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- Nutritional and technological aspects related to the development of functional foods and nutraceuticals.
- Food digestion, bioavailability, mechanism, efficacy, and safety of food ingredients and nutraceuticals.
- Food product development with health benefit
- Characterization of healthy foods and functional constituents
- Preparation of natural and synthetic ingredients for use in foods and supplement
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Journal of Functional Food and Nutraceutical

MINIMUM WATER CONSUMPTION METHOD SCREENING OF VELVET BEAN (MUCUNA SP.) PROCESSINGS TO PRODUCE FUNCTIONAL FOOD INGREDIENTS

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ABSTRACT

Velvet bean (*Mucuna* sp.) has been proven containing many beneficial compounds that can be implemented in pharmaceutical and medicines but less noticed for functional foods even though traditionally it is consumed as daily foods or snacks. The indigenous food preparation such as velvet bean Tempe warrants scientific investigation to help society with better public health management. The objective of the review is to select the best method for functional food ingredient product development using velvet beans and provide hypothetical health-oriented food processing e.g. velvet bean flour as functional food ingredients with a focus on less water consumption during processing. Steaming is the selected method.

Keywords: *Food ingredient, functional food, lower water use processing, safe velvet bean*

ABSTRAK

Koro benguk (*Mucuna* sp.) telah terbukti memiliki banyak senyawa yang menguntungkan yang dapat diterapkan dalam farmasetika dan pengobatan tetapi kurang diperhatikan untuk pangan fungsional meskipun secara tradisional dikonsumsi sebagai pangan sehari-hari atau camilan. Penyiapan pangan secara asli seperti tempe koro benguk memerlukan investigasi ilmiah untuk membantu masyarakat dengan pengelolaan kesehatan masyarakat yang lebih baik. Tujuan dari review ini adalah untuk menyeleksi metode terbaik untuk pengembangan ingridien pangan fungsional menggunakan koro benguk dan memberikan hipotesa pengolahan pangan yang berorientasi kesehatan, misalnya, tepung koro benguk sebagai ingredien pangan fungsional dengan fokus pengolahan yang sedikit memerlukan air. Pengukusan sebagai metode yang dipilih.

Kata kunci: *Ingredien instan, koro benguk yang aman, pengolahan dengan sedikit air, pangan fungsional*

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INTRODUCTION

Velvet beans, Mucuna genus, has recently become important and it seems progressively being explored for potential foods with beneficial health effects so the genus members are potential functional food resources. It has almost been four decades the intensive research on *Mucuna sp.* revealing various aspects, ultimately those of nutritional and health effects, antinutrients, and processing types. The abundant data yet is awaiting to be optimally used to support sustainable healthy living and to fixing the degrading agricultural system for livelihood. Velvet bean is a well-known covering crop to replenish humus and nitrogen fixation as green manures in the farming area. Moreover, mucuna showed the capability to extract >30% of soil hydrocarbon into leaf (Nwaichi *et al.*, 2009) and recommended as decontaminator plant to tackle oil spills in the land (Eucharia and Edward, 2010). Although L-DOPA is released into the soil as a weed controller by mucuna affecting the growth of other plants, its allelochemical effects lose after 12 weeks postharvest period tested using *S. stenocarpa* (Eucharia and Edward, 2010).

There are two velvet bean climbing characteristics, i.e. woody and herbaceous; totally, it is comprised of around 105 species in the world either growing in tropical or subtropical regions (Ingalhalikar *et al.*, 2017). The woody velvet bean is more likely to be newcomers whose different types of pods, seed shapes, and general floral colors. *M. laticifera*, *M. macrocarpa*, and *M. birdwoodiana* are those belong to woody velvet bean whereas herbaceous velvet bean are *M. pruriens, M. utilis*, and many others which have been explored earlier for foods and medicines. Hence, *Mucuna sp.* truly still becomes interesting research subjects besides the abundant research already obtained since the 1970s. Historically, one of the ancient documents about velvet bean in Indonesia was pessimistic about the possibility of good uses of velvet bean along with human's experiences in interactions with velvet bean (Heyne, 1987) because at that moment (Colonialism) they mostly used velvet bean plant for green manures in the farms while the leaves and seeds were consumed or used as part of traditional medicine by native population yet lack of scientific data. Therefore, various experiments were carried out starting from green manure, in vivo for cattle feeds to scientific investigation on traditional medicines. But, now velvet bean has obtained much attention for its previously negative effects on human life through a new concept of functional food or health-oriented food. The compounds responsible for various symptoms such as vomiting, headache, or itchiness have been revealed in studies in many countries.

Indigenous processing methods are the best resources to screen the goals of this research. Vadivel and Bielsaski (2012) evaluate indigenous processing of velvet bean from India for velvet beans collected from around the world (Mexico, India, USA, Zimbabwe, Nigeria, Benin, Guatemala, Ghana, Kenya, and Guinea) and found such processing is safe to be applied continuously as food base products at the household level, not as drugs. Nowadays, in Indonesia velvet bean varieties are still cultivated generally in suboptimal areas where water and/or clean water scarce. The ancients documents indicate velvet bean species found in Indonesia namely: (a) *M. diabolica* Backer, (b) *M. junghuhniana* Backer (*M. blumei* Burck), (c) *M. pruriens* DC (*M. prurita* Hook.), (d) *Spatholobus ferrugineus* Benth, (e) *Spatholobus littoralis* Hassk., (f) *Calopogonium mucunoides* Desv., and (g) *M. bakeri* KDS (Heyne, 1987). This is still apparent to be cultivated.

Nevertheless, until now velvet bean is underutilized crops due to its antinutrients, toxin and itching characteristics of its hairs. Antinutrients in velvet bean include tannin, digestibility enzymic inhibitors (antitrypsin, antiamilase), phytates, and antihemagglutinin. Based on the names, it describes what kind of crops velvet bean is. For instance, devil bean, *pruriens* which is in Latin referring to "itching sensation" from the fine hairs (Sridhar and Bhat, 2007) covering velvet bean pods. The pods have hairs containing 5-hydroxytryptamine (serotonin) and mucunain (a protein) that cause severe itches (erythema) (Giuliano and Allard, 2001). Yet, behind the negative traits, velvet bean suddenly wakes the world up from its contributions for managing health, venoms, malnutrition for marginal people after Ayurvedic messages revealed. Velvet bean's

uniqueness that grows in arid areas, making it is blessed with so many bullets to survive in such poor soils. In contrast to its comparable nutrition to soybean, antinutrients and alkaloids, cyanides and a non-protein amino acid seem complementarily required by velvet bean to facing a battle in environmental challenges. Velvet bean research, especially those herbaceous, started increasing since its content of L-DOPA (3,4-dihydroxy-L-phenylalanine) found to be capable of treating Parkinson's disease (Shaw and Bera, 1993; Prakash and Tewari, 1999; Pierson *et al.*, 2004). Velvet bean exploration is re-born (Buckles, 1995) but in Indonesia, continuous research on velvet bean kept going on including (a) tempe of velvet bean which is continuously carried out in the Javanese society (Sardjono *et al.*, 2012; Wanita and Rahayu, 2011), (b) plantation of velvet bean (Pramono, 2010), (c) food product development of velvet bean (Sardjono *et al.*, 2012; Wanita and Rahayu, 2011; Sudiyono, 2010), and (d) Pharmacology of velvet beans (Sardjono, 1995; Winarni *et al.*, 2011; Sardjono et al., 2018).

A systematic and comprehensive program has not been done for commercialization activities. It is time to transform the research results into an industrial system from agriculture to public health due to its potential safer protection for land from destructive inorganic fertilizers. It is demanded to enrich the soil with organic matters and selective herbicide to control weeds. Among them, the species of *Mucuna pruriens* as a cover crop widely accepted for enhancement of water infiltration into the soil, softening the soil, improvement of soil fertility and to suppress the weeds (*Acanthospermum hispidum, Euphobia hirta, Senescio vulgaris, Oxygonum sinuatum, Schkuria pinnata, Richardia brasiliensis, Bidens pilosa, Sonchus oleraceae*)(Osei-Bonsu *et al.* 1994; Mwangi *et al.*, 2006). Interestingly, all antinutrients which are water-soluble simply leach out during soaking, rinsing, or washing. However, the arid or semiarid areas also mean that there is not much water available to control the antinutrients to the safe level doses during processing in such areas.

Although intensive research has been done on diverse species of mucuna from various points of view, there are still left problems required to be tackled. Hence, there is an urgent call to fix the food processing that saves nutrients and simultaneously reduces the antinutrients at safe levels but retains L-DOPA content at physiologically functional levels for neural health in the way that uses minimum consumption of water. And current society demands convenience food products so instant food product or food ingredients to support instantaneous characteristics is worthy to be investigated and implemented for sustainable agricultural-based livelihood.

The objectives of this review are (a) to screening from publications a candidate to be developed for minimum water consumption type processing of velvet beans including those studied from ancient documents about velvet bean in Indonesia; (b) to estimate the amount of water for each processing at the similar basis of velvet bean feeding quantity in the processing. Finally, it will recommend the processing type which requires less water for further investigations at scaling up to pilot plant for commercial functional food ingredient production in Indonesia.

MUCUNA PLANT

Taxonomically, velvet bean species belongs to the family of Fabaceae, some referring it with the name of *Stizolobium* (Sridhar and Bhat, 2007). Regarding its excellent chemical composition resembling soybean, velvet bean has been developed for combating malnutrition as well as curing particular diseases by usages of alkaloids in the seeds. The most popular species is *M. pruriens* which has several varieties, e.g. in Indonesia *M. hirsuta* W. and A., *M. utilis* Wall, *M. velutina* HASSK., *M. capitata* W. and A., and *M. cochinchinensis* A, Chev., *M. nivea* W. and A., *Stizolobium niveum* O.K. (Asia Nivea) as well as *M. lyoni* Merr (America lyoni) (Heyne, 1987). The varieties in *Mucuna pruriens* var. *pruriens* produce seeds richer in levodopa (Eucharia and Edward 2010) compared to others that can act as a direct precursor of the neurotransmitter dopamine and strongly affect sexual function (Giuliano and Allard, 2001). The characteristics of velvet seeds are described in Table 1.

