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Fermented Soybean Products and Their Bioactive Compounds

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1. Introduction

Soybean (*Glycine max* MERILL), first grown in Eastern Asia thousands of years ago, have long been important protein sources, complementing grain proteins, in Asian countries. In addition to essential nutrients, soybean products, especially fermented soybean products, contain various functional components including peptides, isoflavonoids and more (Davis et al., 2005). Due to these nutritional and functional facts, soybean products were included in the world's top 5 healthiest foods in magazine 'Health (2006)': Due to dozens of studies showing soy is good for your heart; the FDA even allows certain soy products to have a heart-healthy claim on their labels. A number of epidemiological studies have suggested that consumption of soybeans and soy foods is associated with lowered risks for several cancers including breast, prostate, and colon, and cardiovascular diseases (Anderson et al., 1998; P.C. Butler et al., 2007; Messina, 1995; Peterson & Barnes, 1991, 1993) and improves bone health (Bhathena & Velasquez, 2002). Furthermore, some studies have shown that a diet rich in soy can reduce breast cancer risk (Messina, 1999a, 1999b).

Fermentation is one of the major processes used in the production of food from soybeans. Fermented soybean paste is indigenous to the cuisines of East and Southeast Asia. Bibliographically, Korea developed and used its own traditional fermented foods two thousand years ago (Kwon et al., 2011a). Korean soy foods are increasingly present on the worldwide market, and because kochujang (fermented red pepper paste with soybean flour) and fermented soybean pastes (doenjang and chungkukjang; both chungkukjang and chungkookjang are used in the reports) were registered in CODEX in July, 2009, they are now internationally accepted foods (Kwon et al., 2010). Most fermented soybean pastes are salty and savory and some are spicy. They are often used as condiments to flavor foods such as stir-fries, stews, and soups. This fermentation changes the physico-chemical and organoleptic properties of soy products such as color, flavor and active components.

Differences in their color, flavor and active components are due to different production methods such as the conditions of fermentation; the addition of wheat flour, pulverized meju, rice; and the presence of different microflora such as bacteria or yeasts used in their production, as well as whether the soybeans are roasted (as in chunjang) or aged (as in tauchu) before being ground. In addition to physicochemical properties, the fermentation of these soybean products changes the bioactive components, such as isoflavonoids and peptides, in ways which may alter their physiological properties in terms of healthy functions and efficacies.

Although soybean is well known to be good for preventing obesity, diabetes, heart disease and breast cancer, the scientific evidence for these health effects are not well enough documented to satisfy the inquisitiveness of oriental fermented foods. The need to satisfy this curiosity is increasing, so we have investigated the health effect of Korean traditional fermented foods, identified the active compounds produced during fermentation and the possible mechanisms that support and challenge the hypothesis. Also we designed the new noble compounds based on the structure which was isolated from fermented soybeans products by modeling the active conformation of linear peptides. Metabolic diseases such as obesity, diabetes and cardiovascular diseases that are very close related with dietary foods are the focus of this study.

Here we introduced the fermented soybean foods and discussed their compositional changes that occur during fermentation to obtain scientific evidence for the health effects and possible mechanism of action of these fermented soybean products. We reviewed the epidemiologic studies, cellular/animal experiments or metabolomic approached studies about soybeans and fermented soybeans and the most of the research reviewed here was performed by our research teams.

2. Korean fermented soybean products

We already described the typical fermented soybean foods are consumed in Asian countries such as Korea, China, Japan, Indonesia, and Vietnam (Kwon et al., 2010). The most common Korean fermented soybean foods are chungkukjang, doenjang, kanjang (soy sauce), and kochujang. Originally natto and miso were Japanese versions of chungkukjang and doenjang, respectively. China also has various fermented soybean products such as doubanjiang, douche (sweet noodle sauce), tauchu (yellow soybean paste), and dajiang.

Chungkukjang is a short-term fermented soybean product similar to Japanese natto, whereas doenjang, kochujang, and kanjang (soy sauce) undergo long term fermentation as do Chinese tauchu and Japanese miso (Fig. 1).

Traditionally chungkukjang was made by fermenting the cooked but non-crushed soybeans for two or three days in the living room, usually they prepared chungkukjang in autumn and winter after harvesting soybeans. Doenjang and kanjang were prepared by three step fermentations: firstly they prepared the meju (dried soybean block) from cooked beans in late of October or early of November and fermented it for 1 or 2 months like as solid fermentation under the outdoor roof of the Korea traditional house in winter; secondly they aged meju for another 1 or 2 months in large earthenware jars by adding a salt solution as liquid fermentation, and then they decanted the supernatant liquid to prepare kanjang from liquid and doenjang from the remaining soy paste; finally, both liquid and paste were aged for longer periods. In comparison, Japanese style doenjang (miso) and kanjang were prepared by directly fermenting the cooked soybean without making meju individually.

Kochujang is a unique and representative Korean traditional food for more than a thousand years (Kwon et al., 2011a). Kochujang was usually prepared by mixing powdered red peppers, powdered meju, salt, malt-digested rice syrup, and rice flour, and the mixture fermented for more than 6 months.

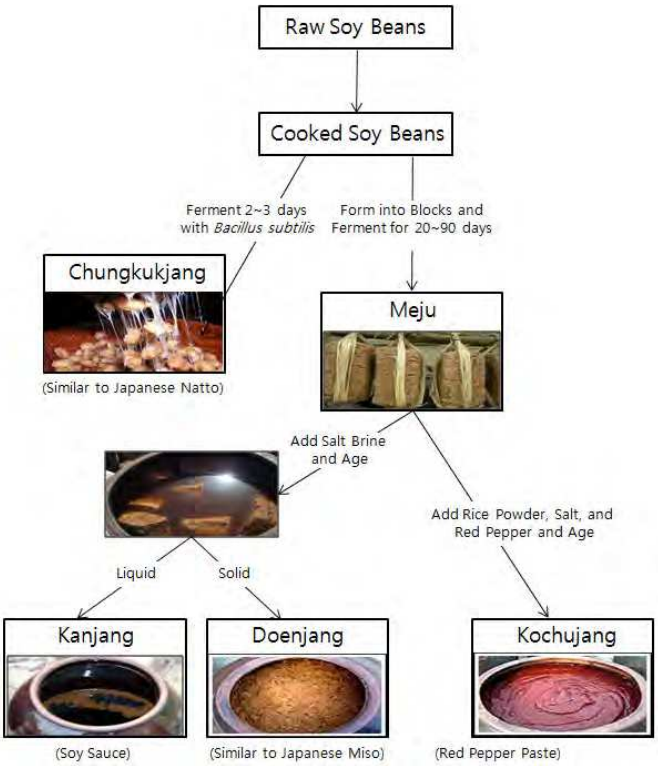


Fig. 1. The preparation of Korean fermented products from soybeans

3. Microorganisms in fermented soybean products

Meju fermentation is the most important step in producing doenjang and kanjang, because many metabolite changes occur during this fermentation, whereas the changes in flavor, color and bioactive components were primarily depends on aging after fermentation. Chungkukjang, doenjang, kanjang and kochujang are fermented with varying microorganisms when traditionally made, because fermentation conditions and ambient microbiota are different among regional environments. To make these products by the traditional method, cooked soybeans are formed into meju and fermented outdoors by micro-organisms naturally present in the environment for 1-2 months. Meju can be fermented by traditional methods, being typically fermented primarily by *Bacillus* species during the early stages of fermentation, followed by *Aspergillus* species, which predominate during the remaining fermentation period. *Aspergillus oryzae* is the major microorganism in the final product of meju when it is made in the traditional method. In traditionally

fermented chungkukjang, many different bacterial strains were identified by rRNA gene sequencing or recA sequence using randomly amplified polymeric DNA-PCR method (G.H. Kwon et al., 2009), *Bacillus subtilis*, *B. amyloliquenfaciens*, *B. licheniformis* and *B. thuringiensis* were representative microorganisms during the chungkukjang fermentation. Among them, *B. subtilis* is the predominant strain in traditional chungkukjang fermentation.

It is well known that poly-γ-glutamate (PGA) and nattokinase (fibrinolytic enzyme) are major components for maintaining a healthy immune system (Messina, 1995) and cardiovascular system (Sumi et al., 1987), respectively. Fibrinolytic microorganisms screened from chungkukjang, doenjang, meju and natto, were identified as *B. subtilis*, *Staphylococcus sciuri*, *Enterococcus faecalis*, and *Citrobacter* or *Enterobac ter* (Yoon et al., 2002). Even in tempeh (Indonesian fermented food), the microorganism producing fibrinolytic enzyme has been screened. We isolated *B. subtilis* from chungkukjang which secreted four different fibrinolytic proteases into the culture medium. Also we cloned the fibrinolytic enzyme gene, aprE2, from this *B. subtilis* into another *B. subtilis* strain to over-express the enzyme successfully using *Esherichia coli-Bacillus* shuttle vector (Jeong et al., 2007). Sometimes chungkukjang became highly viscous due to fibrous PGA polymer accumulation, Ashiuchi et al. (2001) isolated strains of *B. subtilis* from chungkukjang which produced a high contents of PGA.

4. Changes in soybean components by fermentation

4.1 Nutritional and functional compounds in soybeans

Soybean products have been known as healthy foods due to being an excellent source of high quality protein as well as providing various health benefits. The protein content of soybean is 32% to 42%, depending on the variety and growth conditions, of which approximately 80% is composed of 2 storage globulins, 7S globulin (β-conglycinin) and 11S globulin (glycinin), having various functional and physicochemical properties (Garcia et al., 1997; Kwon et al., 2002, 2003). Soybean products are considered as a good substitute for animal protein, and their nutritional value except sulfur amino acids such as methionine and cysteine is almost equivalent to that of animal protein because soy proteins contain most of the essential amino acids for human nutrition.

In addition to high-quality protein, soybeans contain high levels of unsaturated fatty acids, dietary fiber, isoflavones and minerals, which possess numerous health benefits (Young, 1991). In particular, the association of high-quality protein and phytochemicals, especially isoflavones, is unique among plant-based proteins because isoflavones are not widely distributed in plants other than legumes (Velasquez & Bhathena, 2007). Soybeans contain 0.1 to 5 mg total isoflavones per gram, primarily genistein, daidzein, and glycitein. These nonsteroidal compounds, commonly known as soy phytoestrogens, are naturally present as the β-glucosides genistin, daidzin, and glycitin, representing 50% to 55%, 40% to 45%, and 5% to 10% of the total isoflavone content, respectively (Murphy et al., 1999), depending on the soy products (Murphy et al., 1999; Velasquez & Bhathena, 2007; Young, 1991).

4.2 Changes of nutritional compounds and functional components during fermentation

The qualitative and quantitative composition of soybean components is dramatically changed by physical and enzymatic processes during the preparation of soy-based foods (L.M. Baek et al., 2008; Garcia et al., 1997; Jang et al., 2008; Y.W. Lee et al., 2007; Nakajima et

al., 2005; J.S. Park et al., 1994; Yamabe et al., 2007). Fermentation is an excellent processing method for improving nutritional and functional properties of soybeans due to the increased content of small bioactive compounds. The conformation of soy protein (glycinin) is easily changed by heat (steaming) and salt (K.S. Kim et al., 2004). The large protein, lipid, and carbohydrate molecules in raw soybean are broken down by enzymatic hydrolysis during fermentation to small molecules such as peptides, amino acids, fatty acids, and sugars, which are responsible for the unique sensory and functional properties of the final products. Short-term fermented soy foods such as chungkukjang, which are fermented with *B. subtilis*, for less than 72 hr have a much greater concentration of large molecules than do long-term fermented foods including meju and doenjang, which are fermented for more than 6 months with *Bacillus* and *Aspergilus* species from rice straw and koji, respectively (Jang et al., 2008; Y.W. Lee et al., 2007; J.S. Park et al., 1994; Yamabe et al., 2007). Proteomic analysis for soluble proteins from chungkukjang at different fermentation periods suggested that most of the soluble soy proteins were degraded into smaller forms within 20 hr, and many microbial proteins, such as mucilage proteins which, are assimilated into the bacterial biomass, dominated by the soluble protein fraction. The proteomic profile of chungkukjang was very different from that of natto, in terms of the 2-D gel protein profile (Santos et al., 2007) (Fig. 2).

