-Pro	4.8 µM	
Phe-His-Pro	10.1 µM	
Leu-His-Pro-Gly-Asp-Ala-	10.3 µM	
Glu-Arg	880.0 µM	
Trp-Asn-Pro-Arg		
Ala-Trp	10 µg ml^{-1}	[46]
Gly-Trp	30 µg ml^{-1}	
Ala-Tyr	48 µg ml^{-1}	
Ser-Tyr	67 µg ml^{-1}	
Gly-Tyr	97 µg ml^{-1}	
Ala-Phe	190 µg ml^{-1}	
Val-Pro	480 µg ml^{-1}	
Ala-Ile	690	
Val-Gly	1100 µg ml^{-1}	

* Not reported= no numbers were reported in articles.

Moreover, the C-terminal homogeny can increase ACE I potency of the particular peptides [4,52]. Shimakage et al. demonstrated that the higher ACE I activity of Phe-Phe-Tyr-Tyr (the IC_{50} value of 1.9 µM) compared to Trp-Asn-Pro-Arg (the IC_{50} value of 880.0 µM) was due to the C-terminal homogeneity [34]. In addition, Phe-Phe-Val-Ala-Pro sequences showed more ACE I potency, compared to other residues with both Pro-Pro or Ala-Pro [52].

In addition, in one study was reported that peptides with Val-Leu-Ile-Val-Pro (LIVTQ) and Val-Leu-Ile-Val (LIVT) sequences showed inhibition against ACE with the IC_{50} value of 0.087 and 0.110 µM, respectively, while LIV and LI fractions did not show any ACE I. They demonstrated that glutamine and threonine played a key role in ACE-inhibition. The isolated ACE I peptide (Fraction F2 with Leu-Ile-Val-Thr-Gln sequences) was the IC_{50} values of 17 µg ml^{-1}[4].

However, except hydrophobic amino acid, the presence of some other amino acids such as lysine or threonine as a C-terminal amino acid residues could enhance ACE I activity, too. Feng-Juan Li et al. reported that the strongest ACE I activity was observed in a fraction with high contents of glycine, lysine and arginine from fermented soybeans by *B. subtilis* B1 [44]. This is probably because of the profile stability of essential amino acids after fermentation and digestion [39].

3-4- Effects of fermentation on release of low molecular weight and proteolytic proteins

Proteolysis of soybean protein fractions (mainly glycinin and β-conglycinin) via proteolytic enzymes increases several free amino acids and bioactive peptides with functional properties such as the ACE I, anti-thrombotic, surface-active and antioxidant activity during fermentation [2,9,13,41]. Moreover, due to improving the In vitro digestibility of the crude proteins by pepsin and fermentation, the ACE I was strongly increased [45]. Also, the main soybean antigenic proteins (b subunits of β-conglycinin and acidic subunits of glycinin) are catalyzed through the secreted proteinases by microbes during fermentation [17]. The authors observed the significant increase in the small peptide group (< 20 kDa and smaller than 7 kDa, about 60 amino acids) and the reduction in larger peptides (> 60 kDa) in fermented soybean flours [1,45,24]. Teng et al. reported that *B. subtilis* and *A. oryzae* could enhance the concentration of the small-size proteins in fermented soybeans from 5% to 63% and from 5% to 35%, respectively, and reduce the concentrations of large-size proteins from 40% to 2% and from 40% to 8%, respectively [45]. Also, in another study was reported that two-step fermentation by *R. oligosporus* and *B. subtilis* resulted in the increase of the protein hydrolysis degree and the amount of smaller peptides (< 20 kD), which were not obtained from the unfermented soybeans [39]. Short peptides with 2-9 amino acids (mostly di or tripeptides) compared to the complete protein, are resistant to In vitro gastrointestinal digestion and are absorbed more rapidly, then reach with higher concentration to the blood. So they could strongly decrease the ACE activity [41,52]. Puchalska et al. reported that Arg-Pro-Ser-Val- Thr peptide was resistant to gastrointestinal enzymes and high processing temperatures. Moreover, Arg-Pro-Ser-Val- Thr peptide

possessed moderate antihypertensive activity and potent antioxidant activity [54].