Tabel 1. Characteristics of mucuna seeds

	Direct observation	Bhat *et al.* (2008)	Sardjono *et al.* (2012)	Heyne (1987)
origin	Yogyakarta, Indonesia		Yogyakarta, Indonesia	Sumatera, Bali, Java, Molucas, Madura and its small islands, Celebes islands
shape	round-flat	flat or varied	oval and flat	flat, a little bigger than other legumes; very glossy (*M. hirsuta* W. and A.)
color	-grey -yellowish-brown -white -black -white grey spotted -black patterned -brown patterned	black	white, black, white with black dots	white from a purple flower (*M. hirsuta* W. and A.); grey seed coat, white with spots, yellowish pale brown background with brown lines, deep black; big seed and glossy, initially red then dark brown almost black (*M. capita* W. and A.)
weight (g/100 seeds)		43.61±1.06	96.3*	
coat (g)		0.07±0.01		
cotyledon (g)		0.37±0.08		
length (cm)		1.2±0.13	1.491	
diameter (cm)		0.78±0.12	0.682	
thickness (cm)		0.59±0.08		
hilum (cm)		0.44±0.03		
smell	specific velvet beans		characteristics of velvet beans	
taste			bitter	
appearance				parts of plant is covered by fine golden or brown hairs which causes itchiness, glossy in the pods
ethnobotany				many cases indicated headache, vomiting and poor appetite when consumption of velvet bean meals without soaking step; proper processing gives better body weights or good harvest

∗ calculated from single beans

Indonesian sources of velvet bean germplasm is still underexplored and important notes from ancient documents strongly recommend water-intensive uses during food processing. Tempeh making from velvet seeds is still happening in Java in particularly remote areas. Figure 1 shows Mucuna sp. used as tempeh making in Yogyakarta, Indonesia. Exploration for sustainable agricultural-food management in environmentally friendly ways can holistically keep balancing health food requirements and soil maintenance for sustainable agricultural practices.

L-DOPA

All species members of Mucuna sp. have been proven to contain L-DOPA either in seeds, leaves, and other parts of the plant. This compound is grouped into allelochemicals. L-DOPA is a nonprotein amino acid L-3,4-dihydroxyphenylalanine. It is categorized as a product of biotransformation i.e. a single amino acid L-tyrosine is hydroxylated into L-DOPA by tyrosinase (EC 1.14.18.1) in a pathway of melanin biosynthesis (Raval *et al.*, 2012). L-DOPA becomes an active biomolecule different from L-tyrosine. In the velvet bean plant, it is a precursor of many alkaloids, catecholamines, and melanin syntheses and is released into soils, inhibiting the growth of nearby plant species acting as allelochemicals (Soares *et al.*, 2014). As allelochemicals, L-DOPA controls soil and other plant growths (Rogers *et al.*, 2004). L-DOPA resists attacks from insects and thus it can control biological infestation during storage too (Balogun and Olatidoye, 2012). Based on worldwide investigations (India, America, and Africa) on 36-38 mucuna accessions regarding the L-DOPA in velvet eban plant Sridhar and Bhat (2007) concluded that : (1) black seeds contain higher L-DOPA compared to white seeds (Vadivel and Janardhanan, 2000), (2) geographical location of cultivations affect L-DOPA contents, i.e. closer to the equator the seeds contain more L-DOPA (Lorenzetti *et al.*, 1998), and (3) interaction of

genotype and accession is stronger than genotype and geographic location but latitude could affect several accession more (Capo-chichi *et al.*, 2003), furthermore, L-DOPA contents at the early stage of maturation is lower than that of fully mature seeds.

Figure 1. *Mucuna pruriens* currently direct observation found in Indonesia in 2018 (A-C mottled, (D) black, (E) Kaunch, and (F) white.

L-DOPA more likely decreases during the germination of velvet seeds because it is used as N-supply or preparing soil where it is starting to grow. During its growth velvet bean releases L-DOPA into soils and it affects other plants around it as an allelochemical. Then the L-DOPA diffuses out spreading wider due to rains (Ferreira and Janick, 2004) and watering activity thus no weed growths in the area around it. Meanwhile, the allelochemical DOPA can affect other plants through growth suppression decreasing dry matter yields (for *S. stenocarpa* 1.58 g dry matters obtained in control but it became 1.06 g when grows mixed with *M. pruriens* var. *pruriens*), smaller leaf areas, and poor growth rates but not affecting the capacity to germinate (Eucharia and Edward, 2010). L-DOPA suppresses radicle growth but less attacks hypocotyl growth and may ineffectively affect germination (Fujii, 2003). Towards soybean, cucumber, and maize L-DOPA affect its lignin related synthesis in the cell wall because it is effectively absorbed resulting in increased tyrosine, lignin biomarkers, and phenylalanine but its root growth is inhibited. It is categorized as a strong allelochemical with EC_{50} ranges from 5-50 mg/mL. Its herbicidal effects at 1,500 and 3,000 ppm affects wild mustard (*Sinapsis arvense*), creeping thistle (*Cirsium arvense*), filed poppy (*Papaver rhoeas*), and henbit (*Lamium amplexicaule*), but it does not significantly affect wheat and barley through inhibiting root growth (Topa and Kocacaliskan, 2006).

Homeostasis of iron and amino acid metabolism in mammals is also affected by L-DOPA related to ion transporter encoding (Golisz *et al.*, 2011). L-DOPA is converted into dopamine in the brain and body by enzyme L-aromatic amino acid decarboxylase which directly controls the movement of the body (Raval *et al.*, 2012). In patient of Parkinson disease L-DOPA was found to increase incorporation of L-DOPA-protein in lymphocyte cell proteins as a “toxic” situation (Rogers *et al.*, 2004).

Pivotal effects of dose-dependent L-DOPA and its metabolite dopamine on neural-tissues can be described that besides alleviating the disease symptoms, L-DOPA may contribute to disease progression. Acute side effects of L-DOPA, which include nausea, vomiting, and orthostatic hypotension (dizziness), are correlated with plasma levels. It is important to note that dopamine does not pass the blood-brain barrier in sufficient quantities, thus only a small percentage of L-DOPA reaches the brain after systemic administration. Moreover, L-DOPA is quickly metabolized peripherally, therefore high systemic L-DOPA doses are required to achieve the clinical effect (3-4 g of L-DOPA/day). Because immediate

side-effects are directly related to L-DOPA peak plasma levels, L-DOPA was, in recent years, administered in combination therapy with other compounds such as decarboxylase and COMT (Catechol-amine-O-methyl-transferase) inhibitors to prevent peripheral metabolism. To prevent the metabolism of dopamine in the brain, MAO (Mono-amine oxidase) inhibitors were also used. With these additives, it was possible to reduce the daily required dose of L-DOPA to an average of about 600 mg/day. However, these additives were only partially capable of reducing the toxic side effects of the treatment with levodopa and could not prevent disease progression.

In vitro synthesis of L-DOPA from amino acid tyrosine by soil isolate, *Penicillium jensenii* showed the production of alpha methyl DOPA along with L-DOPA. It is proposed that alpha methyl DOPA as a candidate to prevent unmetabolized L-DOPA from intensive L-DOPA metabolism for movement control after transformation into dopamine in the brain. Alpha methyl DOPA will be a delivery facility of unmetabolized L-DOPA so that the injured nerve cells have chances to recover instead of losing L-DOPA for movement intensively (converted into dopamine) (Raval *et al.*, 2012). It may indirectly control the toxic situation by L-DOPA-protein in lymphocyte cells. Fermentation would give a window opportunity to put biotransformation of tyrosine for both L-DOPA and alpha-methyl DOPA for a special goal towards functional foods containing L-DOPA mixed with alpha-methyl DOPA for the diet of Parkinson's sufferers of neural diseases.

VELVET BEAN USES

Velvet bean has intensively been revealed as one of functional food and simultaneously nutraceutical resources from ancient Ayurveda document in India. It is one of the potential pharmaceutical resources for neural related disease, proasidiac, snake bite venom, etc. The potential compounds highly important in pharmaceuticals include 1-methyl-3-carboxy-6,7-dihydroxy-1 2,3,4-tetrahydroisoquinolone, 5-hydroxytryptamine, 5-methoxy-n,n-dimethyltryptamine-n-oxide, 5-oxyindole-3-alkylamine, 6-methoxyharman, Alanine, Arachidic-acid, Arginine, Aspartic-acid, Behenic-acid, Beta-carboline, Beta-sitosterol, Bufotenine, Choline, Cis-12,13-epoxyoctadec-trans-9-cis-acid, Cis-12,13-epoxyoctadec-trans-9-enoic-acid, Cystine, DOPA, Gallic-acid, Glutamic-acid, Glutathione, Glycine, Histidine, L-DOPA, Lecithin, Leucine, Linoleic-acid, Mucunadine, Mucunain, Mucunine, Myristic-acid, N,n-dimethyltryptamine, N,n-dimethyltryptamine-n-oxide, Nicotine, Oleic-acid, Palmitic-acid, Palmitoleic-acid, Phenyalanine, Phosphorus, Proline, Protein, Prurienidine, Prurienine, Saponins, Serine, Serotonin, Stearic-acid, Threonine, Tryptamine, Tyrosine, Valine, Vernolic-acid (Phytochemical and Ethnobotanical Databases at Phytochemical Database, USDA-ARS-NGRL, Beltsville Agricultural Research Center, Beltsville, Md).

The uses of velvet bean are not limited for general foods only because of famine, but also the intentional uses for particular goals regarding health and curing. Traditionally, velvet bean is used as a carminative, hypotensive, and hypoglycemic agent. Moreover, it is also used as anodyne, antidotal, aphrodisiac, diuretic, nervine, resolvent, rubefacient, and vermifuge; used for anasarca, asthma, cancer, cholera, cough, diarrhea, dog bite, dropsy, dysuria, insanity, mumps, pleuritis, ringworm, snakebite, sores, syphilis, tumors, and worms (Divya *et al.*, 2017). Matured beans are consumed widely in Asia such as India, Sri Lanka, Ghana and Nigeria (Sridhar and Bhat 2007), Mozambique and Malawi (Infante *et al.*, 1990; Gilbert, 2002) as well as in Indonesia and, Japan (Higasa *et al.*, 1996) and they are considered as safe (Diallo *et al.*, 2002) food products. These seeds are also reported to be rich in antioxidant properties (Tripathi and Upadhyay, 2001). L-DOPA is also claimed to have a high antioxidant capacity (Raval *et al.*, 2012; Balogun *et al.*, 2017).