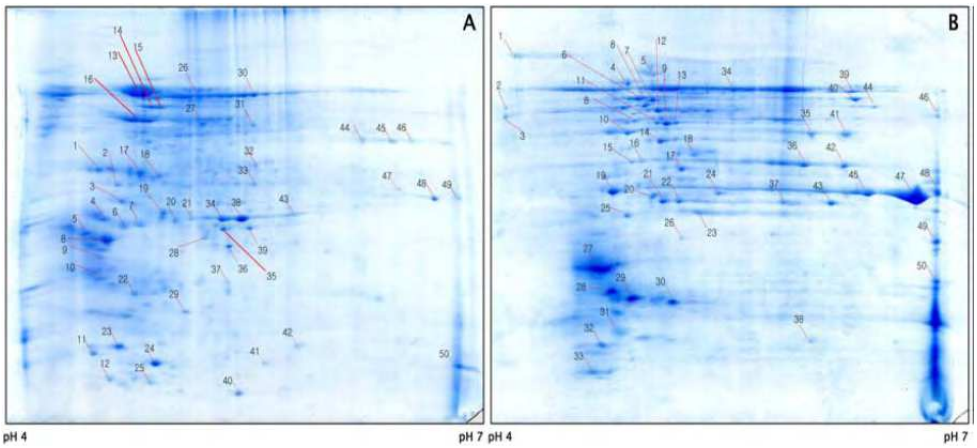


Fig. 2. A comparison of soluble proteins from chungkukjang and natto on a 2-D gel. Phenol extracted soluble proteins from chungkukjang (A) and natto (B) were separated on pH 4-7 IPG strip and 12% SDS-PAGE gel (18×20 cm), and 50 randomly selected protein spots were analyzed by MALDI-TOF MS (Santos et al., 2007).

The degradation of lipids and carbohydrates proceeds especially rapidly during the initial stage of fermentation, since these are used as the major energy sources for the microorganisms (Yamabe et al., 2007). After the initial stage of fermentation, however, soy proteins are rapidly degraded and only a small amount of the crude protein remains at the end stage of long-term fermentation. Soybean isoflavones appear to be the major components responsible for the bioactive functions such as lowering the risks of cancers of breast, prostate, and colon, cardiovascular diseases and osteoporosis.

Isoflavones, which are mostly present as 6-O-malonylglucoside and β-glucoside conjugates and associated with proteins in soybean, are also broken down by heat treatment and fermentation (Barnes et al., 1998; H.K. Choi et al., 2007). During preparation of fermented soy foods, 6-O-malonylglucosides, the most prevalent soybean isoflavones, are converted to 6-O-acetylglucosides or β-glucosides by heating, and β-glucosides are de-conjugated by the action of β-glucosidases secreted by fermentation microorganisms (Murphy et al., 1999). Most isoflavones are not enzymatically hydrolyzed during short-term fermentation, in contrast to long-term fermentation in which 6-o-malonylglucoside content declines with increasing fermentation time with concomitant increases in unconjugated aglycones (genistein and daizein). Total isoflavone content in raw soybeans was about 3,000mg/kg and this decreased by about 50% during cooking prior to chungkukjang preparation. Total isoflavone content of chungkukjang changed slightly during 45 hr fermentation. However, the content of isoflavone glycosides, consisting mainly of daizein, glycitin and genistin, decreased by about 40% during 45 hr fermentation, while the contents of free isoflavones including, daidzein, glycitein and genistein showed a dramatic increase during chungkukjang fermentation, with 2.9-, 54.0-, and 20.6 fold increases in concentration, respectively, by end of fermentation (45 hr) (Jang et al., 2006) (Table 1).

Isoflavone	Fermentation time (hr)										
	raw	0	5	10	15	20	25	30	35	40	45
Daidzin	99±31[1)]	454±17	425±24	401±25	327±28	280±5	245±22	191±5	192±7	212±7	204±10
Glycitin	45±15	101±5	101±5	87±6	77±6	60±6	65±26	69±4	34±5	41±4	35±4
Genistin	184±50	548±22	568±19	513±30	476±32	401±14	333±16	295±12	289±17	281±14	259±22
M-Daidzin	1009±29	129±41	127±29	82±7	97±12	75±9	120±54	96±30	113±22	134±10	120±27
M-Glycitin	157±2	10±5	3±0	12±5	63±5	144±3	217±1	220±0	225±7	244±5	231±1
M-Genistin	1111±8	tr[2)]	tr	tr	tr	tr	tr	tr	tr	tr	tr
A-Daidzin	85±47	75±3	64±8	54±2	56±5	83±2	93±2	91±3	94±2	99±5	97±6
A-Glycitin	101±174	10±2	7±1	5±4	11±5	29±12	44±7	50±10	46±9	38±12	33±12
A-Genistin	tr	43±3	47±3	37±3	35±3	45±0	38±5	41±6	39±5	31±6	27±5
Total glycoside	2818	1370	1341	1191	1142	1117	1156	1020	1033	1079	1006
Daidzein	tr	33±6	50±8	36±2	69±6	79±2	82±2	107±3	112±5	102±6	114±7
Glycitein	tr	4±3	3±4	5±3	43±6	100±0	141±4	152±2	156±2	171±1	165±2
Genistein	7±2	5±4	16±3	9±1	32±9	45±1	39±1	71±4	72±5	52±3	64±4
Total aglycone	7	43	69	51	143	223	262	330	341	325	342
Total isoflavones	2825	1413	1410	1242	1285	1340	1418	1350	1370	1404	1348

[1)]Values are mean ± SD (n-3). [2)]tr : trace.

Table 1. Isoflavone contents (μg/g dry water) of chungkukjang collected at various fermentation times (Jang et al., 2006).

We also investigated changes in isoflavone contents during the fermentation of meju and doenjang (Jang et al., 2008) (Table 2-3). Most of isoflavones in cooked and unfermented soybean existed as glycosides (Table 2), accounting for total isoflavones. While isoflavone

contents were not changed significantly during the first 10 days of meju fermentation, they were changed between the 10 and 90 day from the initiation of fermentation, suggesting that there was the rapid growth of microorganisms and they produced isoflavone-degrading enzymes during those periods.

Isoflavone	Raw	Steamed	Fermented period (day)			
			10	20	40	90
Daidzin	98±18	454±3	367±22	355±19	334±8	110±28
Glycitin	94±51	104±16	93±18	77±37	67±23	13±20
Genistin	148±21	913±11	637±62	623±21	488±9	229±33
M-Daidzin	1,080±44	61±3	280±31	165±14	196±6	92±41
M-Glycitin	233±24	1±1	5±9	12±1	3±5	10±9
M-Genistin	1,244±39	74±17	264±10	Tr[1)]	Tr	Tr
A-Daidzin	23±16	55±5	50±8	47±0	46±8	14±1
A-Glycitin	134±19	26±19	82±8	13±2	11±8	6±2
A-Genistin	57±10	139±3	53±22	45±19	35±17	15±24
Total glycosides	3,111±177	1,827±20	1,830±125	1,337±52	1,181±71	487±220
Daidzein	Tr	Tr	Tr	74±3	68±9	152±19
Glycitein	13±7	6±1	Tr	Tr	4±5	7±2
Genistein	12±2	16±1	6±3	35±3	25±3	170±20
Total aglycones	25±6	22±2	6±4	108±6	97±9	329±56
Total isoflavones	3,137±174	1,849±23	1,836±123	1,445±47	1,278±65	816±276

[1.)]Trace

Table 2. Change in isoflavone contents (μg/g) during meju fermentation (Jang et al., 2008)

Isoflavone	Aging period (day)							
	0	20	40	60	80	120	140	160
Daidzin	Tr[1)]	Tr	Tr	Tr	Tr	Tr	Tr	Tr
Glycitin	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr
Genistin	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr
M-Daidzin	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr
M-Glycitin	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr
M-Genistin	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr
A-Daidzin	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr
A-Glycitin	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr
A-Genistin	27±5	36±7	32±5	42±7	41±8	42±17	29±8	16±2
Total glycosides	27±5	36±8	32±6	42±8	41±9	42±18	29±9	16±3
Daidzein	222±34	246±67	226±23	282±23	271±50	275±47	325±55	273±24
Glycitein	72±14	78±21	72±4	82±5	82±17	71±16	89±22	79±23
Genistein	298±27	366±81	349±42	411±25	361±40	374±36	334±1	292±55
Total aglycones	592±70	689±167	646±64	775±50	714±106	719±75	749±110	644±14
Total isoflavones	618±75	724±175	678±69	817±56	754±114	761±92	778±111	660±12

[1)]Trace

Table 3. Change in isoflavone contents (μg/g) during aging of doenjang (Jang et al., 2008)

The concentration of aglycones in meju was increased to reach to approximately 40% of total isoflavones in 90 day meju fermentation. However, during the aging step of doenjang production, no significant changes in isoflavone content were observed probably due to inhibition of microorganism growth under high salt condition (Table 3).

In addition to the natural isoflavones found in soybeans, novel isoflavonoids such as equol (7-hydroxyisoflavan), dihydrodaidzein, and O-desmethylangolensin (Joannou et al., 1995; Lampe, 2009), known to be produced by intestinal microflora from daidzein, were found in some fermented soy products, which were metabolized by microorganisms (Fig. 3). Equol exhibits a more powerful estrogenic activity than daidzein and has been reported to exert beneficial health effects on various types of diseases such as osteoporosis, cardiovascular disease, hormone-dependent cancer, and post-menopausal syndrome. Therefore, equol production is probably one factor responsible for the decreased risk of certain cancers and other diseases in humans consuming fermented soybean foods. In general, the chemical profiles of various minor components related to health benefits and nutritional quality of products are also affected by fermentation (Izumi et al., 2000; D.J. Kim et al., 2008)

Among the various small metabolites derived from macromolecules, the changes in amino acid and peptide concentrations were especially prominent, although qualitative and quantitative changes in individual peptides were not studied. Some amino acids increased and others remained almost constant with increased fermentation time, but glutamate, the richest amino acid in soybean, was obviously decreased by fermentation, suggesting that microorganisms might use it as a preferred nitrogen source (Kada et al., 2008). Novel bioactive oligopeptides from soybean protein which are produced by fermentative microorganism is an emerging area of research with great promise. It is well known that various tastes and flavors such as umami, bitter taste and savory flavor originate from soybean peptides.

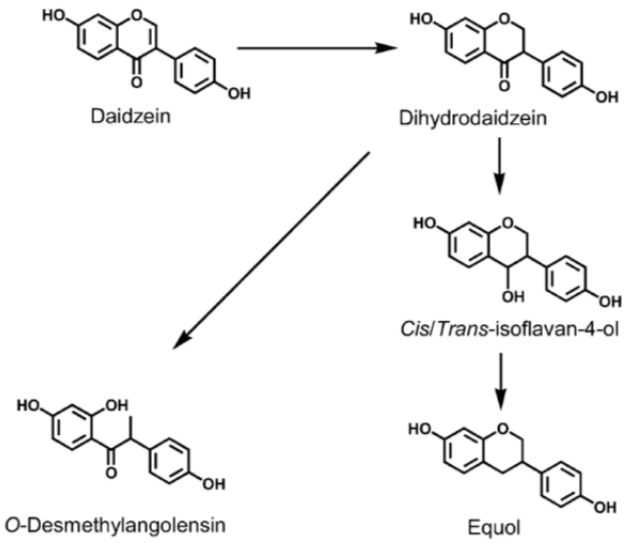


Fig. 3. Metabolism of the soy isoflavone daidzein to O-desmethylangolensin and equol. Adapted from Heinonen et al. (1999).