The content of free amino acid also increased 10-20 folds during soybean fermentation by proteolytic strains of *B. subtilis* [20]. Also, Shimakage et al. deduced that the ACE I compounds of natto were produced because of the degradation of soybean proteins using proteases secreted by *B. subtilis natto* [34]. By the way, fermentation could not hydrolyze the whole soybean proteins because most proteases could not cleave post translationally modified proteins [9]. So some other treatments such as germination should be associated with fermentation to gain the beast results [22].

4. Conclusion

Food derived ACE I compounds are alternatives to synthetic antihypertension drugs due to the lack of unwanted effects on the human body. Many studies have been reported that seeds e.g. legumes could generate bioactive compounds with ACE I properties. The high protein content of soybeans and its profiles/sequences of peptides provide nutritional properties for the consumers. Hydrolysis of these proteins releases a group of short-chain peptides with 3-10 amino acid which show multiple health-promoting functions such as antihypertensive activities. The ACE I potency of these peptides are associated with the presence of hydrophobic amino acids such as proline, alanine, phenylalanine, and etc. Inside of health effects of soybean compounds, some pretreatment e.g. germination and fermentation need to eliminate its anti-nutritional ingredients and release bioactive components with ACE I properties. Based on researches, determining the best germination and fermentation condition is the most important factor to reach this aim. Taking into account, the best germination temperature is at 35-40°C but there were different results for determining the best time. While kind of the starter should be considered for determining the best fermentation time and temperature and improved the bio functional compounds with ACE I properties. Based on studies, *B. subtilis* is the most favor starter for soybean fermentation. In addition, LAB and filamentous fungi via possessing galactosidase and protease enzymes can hydrolyze soybean oligosaccharides and proteins during fermentation, reduce its beany flavor, provide soybean aroma/color and released ACE I peptides.

So, future work connecting to soybean and its products could consider that preparation of soybean and its products by both germination and fermentation processes may show greater ACE activity inhibiting effects than fermentation or germination alone.

5. Conflict of interest

The authors report no conflicts of interest.

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جوانه زنی و تخمیر دانه سویا: دو مرحله سالم برای رهایش ترکیباتی با فعالیت مهار آنزیم تبدیل آنژیوتانسین

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چکیده

سابقه و هدف: سویا یکی از مهمترین دانه های خوراکی به شمار می‌رود که حاوی مقادیر بالای پروتئین، روغن های خوراکی با کیفیت خوب و مقادیر مناسبی مواد معدنی و ویتامین هاست. به دلیل حضور برخی از ترکیبات سویا که بر خواص عطری، طعمی و پایداری آن اثر نامطلوبی دارند انواع و سطوح گوناگونی از فرایندها استفاده می شود تا محصولات مناسبتر با خواص سلامتی ایجاد گردد. فشار خون بالا یکی از بیماری های دنیای مدرن است که خطر مشکلات جدی سلامتی انسان را افزایش می‌دهد. در بدن انسان، چندین روش برای کنترل فشار خون وجود دارد مانند تنظیم کننده آنزیم مبدل آنژیوتانسین. هدف این مطالعه، بررسی اثر جوانه زنی و تخمیر بر غلظت ترکیبات زیست فعال با خواص مهار فعالیت آنزیم مبدل آنژیوتانسین است.

یافته‌ها و نتیجه‌گیری: تحقیقات علمی بسیاری نشان داده اند که جوانه زنی (رویش یا به حالت مالت در آوردن) و تخمیر دو فرایند موثر و ارزان برای بهبود کیفیت دانه سویا می باشند. در حین این دو فرایند، برخی از ترکیبات ضد تغذیه ای و زیست فعال موثر بر سلامتی انسان مانند ترکیبات ضد فشار خون به ترتیب حذف و رها می‌شوند. علاوه بر این، در چندین مطالعه اثر ترکیبات جدا شده از دانه سویا بر مهار آنزیم مبدل آنژیوتانسین نشان داده شده است. بنابراین، جوانه زنی و تخمیر دانه سویا می‌تواند بر غلظت ترکیبات زیست فعال با خواص مهار آنزیم مبدل آنژیوتانسین موثر باشد.

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- مهار آنزیم مبدل آنژیوتانسین
- ترکیبات زیست فعال
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