NUTRITIONAL PROPERTIES OF VELVET BEAN

The pulished nutritional analysis of velvet beans is presented in Table 3. It can be seen that various mucuna varieties would give relatively similar nutritional provisions for human needs: carbohydrates with a sufficient amount of fibers,

proteins, high minerals (ash), and moderate levels of lipids. Any species of mucuna can be useful germplasm worldwide. Further detailed ash, fatty acids, and amino acid compositions listed in Table 4 - 6 strongly recommend balance nutrients required for any age groups and genders can be obtained from mucuna seed-based food products. Iron minerals exist in all mucuna species indicating that the beans can be good iron sources regardless of the antinutrients in them. The potassium contents are high thus it can be good contributions for diet components for people suffering from heart diseases and blood pressure. Calcium and phosphor with low sodium contents may a good composition for osteoporosis controls. Furthermore, micronutrient minerals such as zinc, cuprum, selenium, and mangan also provide better supply for people with diabetes mellitus problems. Fatty acid compositions indicate that the polyunsaturated-saturated ratios >1 would be less risk for health problems for instance people living with heart diseases. Many essential amino acids are available in velvet beans of different species; indeed, methionine is the least existing amino acid in them, thus diverse foods are recommended.

Table 2. Uses of velvet beans

Velvet bean based food products	Locations	References
velvet bean tempe-meal, dish	Indonesia	Pramono (2010) Harmayani *et al.* (2016) Heyne (1987)
"dage benguk" a spontaneous fermentation product	Indonesia, in the past	
"gedebel benguk" (oncom like product, a fermented legume using *Monilia sitophilia*)	Indonesia, in the past	Heyne (1987)
leafy vegetables from young leaves (*M. hirsuta* W. and A.) and seeds were consumed as foods eaten with rice or uses as part of medicines	Indonesia, in the past	
L-DOPA to relief Parkinson's disease symptoms	na	Shaw and Bera (1993) Prakash and Tewari (1999).
decoction of *Mucuna* seeds lowered plasma cholesterol and lipids in rats	na	Iauk *et al.* (1989)
beverage component or consumed as roasted powder	Kenya	Saha and Muli (2000)
beverage of coffee mucuna powder	na	Diallo *et al.* (2002)
yogurt	Indonesia	Wanita and Rahayu (2010)
beef burger	na	Onweluzo *et al.* (2004)
weaning foods	na	Egounlety (2003)
snack	na	Sridhar and Bhat (2007),
fried velvet bean snack	Indonesia	Sudiyono (2010)
paste or oil soup (stew),	Southern Ghana	
roasted snacks (Akpaka Ide)	Southern Ghana	Osei-Bonsu *et al.* (1996)
sauce (Akpoko ji/nkashi/Una)	Southern Ghana	
gel (Opka),	Southern Ghana	
additives or condiment, e.g. as thickener in sauce or soup, stabilizer gum or gel,	na	Onweluzo *et al.* (2004)
Moi-moi, and fried cake	na	Sridhar and Bhat (2007)
porridge	na	Sridhar and Bhat (2007) Diallo *et al.* (2002),
non soya tempe.	Japan	Higasa *et al.* (1996)
young leaves and pods were consumed as vegetables; sometimes also for cattle feeds	Indonesia	Heyne (1987)
immature pods and leaves serve as vegetables, while seeds as condiment and main dish by ethnic groups	Nigeria	Adebowale and Lawal (2003b)
daily meal, cooked and ground like mashed velvet beans *M.cochinchinensis* and *M. utilis*	Southern Ghana	
After draining the cooked water, softened seeds are hulled, ground into paste and mixed with other ingredients (e.g. chillies, egg plant, onions, meat or fish) to prepare soup (Asadua and Nkwan), which is eaten along with starchy staples.	Southern Ghana	Osei-Bonsu *et al.* (1996)

Table 3. Velvet bean diverse nutritional composition based on worldwide researches

Nutrients	Balogun and Olatidoye (2012)	Balogun *et al.* (2017)	Ravindran and Ravindran (1988)	Bhat *et al.* (2008)	Sardjono *et al.* (2012)	Siddhuraju *et al.* (1996)	Ezeagu *et al.* (2003)	Rajaram and Janardhanan (1991)	Ezeagu *et al.* (2003)	Mohan and Janardhanan (1995)	Afolabi *et al.* (1985)	Ezeagu *et al.* (2003)
origin	Ibadan, Nigeria	Ibadan, Nigeria		India	Yogyakarta, Indonesia							
analyses locations	Nigeria	Indonesia		India	Indonesia							
species	*M. utilis* black	*M. utilis* black	*M. utilis*	*M. pruriens*	*M. pruriens* (L)	*M. pruriens*	*M. cochinchinensis*	*M. gigantea*	*M. jaspeada*	*M. monosperma*	*M. solanei*	*M. veracruz* (black)
proximate (%)												
moisture	6.02±0.11	10.33±0.07	-	9.58±0.38	9.14^{shell} 10.80^{kernel}	-	-	-	-	-	-	-
crude protein	25.65±0.14	22.67±0.33	26.40	23.04±0.38		31.44	29.79	30.62	27.56	23.50	24.00	24.50
crude lipid	$14.52±0.05^{ether}$	$2.12±0.11^{hexane}$	4.10	7.13±0.17		6.73	6.51	9.03	4.72	14.39	6.50	6.90
fatty acids$^{0.86xfat}$	12.49		-	-		-	-	-	-	-	-	-
ash	3.60±0.01	3.94±0.02	3.70	4.79±0.72	2.18^{shell} 3.04^{kernel}	4.11	4.16	5.99	3.25	3.21	3.00	3.66
carbohydrate	42.98	60.23	59.50	57.18±0.93	-	52.56	59.54	42.54	64.47	52.20	ND	64.88
crude fiber	7.23±0.05	-	6.30	7.85±0.04	-	5.16	4.19	-	4.43	6.79	5.30	4.27
in vitro protein digestibility	-	-	-	50.65±5.42	-	-	-	-	-	-	-	-

Table 4. Mineral compositions

	Balogun and Olatidoye (2012)	Ravindran and Ravindran (1988)	Bhat *et al.* (2008)	Mary Josephine and Janardhanan (1992)	Siddhuraju *et al* (2000)	Siddhuraju *et al* (2000)	Ajayi *et al* (2006)	Rajaram and Janardhanan (1991)	Ezeagu *et al* (2003)
spesies	*M. utilis* (black)	*M. utilis*	*M. pruriens*	*M. pruriens*	*M. pruriens* var. utilis white	*M. pruriens* var. utilis black	*M. flagellipes*	*M. gigantea*	*M. jaspeada*
minerals (mg/100 g)									
-Ca	148.88±0.2	250	66.53±0.92	247	87.80	104	12.80	518	80
-Na	54.46±0.2	70.00	6.15±2.03	4.10	12.70	25.70	11.10	35.30	-
-K	1,472.33±0.2	11,110	164±2.15	2,537	1,575	1,343	1,322	2,296	8,460
-Mg	23.66±0.3	110	42±0.4	72.40	120	109	58.30	506	170
-P	377.12±0.2	220	245±11.45	459	499	376	-	194	470
-Fe	3.44±0.2	1.30	14.63±1.05	5.19	5.79	7.47	82	9.42	6,800
-Zn	3.46±0.1	1.00	5.7±1.15	1.71	5.26	12.20	7.30	8.24	4.60
-Cu	0.71±0.1	0.60	2.51±0.01	0.47	2.42	1.65	2.60	1.18	1.82
-Mn	5.28±0.1	1.00	3.03±0.12	0.31	1.49	2.41	11.90	2.36	5.17
-Se	-	-	19.43±4.39	-	-	-	-	-	-

Table 5. Fatty acid compositions

	Balogun and Olatidoye (2012)	Bhat *et al.* (2008)	Siddhuraju *et al.* (2000)*	Siddhuraju *et al.* (2000)*	Siddhuraju *et al* (1996)*	Ajayi *et al.* (2006)*	Mohan and Janardhanan (1995)*
species	*M. utilis* (black)	*M. pruriens*	*M. pruriens* var. utilis white	*M. pruriens* var. utilis black	*M. pruriens*	*M. flagellipes*	*M. monosperma*
fatty acids mg/g lipid							
-palmitic	28.8%**	4.17	2.01	2.18	2.02	1.07	2.46
-stearic	18.21**	0.06	0.71	0.74	0.38	0.34	1.17
-oleic	20.12**	6.82	0.83	0.70	2.87	-	3.08
-linoleic	26.40**	-	4.88	4.80	3.71	1.50	2.47
-linolenic	8.71**	1.42	0.65	0.77	0.33	-	0.47
-behenic	2.42**	0.64	0.34	0.34	0.07	0.14	0.35
-myristic	-	0.07	0.02	0.02	-	-	-
-myristoleic	-	0.01	trace	nd	-	-	-
-elaidic	-	2.16	trace	trace	-	6.07	-
-linoelaidic	-	5.82	-	-	-	-	-
-heneicosanoic	-	0.10	0.01	0.01	-	-	-
-lauric	-	-	trace	-	-	-	-
-arachidic	-	-	0.14	0.45	0.18	-	-
-tricosanoic	-	-	-	0.01	-	-	-
-lignoceric	-	-	0.09	0.09	-	0.39	-
-palmitoleic	-	-	0.03	0.03	0.17	-	-
-linolelaidic	-	-	0.27	0.15	-	-	-
-eicosadienoic	-	-	0.01	0.01	-	-	-
-eicosenoic	-	-	-	-	-	0.23	-
-cerotic	-	-	-	0.01	-	-	-
Oleic:linolenic ratio	2.31**	-	-	-	-	-	-
Polyunsaturated:saturated	-	3.20	2.02	1.83	2.67	3.54	1.51

Table 6. Amino acid compositions

Nutrients	Balogun and Olatidoye (2012)	Bhat *et al.*(2008)	Adebowale *et al* (2005)	Siddhuraju *et al* (1996)	Afolabi *et al* (1985)
amino acid composition g/100 g	*M. utilis* (black)	*M. pruriens*	*M. cochinchinensis*	*M. pruriens*	*M. solanei*c
lysine*	5.72	8.98	6.78	6.60	13.47
histidine*	3.13	3.30	2.36	3.14	1.13
arginine*	7.41	9.55	8.05	7.16	11.81
aspartic acid	14.28	17.10	13.6	8.16	6.94
glutamic acid	13.28	19.31	16.8	17.23	10.03
glycine	5.49	6.21	5.43	5.12	3.08
valine*	4.47	7.60	6.98	5.57	3.65
methionine*	0.69	0.78	1.32	1.28	1.18
isoleucine*	7.24	8.77	9.08	4.12	2.70
leucine*	6.14	10.42	7.27	7.85	5.16
tyrosine	3.94	7.51	5.46	4.76	11.16
cysteine	4.52	1.61	1.04	0.84	1.72
phenylalanine*	4.58	6.51	7.69	3.85	14.81
serine	4.53	6.08	3.45	4.10	3.32
proline	3.64	7.38	13.45	nd	3.30
tryptophan	0.81	ND	2.34	1.35	nd
alanine	4.28	4.95	7.45	2.81	3.61
threonine*	3.86	5.21	5.04	3.64	2.04

*** essential amino acids; **calculated**

VELVET BEAN PROCESSING AND WATER REQUIREMENTS

Sterilization of velvet seeds is important (Bhat *et al.*, 2007) that there was fungi growth on the surface of grains including Aspergillus, Fusarium, Eurotium, and yeast. Precaution should be paid for the toxigenic fungi namely *Aspergillus flavus*, *A. niger*, and Fusarium sp. by which irradiation at 10 kGy is sufficiently effective to give better safety levels of fungal contaminations. Hydrothermal treatments, fermentation, and germination are most effective in reducing the antinutrients of velvet seeds (Wanjekeche *et al.*, 2003). Various processing technology has been investigated onto velvet seeds aiming to reduce the toxins.