S.H. Kim & Lee (2003) evaluated the flavor compounds in water-soluble extract from doenjang by fractionating the amino acids and peptides by gel filtration chromatography (Table 4). Umami taste in doenjang and the savory flavor of fermented and non-fermented soy foods, such as soy sauce and hydrolyzed vegetable protein, are the result of the release of small peptides and amino acids from the fermentative digestion or acidic hydrolysis of soy proteins (Aaslyng et al., 1998; Rhyu & Kim, 2011).

In addition to the umami taste, soy protein is reported to have numerous beneficial effects in humans, including improvements in body composition, anti-hypertension, insulin secretion etc. (S.J. Kim et al., 1999; Sites et al., 2007). Many peptides having anti-hypertension and hypocholesterolemic from doenjang and kanjang have been isolated. Detailed structure of bioactive peptides preventing hypertension and hypercholesterolemia will be described in section 7.1.

Amino acids	Fractions						Threshold	Tastes of
(mg/l) (Vt, ml)[a]	2	3	4	5	6	7	value (mg/l)[b]	free amino acid in water[b]
Asp (Na)	0	0	24	36	4	2	1000	Umami
Ser	1	2	2	61	13	2	1500	Sweet
Glu (Na)	1	0	150	65	10	2	300	Umami
Gly	1	0	5	16	4	1	1300	Sweet
His	1	0	2	27	3	1	200	Bitter
Thr	1	1	4	67	12	0	2600	Sweet
Arg	1	1	2	19	14	0	500	Bitter
Ala	1	1	4	120	13	0	600	Sweet
Pro	0	2	9	98	8	2	3000	Sweet, Bitter
Cys	0	18	150	18	0	5	nd[c]	Bitter
Tyr	0	3	11	2	2	50	nd	Bitter
Val	0	1	6	82	9	0	400	Bitter
Met	1	3	4	0	6	0	300	Sweet
Lys	1	3	82	27	6	2	500	Bitter
Ile	1	2	9	70	11	1	900	Bitter
Leu	1	1	3	120	18	2	1900	Bitter
Phe	0	1	1	1	22	25	900	Bitter
Total	11	39	468	829	155	95		

[a]Vt = Total volume of the fractions obtained after chromatography of 10 ml water-soluble extract.
[b]defined as in Kato et al. (1989).
[c] not detected.

Table 4. Concentration of free amino acids identified in each tasted Sephadex G-25 gel filtrate obtained from the water-extract of doenjang (S.H. Kim & Lee, 2003).

5. Metabolite profiling of fermented soybean foods

Thousands of metabolites are among the fermented products produced by microorganism during fermentation. Most of metabolites such as fatty acids, organic acids, amino acids, peptides or isoflavone derivatives as described above are produced from the soybean by degradation, however, some metabolites do not originate from soybean. This means that fermenting microorganisms, especially *Aspergillus* species, might produce new metabolites by themselves, using soybean as a substrate source in the long fermentation period.

Chungkukjang has a characteristic flavor which is generally acceptable to Koreans but hardly tolerable to some foreign people. Volatile organic acids, such as acetic acid, propionic acid, 2-methylpropanoic acid, butanoic acid and 3-methylbutanoic acid, have been identified as the characteristic flavor in chungkukjang by gas chromatography-mass spectrometry (GC-MS) (M.K. Park et al., 2007). The contents of volatile organic acids in chungkukjang were highly dependent on the fermentation period; increasing during fermentation. Moreover, the branched-chain organic acids (2-methypropionic acid and 3-methylbutanoic acid) were formed earlier and were present in much higher concentration than the corresponding straight-chain organic acids during chungkukjang fermentation. We investigated which metabolites were made from different chungkukjang in terms of fermentation time and inoculated strains, *B. subtilis*, *B. amyloliquefaeciens* and *B. licheniformis* by GC-MS: the metabolite profiling of chungkukjang by PCA (principal component analysis) and PLS-DA (partial least square discriminant analysis) showed that some sugars and organic acids including sucrose, fructose, glucose, mannose, succinic acid, and malonic acid, as well as most amino acids, contributed mainly to differentiation of the different chungkukjangs according to fermentation time. The levels of most amino acids decreased in the early stage of fermentation and in increased in the late stage of chungkukjang fermentation with *B. subtilis* (M.K. Park et al., 2010). The levels of fatty acids generally increased throughout the fermentation process and levels of most organic acids, except for tartaric acids, decreased. Tryptophan, citric acid, β-alanine, itaconic acid, 2-hydroxyglutaric acid, γ-aminobutyric acid, leucine, malic acid, and tartaric acid were the major components that discriminated various chungkukjangs according to fermentation period. On the other hand, mannose, xylose, glutamic acid and proline were mainly responsible for differentiating the chungkukjang according to the various inoculated strains (Baek et al., 2010).

In addition to metabolic profiling by GC-MC analysis, metabolomic profiling of chungkukjang during fermentation was also carried by ^{1}H NMR spectrometry and PCA. The major peaks in the ^{1}H NMR spectra of the 50% methanol fraction of chungkukjang corresponded with isoleucine/leucine, lactate, alanine, acetic acid, citric acid, choline, fructose, sucrose, tyrosine, phenylalanine and formic acid (H.K. Choi et al., 2007) (Fig. 4).

The first two principle components (PC1 and PC2) of the ^{1}H NMR spectra of the aqueous fraction (Fig. 5) allowing discrimination of chungkukjang extracts obtained after different periods of fermentation showed that samples obtained after 0 hr and 5 hr of fermentation were separated but relatively close proximity to each other. Similarly, score plots of the samples obtained after 10, 20, and 40 hr of fermentation were separated clearly from each other. The spectra for samples obtained during the later stages of fermentation (20-40 hr) were less pronounced than those that occurred in samples obtained earlier. Therefore, fermentation appeared to occur primarily between 5 and 20 hr after the start of the fermentation process. The major compounds that contributed to discrimination were isoleucine/leucine, lactate,

acetic acid, citric acid, choline, fructose, tyrosine and phenylalanine. Among them, lactate, acetic acid, citric acid, fructose and sucrose contributed mainly to discrimination by PC1, while lactate, acetic acid, citric acid, choline, sucrose, tyrosine and phenylalanine contributed mainly to discrimination by PC2. The GC-MC and NMR results revealed similar metabolic profiling of chungkukjang according to fermentation periods.

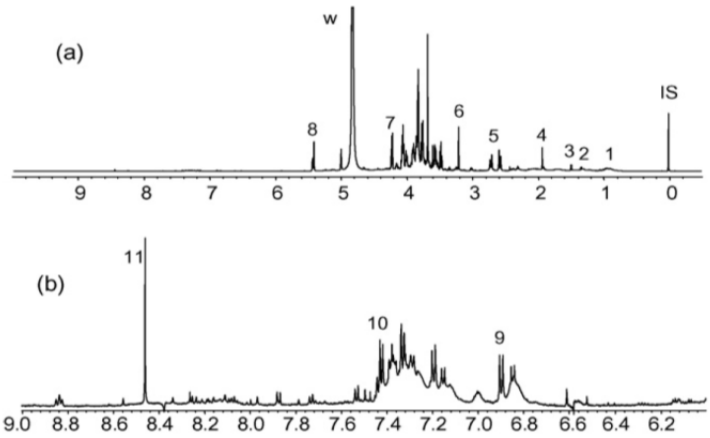


Fig. 4. Representative ^{1}H nuclear magnetic resonance (NMR) spectra of the total (a) and aromatic (b) region of the aqueous fraction of chungkukjang extracts from samples obtained at the start of fermentation. IS, internal standard; 1, isoleucine/leucine; 2, lactate; 3, alanine; 4, acetic acid; 5, citric acid; 6, choline; 7, fructose; 8, sucrose; 9, tyrosine; 10, phenylalanine; 11, formic acid; w, water. Values on the X-axis are the chemical shift in ppm relative to TSP (H.K. Choi et al., 2007).

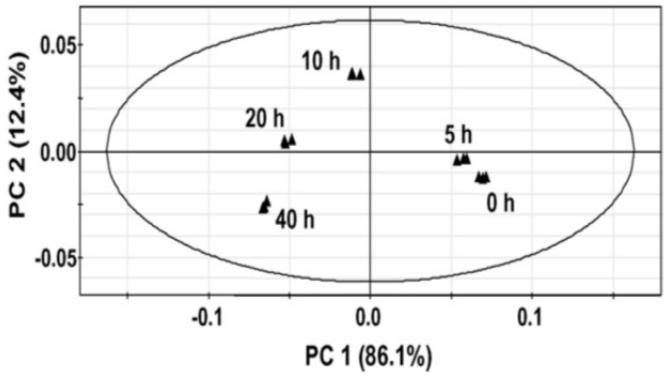


Fig. 5. Principal component analysis (PCA)-derived score plot of the first two principal components (PC1 and PC2) of the aqueous fraction of chungkukjang extracts. (H.K. Choi et al., 2007).

Metabolomic analysis of meju during fermentation by ultra performance liquid chromatography-quadrupole-time of flight mass spectrometry (UPLC-Q-TOF MS) revealed

that various metabolites, including amino acids, small peptides, nucleosides, urea cycle intermediates, and organic acids, responsible for the unique taste and nutritional and functional quality of fermented soy foods, were clearly altered by increasing the fermentation period (Kang et al., 2011). Changes in these metabolites allowed discrimination among meju samples with different fermentation periods (0, 10, 20, 40, and 60 day) on a PLS-DA score plot, and the fermentation was mainly processed between 10 and 40 day of fermentation. Twenty-two metabolites (phenylalanine, glutamic acid, leucine, adenine, citrulline, arginine, glutamine, γ-aminobutyric acid, proline, acetylornithine, valine, pipecolic acid, methionine, citric acid, xanthine, tyrosine, isoleucine, Glu-Tyr, Ser-Pro, tryptophan, Glu-Phe, and Leu-Val-Pro-Pro) with high PLS-DA values over 1.00 were determined to be the major compounds contributing to the discrimination of meju samples. These metabolites, which were positively related to the sensory quality of meju, can be used as fermentation biomarkers for the production of meju and construction of the meju fermentation metabolic pathway. Based on the metabolites found, we constructed a meju fermentation metabolic pathway that shows the overproduction and underproduction of metabolites during fermentation and the several related cycles, including the urea cycle, TCA cycle, glutamine cycle, and methionine cycle (Kang et al., 2011) (Fig. 6).

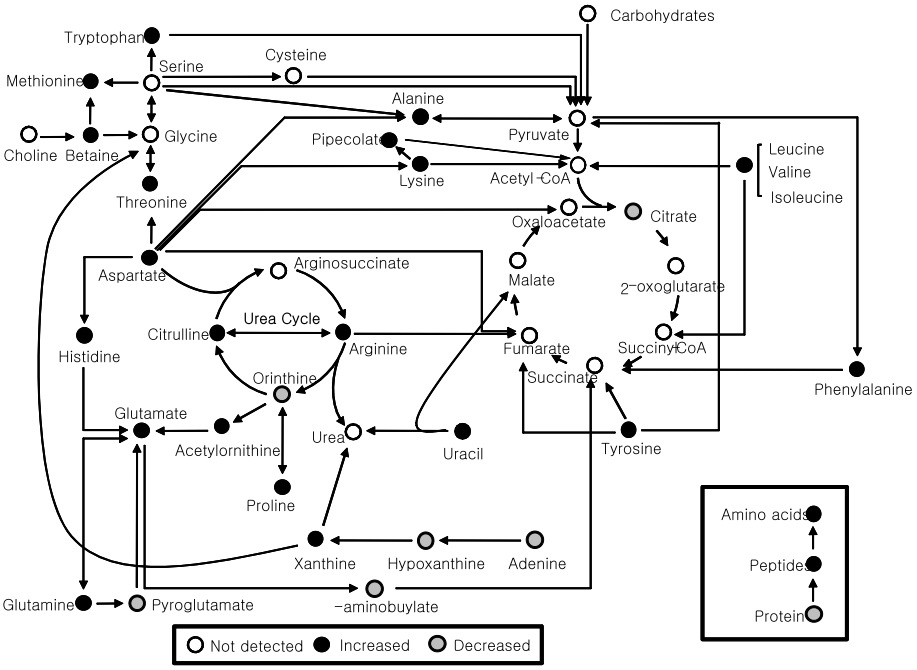


Fig. 6. Schematic representation of metabolites produced during meju fermentation. Metabolites identified by UPLS-Q-TOF are marked with black and grey. Black, grey, and open circles represent increased, decreased, and undetected metabolites, respectively. Most metabolites were physically and enzymatically produced from soybean biomass during fermentation, but some metabolites might originate from the biomass of microorganisms (Kang et al., 2011).

Some microbial metabolites being independent of proteins and other large molecules from soybean were produced by the proposed pathway during meju processing. Some metabolites, such as citrulline, for example, were likely produced via some part(s) of the pathways.

The metabolomic profiling of doenjang by NMR-PCA and GC-MS (Namgung et al., 2010; S.O. Yang et al., 2009) demonstrated that the predominantly produced amino acids included alanine, valine, leucine, isoleucine, proline, glutamine, phenylalanine and lysine, showing remarkable increases in amounts during the later stages of fermentation. Carbonic acid, citric acid, lactic acid and pyrogultamic acid were identified as the major organic acids. Significant amounts of erythrose, xylitol, inositol and mannitol were also detected during fermentation. Among fatty acids, relatively higher amounts of palmitic acid, stearic acid, oleic acid, linoleic acid and linolenic acid were found in the doenjang at each fermentation time point. Our results indicate that monitoring the changes in metabolites during meju fermentation might be important for producing meju-related foods (doenjang and kanjang) with good nutritional and sensory quality.

The study of metabolomics plays a crucial role in determining the changes in metabolites in fermented foods but also detecting bioavailability of metabolites after the consumption of foods. The function of soybeans and fermented soybeans in humans are somewhat different possibly due to their different metabolite in the blood. Thus, it is necessary to study bioavailability of metabolites in the blood using metabolomics methods in humans after consuming soybean products.

6. Functionality of soybean products

6.1 Soybeans

Soybeans are well known for their health-promoting benefits which include antioxidation, anti-obesity, anti-diabetes, properties and prevention of osteoporosis and cancers such as breast and prostate cancer (Anderson et al., 2008; Anderson & Pasupuleti, 2008; Messina et al., 1999). Many studies, but insufficient and inconclusive, suggest that these kinds of health function are primarily due to esterogenic properties of isoflavones but the results are still controversial (Kwon et al., 2010). There are hundreds of *in vitro* studies showing that genistein has anti-oxidant properties, inhibits the growth of a wide range of both hormone-dependent and hormone independent cancer cells, including breast (Pagliacci et al., 1994; Peterson et al., 1996; Zava & Duwe, 1997), prostate (Kyle et al., 1997; Zhou et al., 1999), colon (Kuo, 1996; Kuo et al., 1997), and skin (Rauth et al., 1997) cells (Adlercreutz & Mazur, 1997; Akiyama & Ogawara, 1991; Constantinou & Huberman, 1995) and that genistein also inhibits the metastatic activity of both breast (Peterson & Barnes, 1996; Scholar & Toewa, 1994) and prostate (Santibáñez et al., 1997) cancer cells independent of the effects on cell growth. Daidzein, one of the 2 primary isoflavones in soybeans, exhibits anticancer effects (Jing et al., 1993). Recently, genistein was also demonstrates to have anti-inflammatory properties (Hernandez-Montes et al., 2006; Verdrengh et al., 2003), and we found that genistein decreased cisplatin induced apoptosis by regulation p53 induction in kidney, and reactive oxygen species production in cisplatin-treated normal kidney HK-2 cells (Sung et al., 2008). Our previous review (Kwon et al., 2010) described in detail the anti-obesity effects of soybean for type 2 diabetes. Some studies of the effects of soybeans, including isoflavonoids and soy proteins, on glucose metabolism are inconsistent, and the mechanisms have not been extensively studied. However, other studies showed positive

effects on hypocholesterolemia, for example, 46 postmenopausal women taking isolated isoflavone extracts had significantly increased high-density lipoprotein cholesterol and a decrease in apolipoprotein B, the primary apolipoprotein in low-density lipoprotein particles (Clifton-Bligh et al., 2001; Goodman-Gruen & Kritz-Silverstein, 2001).

In soy protein nutrition, the sulfur amino acids, methionine and cysteine, are the limiting amino acids. However, the relatively low sulfur amino acid content of soybeans may actually provide an advantage in terms of calcium retention ironically. The reported hypercalciuric effect of protein is likely to be at least partially due to the metabolism of sulfur amino acids. The skeletal system serves as one of the main buffering systems in the body; as a result, the hydrogen ions produced from the metabolism of sulfur amino acids cause demineralization of bone and excretion of calcium in the urine (Remer & Manz, 2001). Thus, bean protein may improve calcium retention relative to animal and grain proteins.

Soy protein also has a cholesterol lowering effect in monkey (Terpstra et al., 1984) and in men (Wong et al., 1998), and protein hydrolyzate or hydrolyzed peptides of soybeans decrease blood cholesterol and glucose levels (Kwon et al., 2002; Yoshikawa et al., 1999). Lunasin, a 43-amino-acid peptide from soy, has been shown to have numerous biologic properties including anticancer and anti-inflammatory activities (Galvez et al., 2001; Mejia & Dia, 2009; K.Y. Park et al., 2001). In fact, we identified hypocholesterolemic peptides from fermented soy products, including doenjang (see following section), and soy protein isolates are known to activate peroxisome-proliferator–activated receptors (PPARs) and liver X receptor signaling and to inhibit sterol regulatory element-binding protein-1c signaling, contributing to insulin sensitization (Jhala et al., 2003; Ronis et al., 2009)]. In addition, soy-fed CD-1 mice exhibited enhanced insulin sensitivity, especially in white adipose tissue, due to the potentiation of phosphorylation of AMP-activated protein kinase and acetyl-CoA carboxylase and up-regulation of the expression of genes involved in peroxisomal fatty acid oxidation and mitochondrial biogenesis and in skeletal muscles by increasing glucose uptake (Cederroth et al., 2008).

It is also well-known that substituting vegetable oils, such as soybean oil, for animal fat reduces the risk of high blood pressure, thrombosis, platelet generation, and cholesterol accumulation (Meydani et al., 1991). Soybean oils contain health promoting linoleic, linolenic acids, and phytochemicals.

6.2 Chungkukjang

Recently, there has been growing interest in chungkukjang due to its effects on health. Previous reports indicate that the consumption of chungkukjang decreases blood pressure and serum lipids (J.L. Yang et al., 2003). In addition, dietary supplementation with chungkukjang exerted not only hypoglycemic effects but also antioxidant effects in diabetic rats (Kwon et al., 2007a). After fermentation, isoflavonoid glycones are changed into isoflavonoid aglycones, which seem to have greater anti-diabetic activity than do isoflavonoid glycones (Table 1, 2, 3) (Jang et al., 2006). Kawakami et al. (2005) demonstrated that an isoflavone aglycone–rich diet reduced liver and serum total cholesterol levels, and liver triglyceride levels in rats fed cholesterol. Thus, fermented soybean products may be more effective for ameliorating metabolic disorders due to increased isoflavonoid aglycone content.

We demonstrated that the ethanol extract of chungkukjang inhibits the generation of 1,1-diphenyl-2-picryl hydrazine (DPPH) radicals and prevents LDL oxidation. Chungkukjang

and its constituents (genistein and daidzein) also inhibited H_2O_2-induced DNA damage from NIH/3T3 fibroblasts and exhibited cytoprotective effects against H_2O_2-induced cell death. An *in vivo* study also demonstrated that an oral administration of chungkukjang water extract potently inhibited the formation of malondialdehyde, DNA damage and the formation of micronucleated reticulocytes in $KBrO_3$-treated mice (N.Y. Kim et al., 2008); ethanol extract of chungkukjang showed anti-inflammation activity by inhibiting 5-lipoxygenase from A23187-treated RBL-1 cells, and reducing leukoriene production (Y.H. Choi et al., 2008). Chungkukjang significantly reduced passive cutaneous anaphylaxis in rats at 400 mg/kg/day and also showed *in vivo* anti-inflammatory activity against arachidonic acid-induced mouse ear edema. We found that the methanol extract of 40 hr fermented chungkukjang exhibited the highest degrees of free-radical-scavenging and tyrosinase-inhibition activities, which have been indicated to prevent hyperpigmentation. These results suggest that chungkukjang extracts possess antioxidative anti-inflammatory and tyrosinase-inhibition activities, which are attributable to phenolics other than flavonoids (H.K. Choi et al., 2008).

Anti-diabetic effect was intensively studied with colleagues (D.J. Kim, 2008; Kwon et al., 2006, 2007a, 2007b); several animal studies and a few human studies have evaluated the effects of fermented soybeans on glucose metabolism as reviewed previously (Kwon et al., 2010). We found that chungkukjang improves glucose homeostasis by enhancing hepatic insulin sensitivity and insulinotropic actions in 90% pancreatectomized rats, a type 2 diabetic animal model (Kwon et al., 2007a, 2007b). In addition, chungkukjang enhanced glucose-stimulated insulin secretion in a hyperglycemic clamp study in diabetic rats and increased pancreatic β-cell mass via increased proliferation and decreased apoptosis. D.J. Kim et al. (2008) also showed similar results in C57BL/KsJ-db/db mice. Chungkukjang supplementation induced a significant decrease in blood glucose and glycosylated hemoglobin levels and improved insulin tolerance compared to the diabetic control group via increasing serum insulin and pancreatic insulin contents. Therefore, chungkukjang delayed diabetic symptoms in type 2 diabetic rats more than non-fermented soybeans, and this was related to increased isoflavonoid aglycones such as daidzein and genistein and small peptides.

In addition to anti-diabetic effects of chungkukjang, there is epidemiological evidence that chungkukjang has anti-obesity effects, but insufficient data exist. It is known that soybean and soy components have an anti-obesity and anti-diabetes (Anderson & Pasupuleti, 2008), however, the active compounds from fermented soybeans for obesity and diabetes are not fully identified. The hydrolyzated/fermented peptides or other phytochemicals produced during fermentation will be potential candidates for these actions. Although certain types of peptides isolated from the breakdown of soybeans or fermented by microbes have shown antihypertensive and anti-inflammatory properties (Inoue et al., 2009; Kato et al., 1989; S.H. Kim et al., 1999; Mejia & Dia, 2009), no specific peptides have revealed anti-obesity and anti-diabetic actions due to the difficulties of isolation and identification of bioactive compounds such as peptides.