Accumulated data on velvet bean processing are listed in Table 7 nevertheless literature are scarcely found on an instant ingredient from velvet bean to be modernized based on such huge various processing studied. Indigenous processing often seen as the long successfully tested processing as the prior art for developing instant ingredient oriented for neural diseases already well known obtained from velvet seed products. Besides its prospective and challenging economical values, it is warrants investigation of technological touch to patch a new usage of velvet bean better in environmentally friendly ways.

Aqueous boiling is the best method for L-DOPA removal from velvet seeds (Bressani *et al.*, 2003) and the products developed by International Institute of Tropical Agriculture (IITA), Benin from L-DOPA free velvet beans in Nigeria is accepted by the society (Versteeg *et al.*, 1998). Various processing methods have been tried by investigators to reduce L-DOPA of velvet seeds. Most of the methods employed were based on the use of water, chemicals, and thermal treatments (Bressani, 2002; Diallo and Berhe, 2003; Gilbert, 2002). Dry treatments are the most effective in reducing L-DOPA in velvet seeds and preventing L-DOPA racemization under roasting (Siddhuraju *et al.*, 1996), grilling is considered a better technique than cooking (Dossa *et al.* 1998). Extreme heating is unfavorable nutritionally due to poor protein availability as well as protein digestibility (Kakade and Evans, 1965) although it removes haemagglutinins. Moisture in seeds plays an important role in the destruction of trypsin inhibitors (Liener and Kakade, 1980). Water imbibes into the kernels through soaking, germination, or hydrothermal processing activates enzymes among others is those which can destroy

antinutrients in cereal and legume; for instance, phytase which is also favorable when the metabolites of fermentation provide lactic acid (Sandberg, 2002). Meanwhile, fermentation with inoculums helps us with enzymes obtained from microbes to complement the endogenous activated enzymes in the kernels. The best removal of toxins especially those which are water-soluble such as cyanide can be done simply by soaking and removal of testa before boiling, and generally, cooking significantly eliminate HCN in seeds of *Mucuna utilis* (Ravindran and Ravindran, 1988).

Based on data of estimated water uses in Table 7 soaking treatment would depend on the water-beans ratios. The elimination of toxins would necessarily balance with nutritional retentions, in particular, those water-soluble nutrients. With the challenge of limited clean water in the arid areas where the most common locations of velvet bean growth, a high water-beans ratio make the processing unlikely compatible. Heating in reduced water-beans ratios would be more realistic options. Yet, technology such as microwave heating still expensive, and education about its dangers should be introduced for society. Such an idea would only work in the area with the available electrical supply.

Table 7. Processing of velvet beans

Processing Types	Principal Steps	Results	Minimum water (%v/b seeds)*	References
		Germination		
Germination	i) **Sterilized** (1% mercuri chloride), **rinsed**; **soaked** (distilled water, 4 °C, 12 h); **germinated** (24, 48, 72, 96, and 120 h 30 °C); **moistened** (distilled water 12 h), **rinsed the sprout** (distilled water)		**Total: 350** soaking: 100 moistening: 25 x 10 times=250 rinsing: 100	Gurumoorthi and Uma (2011)
	ii). **Sterilized** (ethanol soaking, 1 min), **soaked** (distilled water 1:10 w/v, 12 h, 25 °C), **drained**, **spread** (thick wet cotton wool), **germinated** (dark 3 d), **dehulled**, **frozen** (-21 °C 12 h) to stop germination, **dried** (oven 50 °C, 24 h), **ground** (≤500°m), **frozen** (-21 °C, 12 h)		**Total: 1,000** soaking: 1,000	Mugendi *et al.* 2010
	Room temperature, humidified cotton beds, 72 h		**Total: 100**	Balogun *et al.* (2017)
Germination to reduce trypsin inhibitor activity	Germinated (5 and 7 d)	Reduction of trypsin inhibitors up to 84.5 and 85.4%.	insufficient information	Wanjekeche *et al.* (2003)
Germination and boiled	**Germinated** (5 and 7 d), **boiled**	Reduced L-DOPA 38.5%	**Total 300%** soaking 100%, boiling: 200%	Wanjekeche *et al.* (2003)
Germination and malting *mucuna* seeds to reduce trypsin inhibitor activity	Germinated (2 vs. 6 d)	Decreased trypsin inhibitor activity 1.88 vs. 0.82 TUI/mg).	Total 300% soaking 100%, :daily weting 200%	Bressani *et al.* (2003)
Sprouting and oil frying (Lambadi Ethnic, India)	Mixed red soil paste (1:5 w/v) – beans ratios of 2:1; **humidified** with wet cloth, **incubated** for 7 d, 25 °C; sprout **separation**, **washed**, **dried** 85-90 °C, 15 min	Increased free phenolics by 4-11% (5,81-9,25% vs. 5.24-8.65%)	wetting: 100%; washing: 100%	Vadivel and Biesalski (2012)
		Fermentation		
Fermentation	*R.oligosporus* fermentation		Total 300% soaking 100%, boiling: 200%	Egounlety (2003)

Processing Types	Principal Steps	Results	Minimum water (%v/b seeds)*	References
	Bacillus sp. Fermentation		Total 300% soaking 100%, boiling: 200%	Egounlety (2003).
	R. oligosporus FNCC 6010, *R. oryzae* FNCC 6011, hybrid of they both 5 days fermentation,	*R. Oligosporus* reduced L-DOPA up to 90%		Balogun *et al.* (2017) Balogun *et al.* (2015)
		Soaking		
Mechanical and physical methods to reduce L-DOPA of *mucuna* seeds	(i) **cracked**, **soaked** in running water (from a faucet, 36 h)	Decreased L-DOPA up to 0.08%	continuous soaking: 75% x1.5 d =112.5%	Diallo and Berhe (2003)
	(ii) **dialyzed-like** whole seeds (cloth bag, **immersed** in a flowing river, 3 d).	Decreased L-DOPA up to 1.60%	immersion: 1,000%	Diallo and Berhe (2003)
	(iii) **leaching** of cracked seed (running water via faucet, 48 h)	Decreased L-DOPA 4.33%	leaching: 75%x2 d = 150%	Diallo and Berhe (2003)
	(iv) **leaching** of whole seeds (running water via faucet, 48 h)	Decreased L-DOPA 4.93%	leaching: 75%x2 d = 150%	Diallo and Berhe (2003)
Physicochemical methods to reduce L-DOPA and trypsin inhibitors of *mucuna* seeds.	Soaked (22 °C, 96.5 h)	70% retention of L-DOPA	Soaking: 100%	Bressani *et al.* (2003)
	Soaked (45 °C, 96.5 h)	Retention decreased to 51%	Soaking: 100%	Bressani *et al.* (2003)
	Soaked (66 °C, 96.5 h)	Retention decreased to 27%	soaking: 100%	Bressani *et al.* (2003)
Soaking and periodically changing water (60°C) toward white and mottled seeds of *mucuna*	Soaked, periodically water changed (60 °C, 48 h)	Reduction of L-DOPA up to 22-30%	Replenished soaking: 200%	Bressani *et al.* (2003)
Acidic soaking and heating	Acidic soaking and mild heating for 48 h		Soaking and heating: 150%	Mugendi (2010)
Soaking in tamarind solution and cooking	Tamarind solution at pH 2.75, **soaking** beans at ratio of 1:10 w/v, **stayed dark** (8 h, 25 °C), **rinsing**, **cooking** 1:10 w/v at 85-90 °C, 45 min (Kanikar tribe, Inda)		Soaking: 1,000% rinsing: 1,000%	Vadivel and Biesalski (2012)
Soaking	Soaked ($Ca(OH)_2$ solution)		Soaking: 100%	Vadivel and Biesalski (2012)
Soaking and cooking	Soaked (freshwater, 48 h), seed coat removal after 24 h, replacing water (12 h); cooked (water, 60-90 min)		soaking: 200% cooking: 100%	Diallo *et al.* (2002).
Soaking and cooking	Soaked (24 h), cooked (60 min)		Soaking: 200% cooking: 100%	Tuleun *et al.* (2009)
Soaking half mature *M. Capita* W. and A.	Peeled, soaked (water or salt solution, 2-3 d, every day water renewal)		Soaking: 100% x 3 d = 300%	Heyne (1987)
Soaking and frying to reduce HCN	Soaked (0.5% natrium bicarbonate, 12 h), fried Soaked (1.5% natrium bicarbonate, 12 h), fried	14.71 – 18.36 ppm	soaking: 100%	Sudiyono. (2010)
Soaking, antinutritional of *mucuna pruriens*.	Soaked (sodium carbonate solution)	Total free phenolics reduction (56%)	soaking: 100%	Vijayakumari *et al.* (1996)
	Soaked (distilled water)	Total free phenolics reduction (47%).	soaking: 100%	Vijayakumari *et al.* (1996)

Processing Types	Principal Steps	Results	Minimum water (%v/b seeds)*	References
Soaking to reduce phytic acid	Soaked (distilled water)	Decreased 27%	soaking: 100%	Sandberg (2002)
	Soaked (sodium bicarbonate)	Decreased 17%	soaking: 100%	Sandberg (2002)
Soaking and cooking to reduce phytic acid	Soaked, cooked (90 min)	Further reduced 18%	soaking and cooking: 150%	Sandberg (2002)
	Soaking, autoclaving (45 min)	Further reduced 44%	soaking and autoclaving: 150%	Sandberg (2002)
Soaking, autoclaving and cooking *mucuna* seeds in to remove phytic acid	Soaked (various solutions), autoclaved and cooked	Decline in phytate content (27-34% and 38-51%)	soaking and autoclaving: 150%	(Siddhuraju and Becker 2001).
Soaking and cooking to eliminate trypsin inhibitor	Soaked (water, 48 h), cooked (30 min)	Complete elimination	soaking and autoclaving: 150%	Udedibie and Carlini (1998)
		Boiling		
Boiling	Boiled up to 40 min		boiling: 100%	Osei-bonsu *et al.* (1996)
Boiling and dehulling to reduce L-DOPA (*m. Pruriens* var. *Utilis*) seeds	Boiled (45 min), dehulled	Decrease L-DOPA up to 6.36% (raw) to 4.71%	boiling: 100%	Egounlety (2003)
	Boiled (45 min), dehulled, soaked (12 h)	Reduced L-DOPA up to 6.36% (raw) to 2.29%	soaking and boiling: 200%	Egounlety (2003)
	Boiled (45 min), dehulled, soaked (12 h), re-soaked (12 h)	Reduced L-DOPA up to 6.36% (raw) to 1.36%	boiling, resoaking: 300%	Egounlety (2003)
	Boiled (45 min), dehulled, soaked (12 h), re-soaked (12 h), re-boiled (45 min)	Reduced L-DOPA up to 6.36% (raw) to 0.64%	boiling, resoaking: 400%	Egounlety (2003)
Boiling 'Magadi soda' (hydrated sodium carbonate) to reduce L-DOPA in whole mature seeds of *mucuna pruriens*	Boiled in alkaline solution	Reduced L-DOPA by 59.3% (5.75% vs. 2.34%),	boiling: 100%	Wanjekeche *et al.* (2003)
	Boiled in cob ash solution	Reduced by 58.1% (5.75 vs. 2.81%).	boiling: 100%	Wanjekeche *et al.* (2003)
Boiling	Boiled in citric acid solution	Reduced by 49.7% (5.75 vs. 2.89%).	boiling: 100%	Wanjekeche *et al.* (2003)
Boiling	Boiled in bean stover ash solution	Reduced by 47.4% (5.75 vs. 3.02%).	boiling: 100%	Wanjekeche *et al.* (2003)
Boiling	Boiled seeds in water	Reduced L-DOPA up to 24.9%	boiling: 100%	Wanjekeche *et al.* (2003)
Boiling of *mucuna cochinchinensis* to eliminate haemagglutinating activities.	Boiled (90 min, 100-105 ºC)	Failed to eliminate all	boiling: 100%	Ukachukwu and Obioha (2000)
Boiling to reduce trypsin inhibitor	Boiled in water	Reduced up to a greater extent 89.7% (27.18 vs. 2.80 TIU/mg).	boiling: 100%	Wanjekeche *et al.* (2003)

Processing Types	Principal Steps	Results	Minimum water (%v/b seeds)*	References
Boiling velvet bean	Soaked (plain water, 24 h, room temperature), washed, put into boiling water, boiled 20, 40, and 60 min),drained, sundried (4 d), ground	Selenium content (0.24 ppm vs. 0.09-0.12 ppm), iron (103 ppm vs. 70-90 ppm), and phosphorous (0.41% vs. 0.26-0.34%) among boiling time course there were relatively insignificant. Crude protein (29.37% dm vs. 27.93-28.27%), lipid (5.9% vs. 3.17-4.53%), and ash (4.43% vs. 6.68-8.78%). Reduced almost half essential amino acids due to the length of boiling time. HCN 33.46 mg/kg DM vs. 29.02-32.04 mg/kg), tannin (1.41 g/kg vs.0.99-1.16 g/kg), trypsin inhibitor activity (33.59 TUI/mg vs. 17.77-23.03 TUI/mg), and oxalates (1.95 g/kg dm vs. 1.38-1.90 g/kg).	soaking, washing, boiling: 300%	Tuleun *et al.* (2009)
		Others		
Pound and ground	Pounded, cracked, drained, hulled, ground		insufficient information	Osei-bonsu *et al.* (1996)
Microwave assisted heating	Microwave heated (130 ºC) overnight soaked, microwave heated (130 ºC)	Increased L-DOPA	soaking: 100%	Kala and Mohan (2012)
Dehulling/soaking and irradiating *mucuna* seeds	Dehulled, irradiated Soaked, irradiated	Reduced total phenolics (up to 80%)	soaking: 100%	Siddhuraju *et al.* (2000)
Gamma irradiation				Bhat *et al.* (2007)
Processing methods suitable for household and community level preparations to reduce L-DOPA of *mucuna* (raw white 3.75%), (raw speckled 3.90%), (raw black 4.36%), (pre-soaked speckled 4.02%)	Boiled (water)	Reduced L-DOPA up to 48.5% (4.02 vs. 2.07%)	boiling: 100%	Nyirenda *et al.* (2003)
	Boiled grits (water)	Reduced L-DOPA up to 57% (4.02 to 1.72%)	boiling: 100%	Nyirenda *et al.* (2003)
	Soaked grits (0.25% sodium bicarbonate (1.5 L), boiled (1.5 L), soaked (1.5 L, 24 h)	Extracted L-DOPA approximately 90% (4.02% vs. 0.39%).	boiling and resoaking: 3,000%	Nyirenda *et al.* (2003)
	Soaked (0.25% sodium bicarbonaten 1.5 L), boiled (1.5 L), soaked (1.5 L, 24 h)	Reduced L-DOPA up to 67%.	boiling and resoaking: 3,000%	Nyirenda *et al.* (2003)
	Soaked grits (24 h, 3 L water)	Reduced L-DOPA up to 54% (4.02 vs. 1.86%).	soaking: 3,000%	Nyirenda *et al.* (2003)
Various cooking treatments to reduce L-DOPA in *mucuna* seeds.	Microwave, vapor, in various water solutions at ph 3, 6, 7, and 11, cooking in alkaline sodium hydroxide/potassium hydroxide/calcium hydroxide	Not effective	soaking an cooking: 200%	Garcia-Echeverria and Bressani (2006)
	Cooked (calcium hydroxide, pH 9), washed in hot water	Reduction up to 80.4%	**total= 300%** cooking: 100% washing: 200%	Garcia-Echeverria and Bressani (2006)

Processing Types	Principal Steps	Results	Minimum water (%v/b seeds)*	References
Cooked yellow powder as soup thickener	Cracked (hitting with a hard object), cooked, hulled, ground, mixed with red palm oil		cooking: 100%	Ezueh (1997)
Cooking and autoclaving to reduce hemagglutinating activity		Reduced hemagglutinating activity up to 89-99%	cooking and autoclaving: 150%	Vijayakumari *et al.* (1996).
Cooking to eliminate haemagglutinin activity oin *M. cochinchinensis* seeds	Cooked (3 h, 100 °C)	Eliminated haemagglutinin activity	cooking: 100%	Onwuka (1997).
Cooking to inactivate trypsin inhibitors in *mucuna* seeds	Cooked (1 h, 96 °C)	Completely eliminated	cooking: 100%	Udedibie and Carlini (1998)
Cooking to decrease antitryptic activity		Totally eliminated	cooking: 100%	Ravindran and Ravindran (1988).
Cooking to reduce cyanide, in mucuna	Cooked	Reduces cyanide up to 46%	cooking: 100%	Montgomery (1980)
Toasted flour	Toasted (5-10 min), ground		sanitary need: 25%	Sridhar and Bhat (2007)
Toasting to eliminate trypsin inhibitors of *M. Utilis* (raw, 2,170 TIU/g)		Elimination of 42% (6,979 vs. 11,865 TIU/g).	sanitary need: 25%	Ravindran and Ravindran (1988)
Roasting	Roasted (100 ºC)	Increased L-DOPA	sanitary need: 25%	Mugendi and Njagi (2010)
Roasting of *mucuna* seeds to reduce trypsin inhibitor activity	Roasted (30 min)	Reduced the trypsin inhibitors (raw 18.90 vs. 1.58 TUI/mg).	sanitary need: 25%	Bressani *et al.* (2003)
Roasting and dehulling to reduce cyanide, (raw 18.6 mg/kg)	Roasted Dehulled, roasted	Complete removal of HCN	sanitary need: 25%	Agbede and Aletor (2005)
Dry heat treatment and autoclaving *M. pruriens* to reduce phytic acid	Dry heated, autoclaved	Reduced the phytic acid (36% and 47%)	**total: 75%** sanitary need: 25% autoclaving: 50%	Siddhuraju *et al.* (1996)
Dry heat treatment to reduce hydrogen cyanide (HCN) *M. Pruriens* seeds	Dry heated	Reduced HCN (67%)	sanitary need: 25%	Siddhuraju *et al.* (1996)
Autoclaving to reduce hydrogen cyanide (HCN) *M. pruriens* seeds	Autoclaved	Reduced HCN (68%)	autoclaving: 100%	Siddhuraju *et al.* (1996)
Autoclaving to reduce cyanide	Autoclaved	Reduces cyanide up to 75%.	autoclaving: 100%	Montgomery (1980).
Autoclaving antinutritional of *M. pruriens* to reduce tannins	Autoclaved (45 min)	Reduced tannins (71%).	autoclaving: 100%	Vijayakumari *et al.* (1996)
Autoclaving, dehulling and roasting, dehulling and soaking	Autoclaved (raw/ dehulled), Dehulled, roasted Dehulled, soaked (urea)	Completely removed trypsin inhibition activity (raw 25.3 mg/g)	autoclaving: 100% autoclaving and soaking: 150%	Agbede and Aletor (2005)
Dehulling and cooking/roasting to reduced lectin *M. pruriens* seed flours.	Dehulled, cooked Dehulled, roasted	Completely eliminated lectin (raw 4.0 HU/mg).	cooking: 100%	Agbede and Aletor (2005)
Dehulling and roasting to reduced phytin and phytin phosphorus *mucuna* seeds (raw 15.3 and 4.3 mg/100 g)	Dehulled, roasted seed flours	Reduced phytin and phytin phosphorus (6.0 mg/100 g).	sanitary need: 25%	Agbede and Aletor (2005)