We tried to identify the anti-obesity effects of chungkukjang by analyzing the hepatic mRNA expressions of enzymes related to fatty oxidation by RT-PCR analysis in high fat-fed mice (Soh et al., 2008). The expression of hepatic ACS (acyl-CoA synthase), CTP-1 (carnitine palmitoyltransferase-1), ACO (acyl-CoA oxidase), and UCP2(uncoupling protein-2) were increased by chungkukjang supplementation. The data demonstrated that chungkukjang supplementation leads to increased mRNA expressions of enzymes and protein involved in

fatty acid oxidation in liver, reduced accumulation of body fat and improvement of serum lipids in high fat diet fed mice (Kwon et al., 2007a; Soh et al., 2008). In addition, the hepatic transcriptional profiles using cDNA microarray showed that several genes involved in fatty acid catabolism (Acaa2, Mgll, Phyh, Slc27a2, Slc27a5), which were the main genes that had altered expressions by high fat diet, were normalized by chungkukjang consumption (Table 5) (Soh et al., 2011). Gene expression profiles obtained by both microarray and RT-PCR analysis were very similar with regard to the direction (up- or down-regulation) and degree of differences in expression. The result means that chungkukjang consumption improves serum lipid profiles and body fat accumulation, probably by modulating transcriptional levels of enzymes for utilization of fatty acids.

Genbank No	Gene	cDNA microarray		Real time RT-PCR	
		HDcon	HDC	HDcon	HDC
BG085346	Acetyl-Coenzyme A acyltransferase 2 (Acaa2)	-4.18	↔[1)]	0.28[2)]	0.94
A277495	Carnitine palmitoyltransferase 1a, liver (Cpt1a)	ND[3)]	ND	1.73	3.41
BF457090	24-dehydrocholesterol reductase (Dhcr24)	2.1	↔	1.45	0.78
BG063933	Dolichyl-phosphate (UDP-N-acetylglucosamine) acetylglucosaminephosphotransferase 1 (Dpagt1)	2.3	↔	3.02	1.91
AA023077	Hydroxysteroid 11-beta dehydrogenase 1 (Hsd11b1)	-8.95	↔	0.23	1.14
AI835105	Monoglyceride lipase (Mgll)	-8.99	↔	0.29	0.62
W82212	Phytanoyl-CoA hydroxylase (Phyh)	-5.78	↔	0.37	1.21
AI893897	Paraoxonase 1 (Pon1)	2.0	↔	1.68	0.69
AA259329	Protein kinase, AMP-activated, gamma 1 non-catalytic subunit (Prkag1)	2.2	↔	2.20	1.41
AA108401	Solute carrier family 27 (fatty acid transporter), member 2 (Slc27a2)	-4.06	↔	0.25	0.86
AA254935	Solute carrier family 27 (fatty acid transporter), member 5 (Slc27a5)	-11.73	↔	0.25	0.83

[1)]The double headed arrow symbol (↔) denotes that there is no significant difference when gene expression in HDcon (high fat diet control) or HDC (high fat diet with chungkukjang) groups were compared with the NDcon (normal diet control) group. [2)]Data are expressed as fold changes, normalized to β-actin mRNA expression, where the values for the NDcon mice were set at 1.00. The analyses were performed in duplicate. [3)]ND, not detected. (Soh et al., 2011)

Table 5. A comparison between cDNA microarray analysis and real time RT-PCR (3-12).

6.3 Meju and doenjang

It is known from *in vitro* studies that doenjang has various beneficial properties, such as anticancer, antimetastatic, antihypertensive and antimutagenic activities (Jung et al., 2006; K.Y. Park et al., 2003; S.O. Yang et al., 2009) reported doenjang showed higher antimutagenic activity than raw soybeans, cooked soybeans, and other fermented soybeans in the Ames test (A.E. Burtler et al., 2003). The active compounds that were identified included genistein, linoleic acid, β-sitosterol glucoside, soya saponin, etc. Anticancer and antimetastatic properties of doenjang were enhanced as aging time progressed (see Fig. 1) (Jung et al., 2006). The 24 month fermentation was the most effective in preventing cancer by decreasing tumor formation and increasing natural killer cell activity in spleens and glutathione S-transferase activity in livers of mice. Unlike chungkookjang, doenjang has not been demonstrated to affect glucose homeostasis because sample preparation is very difficult for studying anti-diabetic effects due to the high content of salt. However, some compounds from doenjang have been shown to have greater activity for reducing blood pressure in terms of angiotensin converting enzyme (ACE) inhibition (Hwang, 1997; S.H. Kim et al., 1999).

The health effects of meju have not attracted much research interest because meju is not the food that is eaten directly. Recently, however, interest in its functional properties has increased gradually because of its impact on functionality when making doenjang and kanjang. Like as doenjang, ACE inhibitory factors associated with its peptides were found in solid meju extract which was fermented with *B. subtilis* (Hwang, 1997). In addition, Min6 insulinoma cells treated with genistein, chungkukjang or meju extract (60 day fermentation) had greater glucose-stimulated insulin secretion capacity and greater β-cell viability than those treated with unfermented soybeans (Kwon et al., 2011b). This improvement was associated with the activation of insulin/insulin-like growth factor-1 signaling; the tyrosine phosphorylation of insulin receptor substrate-2 and serine phosphorylation of Akt was potentiated, and this in turn increased the expression of pancreatic and duodenal homeobox-1 involved in β-cell proliferation. Furthermore, genistein, daidzein, and meju extract stimulated glucagon-like peptide-1 secretion in enteroendocrine NCI-H716 cells, which generated insulinotropic actions, meaning that meju has a better anti-diabetic action than soybeans soybeans (Kwon et al., 2011b).

6.4 Kochujang

Kochujang containing meju and red pepper may affect energy, lipid, and glucose metabolism. Current investigations are concentrating their efforts on investigating the biological and physiological functions of kochujang because red pepper, a major component of kochujang, and its active principle capsaicin are known to enhance energy and lipid metabolism, possibly by increasing catecholamine secretion from the adrenal medulla through the activation of the sympathetic nervous system (Diepvens et al., 2007; Karlsson, 1994). In fact, several studies have indicated that traditional kochujang exhibits anti-mutagenic activity (S.J. Kim et al., 1999), and antitumor effects (K.Y. Park et al., 2001) are reported. Anti-obesity effects were reported in mice and rats fed with high fat diets (Ashiuchi et al., 2001; Choo, 2000; Koo et al., 2008); fermented kochujang supplementation of a high fat diet prevented obesity in mice by reducing fat deposition and decreased circulating levels of cholesterol, triglycerides, and blood glucose, mediated by down-regulating expression of lipogenic enzymes and up-regulating the lipolytic enzymes and the thermogenesis gene UCP-1 (Koo et al., 2008).

The decreased numbers of adipocytes by lipolysis may improve glucose tolerance by the enhancing insulin sensitivity. Although capsaicin content in red pepper was reduced by approximately 50% during the fermentation process, kochujang has been shown to reduce body weight, visceral fat, and serum leptin levels without modulating energy intake in diabetic rats (Kwon et al., 2009). It also improves glucose tolerance by enhancing insulin sensitivity. The improvement in hepatic insulin sensitivity lowered hepatic glucose output and triglyceride accumulation and increased glycogen storage. The possible mechanism is the potentiated phosphorylation of signal transducer and activator of transcription-3 → AMP-activated protein kinase → acetyl CoA carboxylase and the reduced the expression of phosphoenolpyruvate carboxykinase, a regulatory enzyme for gluconeogenesis, in the liver (Choi & Suh, 2004).

7. Bioactive soypeptides and design of new peptides

Many oligopeptides produced from soy protein by digestive endogenous or microbial proteinase during fermentation, demonstrated a range of biological activities – angiotensin converting enzyme (ACE) inhibition, anti-thrombotic, surface tension and antioxidant properties (Gibbs et al., 2004). The biologically active peptides were mostly derived from glycinin, a highly expressed soy protein, therefore some researchers investigated the novel bioactive peptides isolated from soyprotein by treatment with endoproteinase (pronase, trypsin, pepsin, Glu C protease, plasma proteases and kidney membrane proteases). Bioactive peptides were isolated from the fermented soybean products by *Bacillus* species or *Aspergillus* species and identified the structures. We also isolated ACE inhibitory peptides, antithrombotic and hypocholesterolemic peptides and identified the structures from doenjang (S.H. Kim et al., 1999; Kwon et al., 2002).

7.1 Bioactive peptides

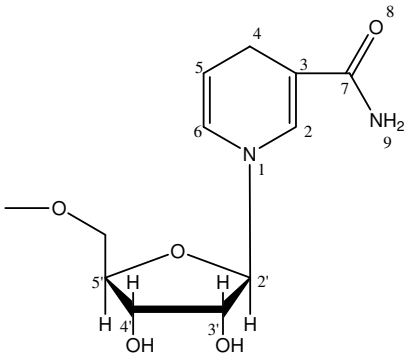
A Chinese group tried to isolate the ACE inhibitory peptides from soy protein by treating it with protease and testing the fraction for hypotensive effects in SHR (spontaneous hypertensive rats) (Wu & Ding, 2001; Gouda et al. 2006) also isolated active fraction from soyprotein peptides hydrolyzed by protease P and its sequential structure was identified as Val-Leu-Ile-Val-Pro (VLIVP). The sequence of VLIVP corresponded to Val_{397}-Pro_{401} of the glycinin subunit G2 of soybean. From kidney bean protein hydrolyzate, ACE inhibitory peptide was isolated and identified as Val-Ile-Pro-Ala-Ala-Tyr (VIPAAY) (J.R. Lee et al., 1999). Fermented soy peptides from doenjang (S.H. Kim et al., 1999) and miso (Inoue et al., 2009) have also been shown to possess ACE inhibitory activity *in vitro* and a dipeptide (Ala-Pro, AP) and tripeptides (Val-Pro-Pro, VPP and Ile-Pro-Pro, IPP) from doenjang and miso are reported to act as antihypertensive agents in SHR. The primary structural and configuration properties of ACE inhibitory peptides from soybean were similar; it starts with an alipathic nonpolar amino acid group such as Val, Ala, and Ile at the N-terminal and ends with Pro at the C-terminus (S.H. Kim et al., 1999). A certain in ACE inhibitory peptide, His-His-Leu (HHL) isolated from doenjang (Shin et al., 1995), exerts a significant decrease of ACE activity in the aorta and leads to lowered systolic blood pressure (SBP) in SHR (Shin et al., 2001). After determining the sequence of these peptides, efficacy studies were done by synthesizing the authentic and analogue peptides and the mechanism was also elucidated (Gouda et al., 2006; Shin et al., 2001). It was not determined which of the peptides in kanjang had ACE inhibitory, anti-thrombotic or antioxidant properties, however, a couple of papers

on the ACE inhibition activity of peptides and nicotinamide in Japanese soy sauce or soy seasoning were reported (Kinoshita et al., 1993; Makahara et al., 2010; Zhu et al., 2008).
Based on the studies that observed hypocholesterolemic effects of soybean protein in humans and monkeys (Terpstra et al., 1984; Wong et al., 1998), hypocholesterolemic peptides were isolated from glycinin hydrolyzate by trypsin and pepsin digestion. The identified primary structure was Leu-Pro-Tyr-Pro (LPYP) (Kwon et al., 2002) and Ile-Ala-Val-Pro-Gly-Glu-Val-Ala (IAVPGEVA) and Ile-Ala-Val-Pro-Thr-Gly-Val-Ala (IAVPTGVA) (Pak et al., 2004, 2005c) from glycinin hydrolyzed by trypsin and pepsin, respectively. Hypocholesterolemic activity was determined by assaying the inhibition of 3-hydroxcy-3-methylglutaryl CoA reductase (HMG-CoA reductase) *in vitro* and by determining the serum cholesterol levels in mice. The sequence (LPYP) was very similar to Yoshigawa's peptide (LPYPR) (Yoshikawa et al., 1999) also isolated from glycinin hydrolyzate, and the sequences including SPYPR correspond to the $A_5A_4B_3$ and A_3B_4 regions of glycinin (Fukazawa et al., 1985; Momma et al., 1985). LPYPR was a little better HMG CoA reductase inhibitor than LPYP *in vitro*, but both reduced serum cholesterol levels by the same amount (30%) (Kwon et al., 2002). Our results indicate a positive correlation between the binding of cholic and deoxycholic acids and the hypocholesterolemic effects of the pepsin hydrolyzate peptide (IAVPGEVA), while peptide hydrolyzates from trypsin digestion (LPYP and LPYPR) had hypocholesterolemic effects and gave a negative correlation for binding of bile acids. Therefore, the degree of interaction of bile acids is not ssential for identifying hypocholesterolemic activity (Pak et al., 2005a, 2005b).

7.1.1 Mode of actions of isolated peptides

Design of more potent compounds based on known bioactive compounds in terms of structure and mode of action is one of the best way to reduce the risks of failure and to save time and expense in the development of bioactive compounds. Using the peptides previously identified, therefore, we tried to design more powerful HMG CoA reductase inhibitors from soy peptide and compared them to statins and statin derivatives. Statins are known inhibitors that effectively lower plasma cholesterol levels. All statins have a chemical structure similar to HMG in their molecules (Istvan, 2003; Istvan & Deisenhofer, 2000a, 2000b; Wilding et al., 2000).
In general, understanding the relationship between structure and activity (mode of action) for two peptide structures, namely LPYP and IAVP are essential for a new peptide design. In cholesterol synthesis, isoprenoids comprise a large group of natural compounds that are formed in living cells from mevalonic acid and are involved in various cellular functions such as growth regulation of higher plants, fungi, and mammals, including sterol synthesis (Bloch, 2009). Investigations of the mechanism of mevalonic acid synthesis using enzymes of HMG-CoA reductase (HMGR) (Frimpon & Rodwell, 1994), which are key enzymes in sterol biosynthesis, established that the conversion of 3-hydroxy-3-metylglutaryl-coenzyme-A (HMGR-CoA) (main substrate) into mevalonate involves two molecules of nicotinamidedinucleotidephosphate (NADPH) (complementary substrate) and occurs through two sequential hydryl shifts. Although isoprenoids are necessary for normal cell function, an excess of certain products synthesized from mevalonate, e.g., cholesterol, can lead to progressive atherosclerosis and associated cardiovascular diseases.
We examined the capability of synthetic LPYP, LPYPR, SPYPR, IAVP and their derivatives to occupy the binding site for NADPH which is one of the substrates of HMGR. We started with the knowledge that these peptides contain a proline unit [2] in the fourth residue from

the N-terminus (this is the same in IAVP and IAVPTGVA or IAVPGEVA). Such a sequence may play an important role in HMGR inhibition (Pak et al, 2005a, 2005b). On the other hand, proline is the only unit with an aliphatic ring containing both the main and side chains. Its presence in the amino-acid sequence makes the conformation of the peptide relatively rigid. Thus, we assumed that proline in the above sequence can form a structure similar to the nicotinamide part [1] of NADPH and, therefore, has similar interactions with the binding site for nicotinamide in HMGR. We compared the peptide structures with those of the nicotinamide part of NADPH using data from an x-ray structure analysis of the enzyme active center with $NADP^+$ (Istvan & Deisenhofer, 2000a, 2000b; Istvan et al., 2000) and selected four atoms (C4, O8, N9, O6′) with the shortest H-bond distances. The peptide structures and their conformations calculated using AM1, PM3 and MNDO (Pak et al., 2005a, 2005b).



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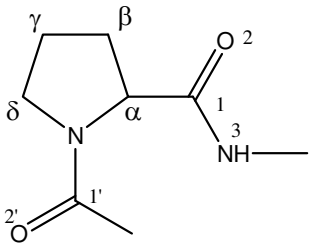

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The steric similarity of these structures were determined by calculating the percent of the projections of the selected bonds of the peptides to the corresponding bonds in the nicotinamide part of NADPH in two mutually perpendicular planes. The first plane passed through the pyridine ring of nicotinamide; the second, through C2′ of the ribose ring. Thus, the results indicate that these peptides have structural features similar to the nicotinamide part in the studied positions and correlate with the inhibitory ability of these peptides. Therefore, they may bind to the active center of HMGR and interact similarly with it as NADPH. Also, LPYP, LPYPR, IAVP, IAVPTGVA and IAVPGEVA may occupy the part of the binding site for NADPH in the active center of the enzyme. According to the mathematical model of the structure - activity relationship for these peptides, a hydrophobic part of these peptides is a required structural element for their biological activity, and the proline acts as a key component for these compounds to be recognized as residues for the nicotinamide part of the NADPH binding site (Pak et al., 2005b, 2006). Kinetic experiments support that these peptides inhibit HMGR competitively with respect to HMG-CoA and NADPH and interact with this enzyme like a bisubstrate (Pak et al., 2005d).

7.2 Peptide modeling and designing

7.2.1 Peptide design for competing with HMG-CoA

Hypocholesteroemic peptides with HMGR (HMG–CoA reductase) inhibiting activity were isolated from soybean, and found to act as inhibitors by competing with NADPH binding at

the active site of HMGR due to the structural and conformation similarity between peptides and NADPH. Based on the structures and reaction mechanisms of isolated peptides and the bioactive conformation of statins (commercial simvastatin and rosuvastatin) that also act as competitive inhibitors of HMG-CoA for HMGR (Istvan & Deisenhofer, 2000a, 2000b), design of another type of peptide sequence was proposed competing with HMG-CoA rather than NADPH. The active space in peptide design was defined by using bioactive conformations of simvastatin and rosuvastatin extracted from the crystal structure of HMGR–statin complex as shown in Fig. 7 (Pak et al, 2006).

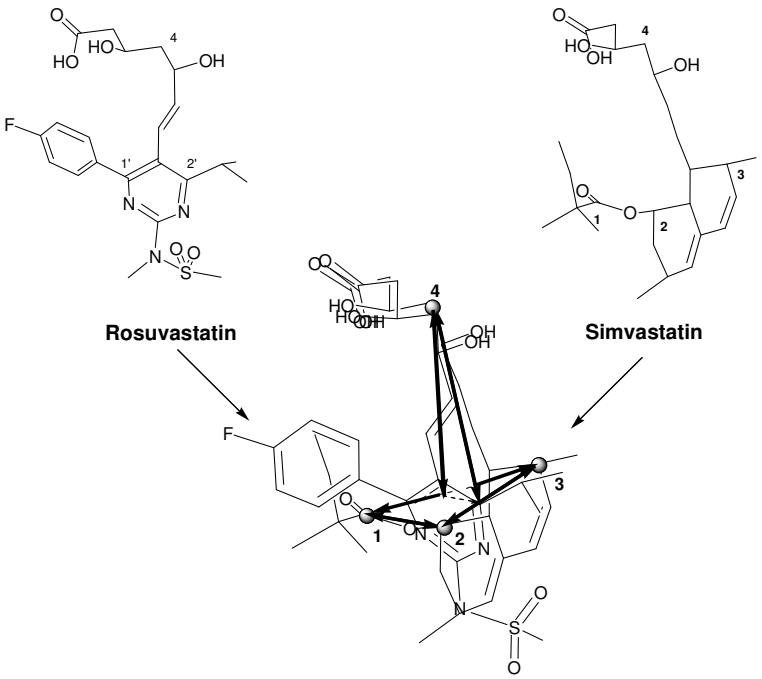


Fig. 7. Modeling of an ''active space'' in the binding site of HMGR using the bioactive conformations of simvastatin and rosuvastatin extracted from the crystal structure of HMGR–statin complexes (PDB codes: 1HW9 (simvastatin), 1DQA (rosuvastatin)). The tetrahedron 1-2-3-4 was used as a model of the ''active space'' in the present study (Pak et al., 2006).

A conformational aspect relating to an analysis of the flexibility of the peptide molecules and their occupied volumes was applied to the peptide design by extrapolation from the bioactive conformation of statin molecules. The design criterion was formulated in terms of a proximity parameter (Pr), reflecting the probability of an active peptide conformation to approximate the statin (Pak et al., 2006). It led to the proposed modeling of the peptide sequence as a tetrapeptide with an E-residue in C-terminus and L-, I-, or Y-residues at the N-terminus (Pak et al., 2005b, Istvan et al., 2000). A- and V-residues have at least a steric effect due to their aliphatic side chains and were selected for introduction into positions 2 and 3. Based on these considerations, nine peptides IAAE, LAAE, YAAE, IAVE, LAVE,

YAVE, IVAE, LVAE, and YVAE were chosen as candidate peptides for the peptide library. To elucidate a role of the E-residue in biological activities, the IAVA peptide was additionally selected. IAVP, having the highest inhibitory activity against HMGR among previously synthesized peptides (Pak et al., 2004), was chosen as a control compound to compare the biological activities of the newly designed peptides. Comparing the calculated Pr, four peptides (IAVE, YAVE, IVAE, and YVAE) from the library were selected and synthesized. This finding proposes that the obtained configurations of the peptide backbones can be seen as a basis to extend the peptide library in search of more active competitive inhibitors of HMG–CoA. Among all peptides, YVAE showed the greatest ability to inhibit HMGR. A kinetic analysis (Pak et al., 2006) revealed that this peptide is a competitive inhibitor of HMG–CoA with an equilibrium constant of inhibitor binding (K_i) of 15.2±1.4 μM. The HMGR inhibitory activity of YVAE was about 10 times greater than LPYP: IC_{50} of YVAE for HMGR is 41.8 μM, whereas the IC_{50} for the original peptide LPYP is 484 μM; a kinetic study showed that YVAE is a competitive inhibitor of HMGR. The calculated coefficient correlation (R) between log (IC_{50}) and the inverse value of proximity parameter (1/Pr) was found to be 0.99, indicating a high degree of correlation and efficacy of the given approach in the peptide sequence design.

7.2.2 Design of peptides conserving recognized residue

Previously, the structure-functional analysis in IAVPTGVA and IAVPGEVA peptides by making mutant peptides by substituting some amino acids for alanine (A-substitution) revealed that the activities suggest that the P-residue (A-substitution with P) is a recognized residue for the nicotinamide part of the NADPH binding site, and that the T- and E-residues can be seen as mimics of an HMG-moiety for the HMG-CoA binding site (Pak et al., 2005b, 2006). A conformational analysis revealed the existence of a 'β-turn' structure in these peptide sequences (Pak et al., 2004). Thus, VPTG and VPGE fragments in IAVPTGVA and IAVPGEVA peptides isolated from soy protein by pepsin treatment were shown to play an important role as a recognition site for peptide activity. Based on the presuppositions, the maintained conformation close to the bioactive conformations of VPTG and VPGE fragments was the focus for developing lead peptide candidates, which include constrained structures (maintaining VPTG and VPGE fragments) in a recognized sequence through a number of peptides in the design of a competitive inhibitor.

Location of the side chain of peptides was compared to that of iso-butyl (simvastatin) and the benzene ring of the 4-fluorophenyl radical of statins (rosuvastatin). The design criterion was formulated in terms of a 'V' parameter, reflecting an occupied volume in conformation space by an individual peptide adduced to the conformation space occupied by all peptide candidates from a library (Pak et al., 2007). Twelve peptide cycles were selected for the peptide library. Comparing the calculated 'V' parameters, two cyclic peptides (GLPTGG and GFPTGG) were selected as lead cycles from the library (Table 6). The constrained GLPTGG and GFPTGG peptides were designed according to obtain the most rigid peptide backbone (Pak et al., 2007). Based on sequences GLPTGG and GFPTGG, six linear peptides obtained by breaking the cycle at different positions were selected as lead peptide candidates. It is proposed that activities of the linear peptides based on an identical amino acid sequence, which are obtained from a less flexible peptide cycle, would be relatively higher than those obtained from more flexible cyclic peptides. The linear GFPTGG peptide, showing the highest inhibitory activity against HMGR, increases the inhibitory potency nearly tenfold

(Table 6). Kinetic analysis reveals that the GFPTGG peptide is a competitive inhibitor of HMG-CoA with an equilibrium constant of inhibitor binding (K_i) of 6.4±0.3 μM and IC_{50} of GFPTGG for HMGR is 16.9 μM. CD spectra GFPTGG peptide in the TFE/water mixture, type II of the β-turn was considered as a major structural element in these peptides (Pak et al., 2007).