Processing Types	Principal Steps	Results	Minimum water (%v/b seeds)*	References
"Gedebel benguk" making	Boiled completely cooked, removed peel, placed into a bamboo basket for 'dialyzing like' process using the flowing clean water, chopped finely, steamed, mashed, put into a mould and placed onto banana leaves, covered with particular bamboo leaves for spontaneous fermentation (uncovered area mixed with Rhizopus sp. fermentation).	no data on chemical effects	**total: 650%** boiling: 100% dializing-like: 500% steaming: 50%	Heyne (1987)
"Dage benguk" making	Steamed completely cooked, removed peel, soaked (water, 48 h), placed into a small basket (covered with banana leaves, 3-4 d), re-steamed	no data on chemical effects	**total= 300%** steaming: 50% cooking: 100% soaking: 100% resteaming: 50%	Heyne (1987)
Steaming and foaming	Steamed (lipidic foam)	Safe (tasty)	steaming: 50%	Heyne (1987)

In Indonesia, mucuna seeds were consumed depends on the variety. *M. hirsuta* W. and A. indicate the needs of a lot of water to make the seed edible: (a) the young seed is boiled, peeled, and washed thoroughly with fresh water and then steamed; (b) mature seed is broken to separate the seed from the peel and the kernel can be eaten directly (*M. hirsuta* W. and A.) or the mature seed is boiled, its peel is removed, soaked into water for 24 h with water is renewed every 8-12 h. Any processing should involve water to soak up to 48 h. Generally, old documents have recorded that soaking is the key processing to avoid a headache from improper removal of velvet bean components during processing (Heyne, 1987).

Antioxidant capacity measured by DPPH methods for the velvet bean flour obtained from germination is superior compared to Rhizopus spp FNCC 610 and 6011 fermentation, as well their hybrid (Balogun *et al.*, 2017). Meanwhile, *Rhizopus oligosporus* has acted well-reducing L-DOPA in the velvet bean after 5 days fermentation (Balogun *et al.*, 2015) which reached 90%. In this case, the seed coat of black velvet bean does not only contribute more scavengers (Balogun *et al.*, 2017) but also becomes important provision to supply mineral intakes (iron 7.9 mg/100 g dry weights, and other minerals comparable to those of soybean) (Mugendi *et al.*, 2010a) and dietary fibers for human as well. L-DOPA is concluded existing in the seed coat higher (6.98%) in the unhulled raw velvet beans than dehulled one (5.71%) (Mugendi *et al.*, 2010b). Therefore, it is recommended to involve the seed coat of the velvet bean in commercial production of health ingredients from the bean.

Then, germination is outnumbered other methods for other choices. It resulted in mild eradication of antinutrients, phenolics, and L-DOPA (6.77-43.78%). Indeed, there are several processes capable of eliminating L-DOPA or antinutrients at the safe levels recommended by WHO/FAO (≤0.1%) (Mugendi *et al.*, 2010b). Concerning priority of the Parkinson disease prevention and long term preventive-curative uses, the health ingredients expected to be produced is flour containing sufficient levels of L-DOPA i.e. 4-6% or 30 g mucuna seed powder (Katzenschlager *et al.*, 2004) Because L-DOPA is thermally resistant (Mugendi *et al.*, 2010b), to decreasing other antinutrient factors (i.e. the well-known L-DOPA, total free phenolics, tannins, haemagglutinin, trypsin and chymotrypsin inhibitors, antivitamins, protease inhibitors, phytic acid, flatulence factors, saponins, and hydrogen cyanide) more after germinating, it is easier to treating further by any types of food processing. Nevertheless, it is not recommended to have a monotonous diet based on velvet bean food products because histological examination indicates inflamed liver and kidney even though the L-DOPA has reached safety levels (Mugendi *et al.*, 2010a). A study using velvet bean as part of chicken feeds (Tuleun *et al.*, 2009) show changes due to boiling resulted in broiler

biochemistry effects of higher weight gain (26.32 g/d vs. 34.68-39.79 g/d), protein intake (16.79 g/d vs. 17.50-18.93 g/d) and PER (1.55 vs. 1.99-2.10). Soaking the beans for 24 h and cooking for 60 min is recommended as adequate to improve the nutritional quality of velvet beans. In an attempt to reduce water uses, germination and fermentation are the best to simultaneously change several anti-nutritional factors. To make it standardized processing then the use of the controlled instrument is important therefore the temperature and humidity are at the right levels to make sure biochemical changes consistently.

LOWER WATERED PROCESSING ALTERNATIVES

Malting technology for preparing malt in beer making is the most established technology of seedling. Germination was adopted from malting technology (Dabija 2012): velvet beans were soaked in 1:2 ratios of beans: water for 4-6 h until moisture content of 30%; sprayed wet for 16 h until moisture content (using infrared moisture tester) MC 31-32%; soaked for 2-4 h until 38% MC; sprayed wet for 14-18 h to reach 40%. Then the beans entered germination step at humidity levels of 75% and 85%, temperature 15 °C and 20 °C steeping until it started to be rootlets. At this stage, cold water was introduced to reduce the temperature and MC content increased to 50-52%. Total water estimated in malting technology is presented in Figure 2 (written in bold letters) and the total water requirement is 370%. In wheat milling technology, to moisten grains before commencing grinding/milling is important and at this stage variations of kernel characteristics become crucial.

Another alternative is steaming of velvet bean: to soak velvet bean at MC of 40% was steamed at 80 °C for 20 min, 30 min, and 45 min which was placed making bed beans against the steam flow. Steaming has limited water supply for gelatinization therefore L-DOPA leaching will be controlled. The effect of processing and water needs is listed in Table 8.

It has not been available research on instant food ingredients aiming to service Parkinson's disease sufferers. Meanwhile, relationship between L-DOPA and germination time course is expressed as $Y = 0.175 + 2.057\ X - 1.548\ X^2$ (R^2=0.791) (Gurumoorthi *et al*., 2011). From Table 8 the priority is that L-DOPA removal is controlled to the optimal levels for dietary L-DOPA at safe levels. The second priority is antitrypsin and cyanides compulsorily reach maximum reduction. Thus alternatives mucuna processing is combinations of aquadest soaking, germination, boiling, cooking especially those involving oils and foaming (Heyne, 1987), roasting, oil frying (Sudiyono, 2010). To achieve the goal of instant ingredients thus foaming and steaming must be involved during processing. Possible products developed are suggested in the present paper include mayonnaise, ice cream, marshmallow, meringue filler, oil soups, or foamed dried flour.

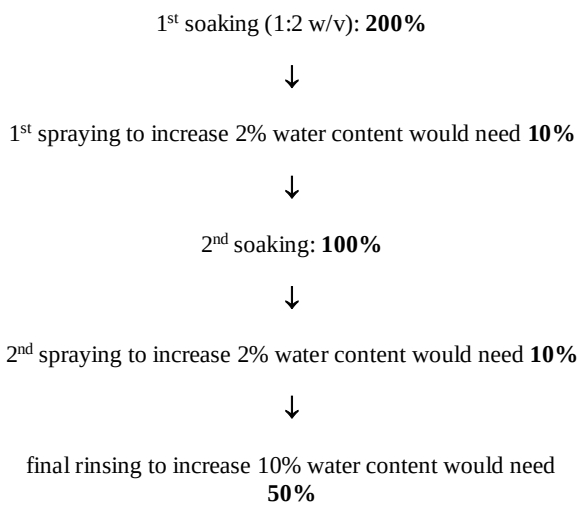

Figure 2. Estimation of malting technology of wheat

Table 8. Processing effects on mucuna seeds components and water needs during processing

No.	Processing	Components	Reduction (%)	Water (%)
1.	Soaking-germination	Antitrypsin	85.4	350
2.	Germination-malting	Antitrypsin	43.62	370
3.	Water boiling	Antitrypsin	89.7	100
4.	Soaking-cooking	Antitrypsin	100	150
5.	Cooking	Antitrypsin	100	100
6.	Cooking	Antitrypsin	100	100
7.	Toasting	Antitrypsin	42	0
8.	Roasting	Antitrypsin	8.36	0
9.	Autoclaving (raw/dehulled)	Antitrypsin	100	50
10.	Dehulling-roasting	Antitrypsin	100	0
11.	Dehulling-urea soaking	Antitrypsin	100	100
10.	Fermentation	L-DOPA	90	100
12.	Cracking-continuous soaking (in the river)	L-DOPA	0.08	unlimited
13.	Dialyzed-like	L-DOPA	1.6	500
14.	Cracking-continuous leaching	L-DOPA	4.33	112.5
15.	Leaching	L-DOPA	4.93	1,000
16.	Soaking 22 ºC	L-DOPA	30	150
17.	Soaking 45 ºC	L-DOPA	49	100
18.	Soaking 66 ºC	L-DOPA	73	100
19.	Soaking, changing water 60 ºC	L-DOPA	30	200
20.	Soaking-germination-boiling	L-DOPA	38.5	1,000
21.	Boiling-dehulling	L-DOPA	24	100
22.	Boiling-dehulling-soaking	L-DOPA	64	200
23.	Boiling-dehulling-resoaking	L-DOPA	79	300
24.	Boiling-dehulling-resoaking-reboiling	L-DOPA	90	400
25.	Alkaline boiling	L-DOPA	59.3	100
26.	Cob ash boiling	L-DOPA	58.1	100
27.	Citric acid boiling	L-DOPA	49.7	100
28.	Bean stover ash boiling	L-DOPA	47.4	100
29.	Water boiling	L-DOPA	24.9	100
30.	Water boiling	L-DOPA	48.5	100
31.	Water boiling-grits	L-DOPA	57	100
32.	Na(bicarbonate) soaking-grits-boiling-resoaking	L-DOPA	90	3,000
33.	Na(bicarbonate) soaking-boiling-resoaking	L-DOPA	67	3,000
34.	Water soaking-grits	L-DOPA	54	3,000
35.	Ca(hydroxide, ph 9) cooking, hot water washing	L-DOPA	80.4	300
36.	Soaking-Na(carbonate)	Total phenol	56	150
37.	Soaking-aquadest	Total phenol	47	100
38.	Dehulling/soaking-irradiating	Total phenol	80	100
39.	Soaking-aquadest	Phytic acid	27	200
40.	Soaking-Na(carbonate)	Phytic acid	17	150
41.	Soaking-cooking	Phytic acid	18	150
42.	Soaking-autoclaving	Phytic acid	44	150

No.	Processing	Components	Reduction (%)	Water (%)
43.	Soaking-autoclaving-cooking	Phytic acid	51	150
44.	Dry heating	Phytic acid	36	0
45.	Autoclaving	Phytic acid	47	50
46.	Dehulling-roasting	Phytic substances	70.4	0
47.	Autoclaving	Hemagglutinating activity	99	50
48.	Cooking	Hemagglutinating activity	89	100
49.	Cooking	Hemagglutinating activity	100	100
50.	Cooking	Cynide	46	100
51.	Roasting	Cynide	100	0
52.	Roasting-dehulling	Cynide	100	0
53.	Dry heating	Cynide	67	0
54.	Autoclaving	Cynide	68	50
55.	Autoclaving	Cynide	75	50
56.	Dehulling-cooking	Leptin	100	100
57.	Dehulling-roasting	Leptin	100	0
58.	Soaking-washing-boiling-draining-drying	Micronutrients	50	300
59.	Autoclaving	Tannin	71	50

PHYSICOCHEMICAL PROPERTIES OF VELVET BEAN FLOUR FROM VARIOUS PROCESSING

Data in Table 9 indicate the effects of the cooking of various techniques. Dehulling increased water absorption capacity as well as oil absorption capacity, foam stability, and viscosity but it reduced gel, emulsion, and foaming capacity, swelling power, bulk density, and pH (Balogun and Olatidoye, 2012). Defatting treatment on various mucuna members indicated increasing water absorption capacity and foam capacity but reducing emulsion capacity (Adebowale *et al.*, 2005). Heating increased water and oil absorption capacity but reduced emulsion and foaming capacity whereas foam stability are relatively similar (Ahenkora *et al.*, 1999). Acetylating of velvet bean flour showed an increase in water and oil absorption capacity while oxidizing only increased solubility of the flour quite profoundly (Adebowale and Lawal, 2003).

Irradiation can be considered to preserve almost all physicochemical properties with better cooking time. Irradiation reduces the texture of raw velvet beans at dosages >7.5 kGy might be due to polymer degradations, solubility, turgor, and moisture losses (Bhat *et al.*, 2007). Irradiation gives better sterilization on microbial contamination on the seed surfaces (Bhat *et al.*, 2007) using ionized irradiation and electron beam irradiation. These technologies are expensive and several important properties similar to those of whole velvet bean in the study of Balogun and Olatidoye (2012). Shorter cooking time after irradiation treatments may reduce energy for cooking but it is unclear if the water requirements for cooking would also be reduced. The availability of lipid/oil in velvet bean food preparation that is considered safe (Vadivel and Bielsaski, 2012) gives clues that defatting is unnecessarily carried out. Lipid is required to 'tame' itching component and to make better palatability (Heyne, 1987). Chemical modification through acetylating and oxidizing may be useful to improve the solubility of velvet bean products during processing. However, washing steps after treating the beans with the chemicals would consume a lot of water. The left problem using the whole bean is more likely to get a long time cooking due to the whole beans to have a high bulk density (Seena and Sridhar, 2005). Therefore, it is noteworthy to keep

Table 9. Physicochemical properties

Physicochem. properties	Balogun and Olatidoye (2012)		Adebowale *et al.* (2005)		Ahenkora *et al.* (1999)		Adebowale and Lawal (2003)			Bhat *et al.* (2008)	
species	*Mucuna utilis*,		*M.cochinchinensis, M. deerigeana, M. pruriens, M. rajada, M. veracruz* (mottle) and *M. veracruz* (white)							*Mucuna pruriens*	
	whole	dehulled	full fat	defatted	raw	heated	native	acetylation	oxidized	raw	irradiated (2.5-30 kGy)
WAC	2.05	2.45	1.40-2.20	higher	140%	156%	1.71	121		ca. 2.2 mL/g	slightly increased
OAC	1.19	1.45			76%	86%		increased	reduced	ca. 1.5 mL/g	slightly increased
GC (%)	4.12	3.85	14-20								
EC	24.42	22.40	78-90%	56-68%	60%	50%				ca. 50%	relatively similar
ES										ca. 90%	slightly increased
SP	2.68	2.28					2.7-13.3	3.6-15.6	2.3-9.9		
FC (%)	21.52*	16.60*	9.6-19.23%	50-84.30%	53%	4%				ca. 39%	slightly increased
FS	21.00^{30min} 18.00^{60min}	26.00^{30min} 21.00^{60min}			10%	9%				ca. 60%	relatively similar
viscosity (Ns/m^2)	17.52	22.18									
ρbulk (g/cm^3)	1.25	0.94								0.649±0.05	0.649±0.05
pH	6.72	6.51									
pI			4-5		4.5						
solubility (g/g)							21-143	36-147	52-200		reduced
t_{min} cooking (min)										22	14-21
WUR										1.43±0.002	1.45-1.56
elongation ratio										1.26±0.08	1.23-1.4
GSL (%)										20.43±0.006	12.13-20.10
-L/B ratio										1.62±0.21	1.59-1.62

* in mL, WAC: Water absorption capacity, OAC: oil absorption capacity, GSL: Gruel solid loss, GC: gelation capacity, FC: foaming capacity; FS: foaming stability, EC: emulsion capacity, ES: emulsion stability, SP: swelling power, WUR: water uptake ratio

a balance between low water consumption while processing the velvet bean also saving energy.

POTENTIAL COMMERCIALIZATION OF VARIOUS PROCESSING

Commercial velvet bean powder has been available to cure Parkinson's disease. This is under the protection of US Patent No. 7470441B2 and US7470441B2, WO2017126959A1, and EP1567177A2. The most commercialization of velvet bean use is in the form of a medicine regime. As far as it is concerned, the patent or other intellectual properties of instant velvet bean flour as a food ingredient to be raw materials of functional foods is not found. Another commercialization potential is the use of the flour as protein-lipid rich food ingredients for daily menu components. A holistic value chain from agriculture to end-users of velvet bean flour also considered as environmentally friendly food chain because the mucuna plant is excellent green manure, controlling weed in the soil through its allelochemicals, DOPA; capable of nitrogen fixation in the soil, a good protein-lipid balance provision comparative to soybean, and can optimally support food sovereignty by optimal use of unfertile soils.

The patent No. 7470441B2 is about an extraction method for *M. pruriens* seeds to produce fractions or crude extract that contain at least one of active compounds, substance, ot their mixtures for pharmaceutical uses capable of preventing, reducing, and curing Parkinson's disease. The successive method uses solvents comprised of hexane, acetone, water-ethanol which contains vitamin C at particular levels. Pharmaceutically, the extract gives broader therapy including L-DOPA therapy, slowing down the development of the disease even though it onsets earlier yet the development can be retarded as well as to prevent or eliminate acute and chronic L-DOPA toxicity.

Patent application No. WO2017126959A1 is about food products containing fermented starch from diverse starch resources with claims of fermented starch content at 15% and inoculum levels of 0.1-10% providing health benefits and the food products are organoleptically well-liked by consumers. The claim covers up the processing methods for commercial productions i.e. the uses of particular moisture content, heating, cooling for appropriate fermentative temperature, inoculation for the claimed method.

Patent application No. EP1567177A2 is pointing out *M. pruriens* for neuroligical disease treatments focusing on pharmaceutical compositions that containing *M. pruriens* seeds or one or more of its components, substances, fractions, as well as their mixtures to prevent, remove or to treat the diseases. The claims also include methods to prepare them from *M. pruriens* for the aforementioned goals. The uses of this product include a wider spectrum of Parkinson's disease from retarding the need to give combination treatments initial onset, and the time course of L-DOPA effication as well as to prevent or to eliminate acute or chronic L-DOPA treatments. The focused components are bipolar-lipophilic fraction compounds either in the form of an infusion, injection solutions, gelatin capsule, tablets, and slow-released tablets. The targeted compounds under the claims have neurostimulator and neuroprotective functions obtained from particular *M. pruriens* (M-PL0100, M-EL100, M-BL0100, LAT543-0 dan M-W-EL1299, M-W0100, MWEL0700, M-ML0100). The scope of pharmaceutical preparation composition is (1) to inhibit L-DOPA metabolism or dopamine, (b) to improve L-DOPA absorption to generate initial L-DOPA effication and (c) to lengthen L-DOPA effication. Patent No. US7470441B2 claims extraction method of *M. pruriens* seeds using hexane, the mixture of water and ethanol containing 0.5% vitamin C for pharmaceutical goals.

Such pharmaceutical preparations can have environmental risks because of organic solvent waste. Moreover, the area where velvet beans produced is categorized by a fragile environment in sustainable viewpoints, which warrant another processing method that more environmentally friendly. Clean water is an obstacle in this area to make the velvet beans safe from high levels of L-DOPA and cyanides. To be positive towards green manure contributions of velvet bean planting, thus the commercialization model is important to be integrated commercialization system.

Commercialization at small-medium scale enterprises producing foods or food ingredients is considered as more realistic. Therefore, food processing oriented for health ingredients is important. Such food processing technology with less water consumption is investigated to achieve a system of food commercialization for velvet beans and oriented to get health-related food materials. It is expected that less water consumption processing would give better economic feasibility.

CONCLUSION

Velvet bean uses for health-related food product development warrant research on a more holistic approach to achieving sustainable commercialization in a way that environmentally friendly, by reducing the use of organic solvents, less water consumption during processing, while during its farming the mucuna plant could give healthier soils. Consequently, the healthier the soils, the better the agricultural productions and finally, and hopefully, society gets benefits for their well being and welfares.

Based on this literature reviews it is concluded that steaming might the best compromising of functional food ingredients where low water consumption, safe and effective energy uses because of the power of steam and latent energy involved. The expectation is capable of producing safe functional food ingredients for diabetes mellitus and/or Parkinson by the use of L-DOPA, cyclic-D-chiro inositol, and fibers also of the basic nutrients. The protein content of velvet bean comparable to that of soybean yet growth values (protein effective ratio) is poor so monotonous diet based velvet beans is not recommended. As functional ingredient, it can be managed to reach safe dosages in daily intakes. However, all hypotheses were currently being proven in our research group. Using steaming and germinating to characterizing steamed-whole flour velvet beans.