Peptide		Position										IC_{50} [μM]
	Set	5	4	3	2	1	1′	2′	3′	4′	5′	
Cyclic				G	L	P	T	G	G			46
				G	F	P	T	G	G			47
				G	W	P	T	G	G			82
Linear					L	P	T	G	G	G		84
	1				F	P	T	G	G	G		60
					W	P	T	G	G	G		357
				G	L	P	T	G	G			19
	2			G	F	P	T	G	G			17
				G	W	P	T	G	G			137
			G	G	L	P	T	G				54
	3		G	G	F	P	T	G				48
			G	G	W	P	T	G				297
			G	G	L	P	T	G	G	G		328
	4		G	G	F	P	T	G	G	G		299
		G	G	G	L	P	T	G	G	G	G	801
		G	G	G	F	P	T	G	G	G	G	698
Control[a]			I	A	V	P	T	G	V	A		152
			I	A	V	P	G	E	V	A		201
Negative control			I	A	V	T	P	G	V	A		Inactive

[a]The previously isolated peptides (Pak et al., 2005c).

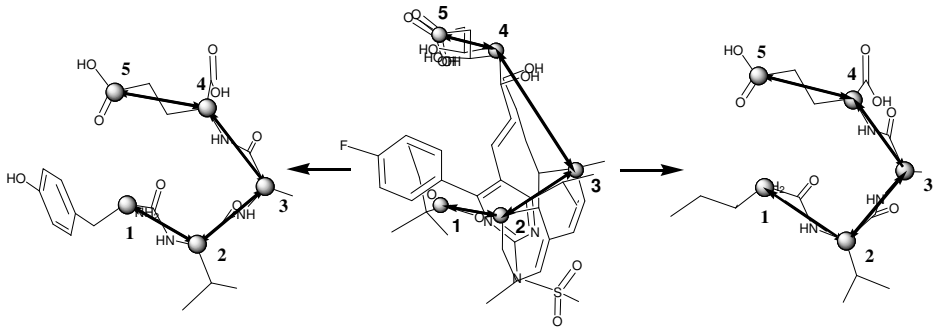
Table 6. Sequence structures and inhibitory activities (IC_{50}) of the synthesized peptides used in this study

7.2.3 Modeling of an active backbone of peptide

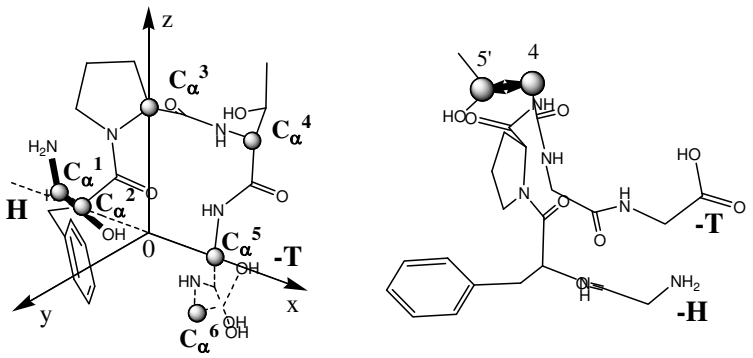
A structure-function analysis of synthesized peptides proposes that a competitive inhibitory peptide can be designed by maintaining bioactive conformation in a recognized sequence as an aspect of the structure-based approach. A two-stage approach was applied in the peptide library design: The first was the modeling of the peptide backbone of a competitive inhibitory peptide in the active site of HMGR using previously designed peptides; The second stage was the design of new peptide libraries in order to evaluate the effects of the functional residue on peptide affinity. These applications utilized two different approaches in the design of constrained and unconstrained peptides in the investigation of the peptide binding effect for HMGR.

Based on the modeling of an active peptide backbone in the active site of HMGR, two peptide libraries for constrained and unconstrained peptides were designed using different amino acids varying in hydrophobicity and electronic properties. IAVP and the peptides IAVE, YAVE, IVAE, and YVAE as competitive inhibitors of NADPH and HMG-CoA,

respectively, were selected by the design approach for unconstrained peptides (Pak et al., 2006). GLPTGG and GFPTGG, acting as a bisubstrate-mimic, were chosen among designed peptides, having the most constrained structures (Fig. 8) (Pak et al., 2008a). Active peptides were selected by the design parameter 'V' or 'Pr', which reflects the probability of active peptide conformations for constrained and unconstrained peptides, respectively. (Table 7) (Pak et al., 2006, 2007). According to the 'Pr' value, FVAE and F(4-Fluoro)VAE have relatively higher values compared with WVAE and halogen-containing peptides,



a



b

Fig. 8. Models of HMG-CoA competitive inhibitory peptides (a) and peptide acting as a bisubstrate (b). (a) Model of an 'active space' (tetrahedron 1-2-3-4) based on a superposition of the bioactive conformations of simvastatin and rosuvastatin extracted from the crystal structure of HMGR–statin complex (PDB codes: 1HW9 (simvastatin), 1DQA (rosuvastatin)), and the average structures determined for IVAE and YVAE peptides. (b) Model of the type II β-turn in peptide conformer (-H:-T) with an arrangement of peptide head (H-bold) and tail (T-short-dashed) relative to the plane passing through the three α-carbon of 2, 3, and 5 atoms of the peptide backbone, and the peptide conformation (-H:-T) used in this study. The 4–5 and 4–50 fragments in the peptide models were fixed as an HMG-mimic during modeling (Pak et al., 2008a).

respectively (Pak et al., 2008a). By considering the 'V' parameter, the GFPDGG and GFPEGG sequences are the most rigid structures compared to the other peptides, respectively. By considering the 'V' parameter, the GFPDGG and GFPEGG sequences are the most rigid structures compared to the other peptides (Pak et al., 2008a). Each of the synthesized peptides showed an ability to inhibit HMGR, with the exception of the GVAE and GGPTGG peptides (Table 7). The activities of the GVAE and GGPTGG peptides were not detectable, even at high concentrations. This indicates that the location of the I-, L-, F- and Y-side chains plays an important role in the peptide–protein interaction in the active site of HMGR. The GFPDGG peptide (IC_{50} = 1.5 μM), designed on the basis of the rigid peptide backbone, increases the inhibitory potency more than 300 times compared to the first isolated LPYP peptide (IC_{50} = 484 μM) from soybean. The obtained data suggest the possibility of designing a highly potent inhibitory peptide for HMGR and confirms that changes in the secondary structure of the enzyme play an important role in the mechanism of HMGR inhibition.

Peptide sequence	Peptide description	IC_{50} (μM)
LPYP	Peptide isolated from soybean by trypsin	484.7
IAVPGECA	Peptide isolated from soybean by pepsin	152.1
Unconstrained peptide		
IAVP	NADPH competitive inhibitor	97.1
IAVE	HMG-CoA competitive inhibitor	75.2
YAVE	HMG-CoA competitive inhibitor	52.6
IVAE	HMG-CoA competitive inhibitor	44.1
YVAE	HMG-CoA competitive inhibitor	41.8
FVAE	HMG-CoA competitive inhibitor	43.8
F(4-Fluore)VAE	HMG-CoA competitive inhibitor	3.8
GVAE	Negative control (I- and Y- side chains)	Inactive
Constrained peptide		
GLPTGG	NADPH and HMG-CoA competitive inhibitor	19.4
GLPDGG	NADPH and HMG-CoA competitive inhibitor	22.3
GLPEGG	NADPH and HMG-CoA competitive inhibitor	27.2
GFPTGG	NADPH and HMG-CoA competitive inhibitor	16.9
GFPDGG	NADPH and HMG-CoA competitive inhibitor	1.5
GFPEGG	NADPH and HMG-CoA competitive inhibitor	1.7
GGPTGG	Negative control (I- and F- side chains)	Inactive

Table 7. Summary of the sequence structures and inhibitory activities (IC_{50}) of the isolated and designed peptides used in this study (Pak et al., 2008a).

7.2.4 Design of an active peptide using a model with fixing recognized residues

In the proposed design, two binding site points were used: the first was derived from a relative assessment of the ''region bioactivity,'' in HMGR, through an analysis of the active structures of statins; the second from binding of the designed peptides inside the ''region bioactivity'' through a common point/site of the bioactive compounds and peptide structures. Under these points, the peptide conformations fixed by active/recognized residues can be seen as an approach to model the restricted flexibility of the peptides in accordance with the conformational requirements imposed by the ''region bioactivity' (Pak et al., 2008b).

In order to design a linear peptide with an unconstrained structure in a recognition sequence, two subsequent stages were applied based on the YVAE peptide as a basis of the recognition sequence: the first stage was to extend the peptide length for YVAE sequence in accordance with statin structures. For this purpose, structures of all statin molecules extracted from the crystal structures of HMGR–statin complexes were analyzed while focusing on the structural diversity of the rigid hydrophobic groups. The second stage was to investigate the conformational behavior of the peptide models. A principle component analysis (PCA), which projects multidimensional data on low-dimensional subspace, was used to evaluate a head-to-tail peptide cycle as a model of linear analog in order to select a lead peptide candidate. Using the space obtained (V parameter) by an analysis of the bioactive conformations of statins, eight cyclic peptides were selected for a peptide library based on the YVAE sequence as a recognized motif (Fig. 8). For each cycle, the four models were assessed according to the design criterion applied for constrained peptides. Three cyclic peptides (FGYVAE, FPYVAE, and FFYVAE) were selected as lead cycles from the library. The linear FGYVAE peptide (IC_{50} = 0.4μM) showed a 1,200-fold increase in inhibitory activity compared to the first isolated LPYP peptide (IC_{50} = 484μM) from soybean (Table 8). Experimental analysis of the modeled peptide structures confirms the appropriateness of the proposed approach for the modeling of active conformations of peptides (Pak et al., 2008b).

Peptides	Peptide sequence	Fixed residues in set				IC_{50} (μM)
		1	2	3	4	
Design	GGYVAE	E	EA	EAV	EAVY	7.4
	FFYVAE	E	EA	EAV	EAVY	2.5
	FPYVAE	E	EA	EAV	EAVY	1.4
	FGYVAE	E	EA	EAV	EAVY	0.4
	FWYVAE	E	EA	EAV	EAVY	29.5
	GGGGYVAE	E	EA	EAV	EAVY	760.7
Control	IAVPTGVA	T	TG	TGV	TGVA	152.1
	IAVPGEVA	E	EV	EVA	EVAI	201.3
	LPYP[a]					484.7
	YVAE[b]					41.9
Negative control	FGYVAA					Inactive

[a]The LPYP peptide was found by analysis of a digested soy glycinin using trypsin (Kwon et al., 2002).
[b]The YVAE peptide was used as a basis of the recognized sequence, which presents an unconstrained peptide structure (Pak et al., 2006).

Table 8. Peptide sequences, peptide models and inhibitory activities (IC_{50}) of the synthesized peptides used in this study

7.2.5 Peptide fragmentation for design of peptides

In a previous design, while searching for lead peptide candidates, the efficacy of a design approach based on the use of a cyclic peptide as a model of linear analog was demonstrated (Valero, et al., 2000). The conformational behavior of the cyclic peptides showed that the 6-membered cyclic peptide was relatively stable compared to the 8-, and 10-membered cyclic peptides (Pak et al., 2010). Analysis of the conformational behavior of the peptide models showed that an analogical approach could be applied in order to assess the conformational

Peptide sequence	Peptide fragment					IC_{50} (μM)
	1	2	3	4	5	
Control[a]						
LPYP	LPYP					484.72
IAVPGEVA	IAVP	AVPG	VPGE	PGEV	GEVA	201.12
IAVPTGVA	IAVP	AVPT	VPTG	PTGV	TGVA	152.19
Set 1[b]						
GLPTGG	GLPT	LPTG	PTGG			19.43
GLPDGG	GLPD	LPDG	PDGG			22.31
GLPEGG	GLPE	LPEG	PEGG			27.28
GFPTGG	GFPT	FPTG	PTGG			16.94
GFPDGG	GFPD	FPDG	PDGG			1.52
GFPEGG	GFPE	FPEG	PEGG			1.78
GXPTGG[d]	GXPT[d]	XPTG[d]	PTGG			4.82
GXPDGG[d]	GXPD[d]	XPDG[d]	PDGG			6.95
GXPEGG[d]	GXPE[d]	XPEG[d]	PEGG			0.75
GXPGGG[d]	Negative control					Inactive
Set 2[c]						
IAVE			IAVE			75.23
IVAE			IVAE			52.67
YAVE			YAVE			44.81
YVAE			YVAE			41.21
FFYVAE	FFYV	FYVA	YVAE			2.56
FPYVAE	FPYV	PYVA	YVAE			1.47
FGYVAE	FGYV	GYVA	YVAE			0.41
XVAE[d]				XVAE[d]		3.84
FGXVAE[d]		FGXV[d]	GXVA[d]	XVAE[d]		8.52
GFGYVAE	GFGY	FGYV	GYVA	YVAE		0.27
TFGYVAE	TFGY	FGYV	GYVA	YVAE		0.26
DFGYVAE	DFGY	FGYV	GYVA	YVAE		0.16
EFGYVAE	EFGY	FGYV	GYVA	YVAE		0.24
DFGYVAG	Negative control					Inactive

[a] 'Control' contains the peptides isolated from soy protein (Kwon et al, 2002; Pak et al., 2005b, c).
[b] 'Set 1' contains the peptides, which includes the recognized T, D or E residue as a corner residue of the β-turn structure (Pak et al., 2007, 2008a).
[c] 'Set 2' contains the peptides, where the E residue is a recognized residue for the HMG-binding pocket, and a β-turn structure is located in the N-terminus of the hexapeptides (Pak et al., 2008b).
[d]The substituted (4-fluoro)phenylalanine residue is indicated as 'X'.

Table 9. Peptide sequence, peptide fragment, and inhibitory activities (IC_{50}) of the synthesized peptides (Pak et al., 2010).

space that was occupied by a peptide by using peptide fragments. In order to assess the proposed method, a competitive inhibitor for HMGR was designed by using two starting points: (1) determined recognition residues and (2) the structural preference of a peptide, such as a β-turn conformation in the present design. For testing the proposed design, 13

peptides that were designed in previous studies were used (Pak et al., 2007, 2008a, 2008b). These peptides were divided into two sets in accordance with the location of a 'turn' structure relative to the recognized residue that is essential for binding. Set 1 comprised six peptides: GLPTGG, GLPDGG, GLPEGG, GFPTGG, GFPDGG, and GFPEGG (Table 9).
A common structural element of these peptides was a turn conformation, which included T, D, or E as a corner residue of the β-turn structure and was recognized by a HMG-binding pocket. All peptides inhibited HMGR in a competitive manner. Set 2 included seven peptides, in which E residue was also a recognition residue for the HMG-binding pocket. A conformational analysis of the IAVE, IVAE, YAVE, and YVAE peptides revealed no observable patterns that were related to a well-populated secondary structure conformation (Pak et al., 2006). For the FFYVAE, FPYVAE, and FGYVAE peptides, a turn conformation was determined at the N-terminus of these peptides. All of these peptides presented as HMG-CoA competitive inhibitors (Pak et al., 2008b) (Table 9).

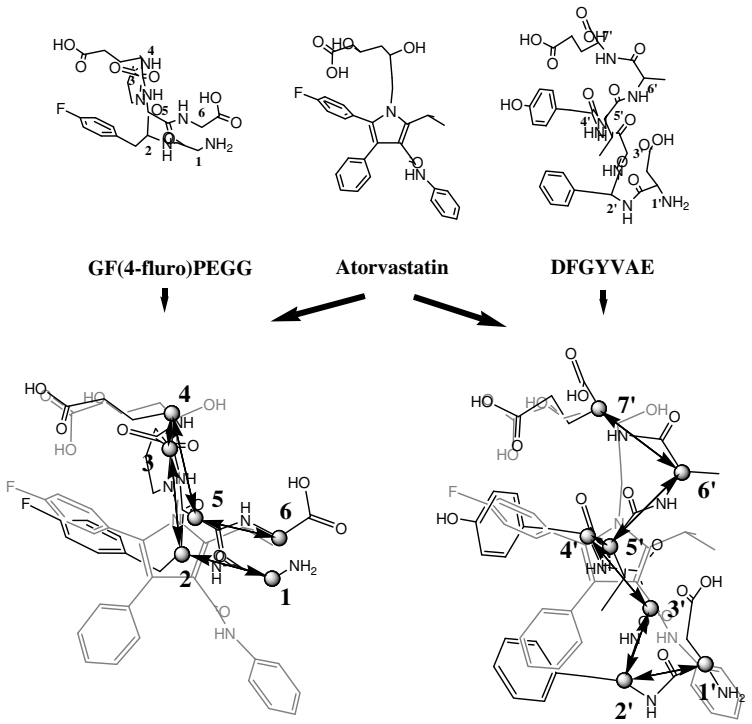


Fig. 9. Spatial compatibility of the linear GF(4-fluoro)PEGG and DFGYVAE peptides with atorvastatin. The alpha-carbon atoms are indicated by marks from 1 to 6 for the GF(4-fluoro)PEGG peptide and from 10 to 70 for the DFGYVAE peptide. The model of GF(4-fluoro)PEGG was built as type II of the β-turn on the basis of the fixed backbone dihedral angles adopted by the two corner proline and glutamic acid residues (φ_{i+1} = -60°, ψ_{i+1} = 120°, φ_{i+2} = 80°, and ψ_{i+2} = 0°). For the DFGYVAE peptide, the model was constructed as type I of the β-turn by using the fixed backbone dihedral angles adopted by the two corner glycine and tyrosine residues (φ_{i+1} = -60°, ψ_{i+1} = -30°, φ_{i+2} = -90°, and ψ_{i+2} = 0°). The orientations of the side-chains and other residues were determined by an optimization procedure.

Two sets of peptides were developed based on the different locations of a β-turn structure relative to a recognition residue (Table 9). Set 1 contains peptides in which a recognition residue is included in turn conformation. In Set 2, the β-turn structure is located distantly from the recognition residues. The design parameter 'V' that was applied in previous studies was slightly modified for the purpose of the current research. The 17 previously and 8 newly designed peptides were estimated by this parameter. In each set, one sequence was selected as a lead peptide candidate for each set: GF(4-fluoro)PEGG for Set 1 and DFGYVAE for Set 2. The inhibitory activities improved in each set, the proposed contribution of the fluorine atom of GF(4-fluoro)PEGG peptide during binding led to an increase in the inhibitory activity for this peptide compared to the other members of Set 1 (Fig. 9). This was explained by a similar location of the 4-fluorophenyl group of atorvastatin and GF(4-fluoro)PEGG peptide. Probably, the increase of inhibitory activity of the DFGYVAE peptide can be interpreted in terms of the contribution of the oxygen atom of the carbonyl group of the D side chain, while the increase of inhibitory activity of the GFGYVAE peptide by the contribution of the oxygen atom from the amide bond between N-terminus' G and F residues in binding. The IC_{50} for the GF(4-fluoro) PEGG peptide was found to be 0.75μM, while the linear DFGYVAE peptide (IC_{50} = 0.16μM) showed a 3,000-fold increase in inhibitory activity compared to the first isolated LPYP peptide (IC_{50} = 484μM) from soybeans. The comparison of the structure–activity relationship (SAR) data between Set 1 and 2 provided an opportunity to design the peptides in terms of peptide selectivity.
In conclusion, the present study not only shows the design of a more potent inhibitor for HMGR, but also defines a design tool to model active conformations for linear peptides.

8. Conclusions

One possible reason for the lower incidence of some diseases such as obesity, diabetes, or even breast cancers among Asians is that they consume fermented soybean products, which are unique to traditional Asian diets. Asians have prepared and eaten their own soy products such as tofu and fermented soy products for thousands of years. Soybean fermented foods such as doenjang (long-term fermented soy products), chungkukjang (short-term fermented soy products), kanjang (fermented soy sauce) are highlighted due to their healthy functionalities. Both short- and long-term fermented soybeans contain more beneficial components to ameliorate metabolic disorders than unfermented soybeans. The changes in nutritive and non-nutritive biofactors during fermentation and their capacity for ameliorating metabolic disorders were reported in Asian countries, especially Korea. The changes in metabolomic profiling were analyzed using GC, LC, and NMR with mass spectrometry for the metabolites produced during the fermentation by *Bacillus* for a short-term fermentation and *Aspergillus* for a long-term fermentation. Fermentation of soybeans increased isoflavonoid aglycones, modified isoflavonoids such as equol and small peptides and these changes enhanced the prevention of metabolic disorders. Chungkukjang and meju improved anti-diabetic action by improving β-cell function and mass via potentiating insulin/IGF-1 signaling in the islets. Thus, daily consumption of fermented soybean products can prevent and/or delay metabolic disorders in humans. From fermented soybeans we found some effective peptides for reducing the hypertension and hypercholesterolemia by analyzing ACE (angiotensin converting enzyme) inhibition and HMG(3-hydroxy-3-methylglutaryl)-CoA reductase inhibition, respectively. Active hypotensive and hypocholesterolemic peptides were isolated and structures identified as HHL for hypotensive, and LPYP and IAVPGEVA for hypocholesterolemic peptide, respectively. Based on the conformation of these peptide structures and mechanisms of the

reactions, new peptides were designed using peptide modeling to strengthen their activity as candidates for a new drug and nutraceuticals. Novel peptide (DFGYVAE) was designed to increase the hypocholesterolemic effectiveness to three thousand times higher than origin peptide (LPYP). Currently the peptides from soy or other beans can be the subject of investigation for new drugs and functional food ingredients for gut health and modulating the intestinal absorption of nutrients. Research into bioactive soy peptides is still in its infancy, but holds great promise.

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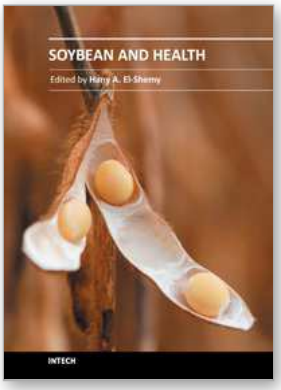

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Worldwide, soybean seed proteins represent a major source of amino acids for human and animal nutrition. Soybean seeds are an important and economical source of protein in the diet of many developed and developing countries. Soy is a complete protein, and soy-foods are rich in vitamins and minerals. Soybean protein provides all the essential amino acids in the amounts needed for human health. Recent research suggests that soy may also lower risk of prostate, colon and breast cancers as well as osteoporosis and other bone health problems, and alleviate hot flashes associated with menopause. This volume is expected to be useful for student, researchers and public who are interested in soybean.

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