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Journal of Functional Food and Nutraceutical

SOYBEAN (*GLYCINE MAX* (L.) MERR.) AND WILD SOYBEAN (*G. SOJA* SIEB. ET ZUCC) GENETIC DIVERSITY FOR FUNCTIONAL FOOD

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ABSTRACT

Soybean has been a major protein source for people in East and Southeast Asia. It contains numerous secondary metabolites that are useful for human and animal health, such as isoflavones, polyphenols, tocopherols, lutein, carotenoids, and saponins. Isoflavones are the most popular nutraceutical compounds of soybean. Several soybean varieties having high isoflavones have been developed and utilized as functional food resources. Tocopherols, lutein, and saponins are also antioxidants present in soybean seeds. Tocopherols are known as vitamin E, whereas lutein has potential to prevent age-related macular degeneration. DDMP saponins in soybean is reported to inhibit HIV infection in vitro and prevent colon cancer. Screening of soybean germplasm showed that soybean holds a large genetic diversity of content and composition of tocopherols, lutein and saponins that can be utilized to breed new soybean cultivars. In addition, the wild counterpart of soybean (wild soybean) can also be source of genetic variations useful for soybean breeding. This review will summarize studies on genetic diversity of tocopherols, lutein, and saponins found in soybean and wild soybean.

Keywords: *DDMP saponin, lutein, soybean, vitamin E, wild soybean*

ABSTRAK

Kedelai telah menjadi sumber protein utama bagi masyarakat di Asia Timur dan Tenggara. Kedelai mengandung banyak metabolit sekunder yang bermanfaat untuk kesehatan manusia dan hewan, seperti isoflavon, polifenol, tokoferol, lutein, karotenoid, dan saponin. Hasil skrining plasma nutfah kedelai menunjukkan bahwa kedelai memiliki keragaman kandungan metabolit sekunder yang besar dan dapat dimanfaatkan untuk pembiakan kultivar kedelai baru. Selain itu, kerabat kedelai atau kedelai liar (wild soybean) juga dapat menjadi sumber variasi genetik yang berguna untuk pemuliaan kedelai. Review ini merangkum studi tentang keragaman genetik zat gizi yang ditemukan pada kedelai dan kedelai liar, dengan fokus pada tokoferol, lutein, dan saponin.

Kata kunci: *Kedelai, kedelai liar, lutein, saponin DDMP, vitamin E*

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I. SOYBEAN AND WILD SOYBEAN

Soybean (*Glycine max* (L.) Merrill) has been a major protein source for human consumption, particularly in East and Southeast Asia. Traditional soy-based food products can be found in East Asia and Southeast Asian countries. Worldwide, soybean is grown in a large scale in Brazil, US, Argentine, China (http://soystats.com/international-world-soybean-production/, accessed July 12th, 2020), mainly for oil and feed. Soybean seeds vary in shape, size, seed coat color, and cotyledon color (Figure 1), as well as in oil content, fatty acid composition, protein, and sugar content.

The ancestor of soybean is wild soybean or *G. soja* (Siebold and Zucc.). It can be found naturally in wide region of China, Japan, Korean Peninsula, and Russia. Wild soybean has small, ellipse black seeds, and the plants form vine (Figure 1). The genetic diversity analyses showed that wild soybean has larger genetic diversity analysis than that of soybean (Kofsky et al. 2018). Moreover, wild soybean also has unique genes that are not present in cultivated soybeans (Xie et al. 2019). Alleles related to soybean cyst nematode resistance; aphid resistance; salt, alkalinity and drought tolerance; yield, seed oil, protein and unsaturated fatty acid have been identified from wild soybean (Xie et al. 2019). Crossing between wild soybean and soybean produces fertile progenies, thus wild soybean has a large potential to be a donor parent for soybean breeding.

The Japan National Institute of Agrobiological Sciences (NIAS) Genebank holds a collection of approximately 11,300 accessions consisting of Japan and overseas landraces, improved varieties and breeding lines developed by agriculture research centers, and also wild soybean accessions (Kaga et al. 2012). In addition, U.S. Departement of Agriculture (USDA) genebank holds a collection of nearly 22,000 accessions of soybean, wild soybean, and perennial soybeans (Bandillo et al. 2015). After filtering out duplicates, there were 14,430 unique accessions originated from 85 countries. Of these, 14,000 soybean and wild soybean accessions have been genotyped using 50,000 single nucleotide polymorphism (SNP) chip and they were evaluated for their oil and protein content (Bandillo et al. 2015).

There are growing interests in developing soybean as functional food. The most popular compounds of soybean that have been utilized in functional food, supplements, or cosmetics are isoflavones. However, soybean and wild soybean also contain many other secondary metabolites that can be developed further for new products. Here, three compounds selected: tocopherols, lutein, and saponins. This review will discuss about the genetic diversity that found in soybean and wild soybean, the impact of crop management and the environmental condition to the content and composition of the three traits.

Figure 1. Seed and plant types of soybean and wild soybean. left: example of seeds of soybean and wild soybean. right: example of plants of wild soybean and soybean.

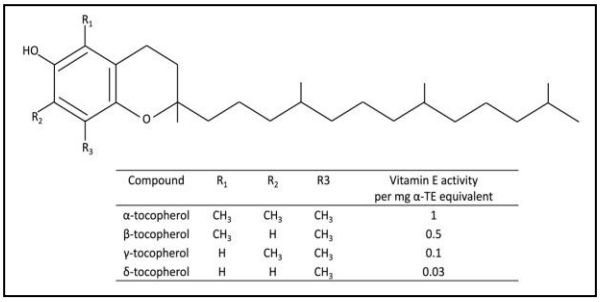
II. STUDIES ON GENETIC DIVERSITY OF NUTRITIONAL COMPOUNDS

2.1. Tocopherols

2.1.1. Tocopherol in soybean and its biosynthesis regulation

Tocopherols are lipophilic antioxidants belong to tocochromanol group, which is known to possess vitamin E activity (Bramley et al. 2000). The structure consists of a chromanol head and a phytyl tail. Based on the number and position of methyl group on the chromanol head, tocopherols are divided to four isoforms (α-, β-, γ-, and δ-tocopherol) (Figure 2). Natural isoforms of tocopherols have *RRR*-forms, thus tocopherols

from natural sources are designated as *RRR*-α-, β-, γ-, and δ-tocopherol, respectively. Among four isoforms, α-tocopherol has the highest vitamin E activity because it is preferentially absorbed by α-tocopherol transfer protein in liver and it is retained at high level in human blood plasma. When *RRR*-α-tocopherol vitamin E activity is 1 mg α-tocopherol equivalents (α-TE mg-1 compound), the vitamin E activities of *RRR*-β-, *RRR*-γ-, and *RRR*-δ-tocopherol are 0.5, 0.1, and 0.03 mg, respectively (Figure 2; Bramley et al. 2000). Besides tocopherols, there are tocotrienols which differ from tocopherols by the presence of unsaturated hydrogen bonds in the phytyl tail. Rice bran contains high level of tocotrienols, however, soybean contains no tocotrienols.



Compound	R_1	R_2	R3	Vitamin E activity per mg α-TE equivalent
α-tocopherol	CH_3	CH_3	CH_3	1
β-tocopherol	CH_3	H	CH_3	0.5
γ-tocopherol	H	CH_3	CH_3	0.1
δ-tocopherol	H	H	CH_3	0.03

Figure 2. Tocopherol isoforms and its vitamin E activity

Tocopherols are commonly added in food and cosmetics to prevent oil oxidation. Soybean oil is also one of the sources of vitamin E supplement sold in markets. Soybean oil contains about 1,000 mg tocopherols per kg edible portion (Bramley et al. 2000). This value is higher compared with oils sourced from canola (253.4 mg tocopherols per kg edible portion), sunflower (546 mg tocopherols per kg edible portion), or palm (386 mg tocopherols per kg edible portion) (Bramley et al. 2000; Sheppard et al. 1993). However, the vitamin E activity as α-TE (mg) of soybean oil is lower than that of canola, sunflower, or palm, because soybean seeds contain α-tocopherol only less than 10% of total tocopherol content. The predominant form of tocopherol in soybean seeds is γ-tocopherol (60-70% of total tocopherol content), followed by δ-tocopherol (20-30% of total tocopherol content). The remaining isoform, β-tocopherol is usually present at negligible amount (1-2% of total tocopherol content).

It has been great interest to increase vitamin E content in soybean seeds by increasing its α-tocopherol ratio. Tocopherol biosynthesis pathway is already elucidated (Figure 3), and genes involved in the tocopherol biosynthesis have been characterized in model plant *Arabidopsis thaliana*, and several oil crops such as soybean, maize, rapeseed, and sunflower (Fritsche et al. 2017). The first step in tocopherol biosynthesis is the creation of 2-methyl-6-phytyl-1,4-benzoquinol (MPBQ) by joining homogentisic acid (HGA) and phytyl-diphosphate (PDP). This step is catalyzed by homogentisic phytyltransferase (HPT). MPBQ-methyltransferase enzyme (MPBQ-MT) adds a methyl group to MPBQ, creating 2,3-dimethyl-5-phytyl-1,4-benzoquinone (DMPBQ). The chromanol heads of MPBQ and DMPBQ were cyclized to create δ-tocopherol and γ-tocopherol, respectively. This step is catalyzed by tocopherol cyclase (TC). The last step of tocopherol biosynthesis is the addition of a methyl group to δ-tocopherol and γ-tocopherol, creating β-tocopherol and α-tocopherol, respectively. This step is catalyzed by γ-tocopherol methyltransferase (γ-TMT). In *A.thaliana*, *VTE3* and *VTE4* genes encode MPBQ-MT and γ-TMT, respectively. Overexpressing *VTE4* in soybean increased α-tocopherol ratio to 50~75% of total tocopherol content, whereas overexpressing both *VTE3* and *VTE4* in soybean increased α-tocopherol ratio to 60~91% of total tocopherol content (Van Eenennaam et al. 2003).

2.1.2. Genetic diversity of tocopherol composition and content in soybean germplasm

Soybean has a considerable diversity of tocopherol composition and tocopherol content. Studies from India (Rani et al. 2007), Brazil (Carrão-Panizzi and Erhan 2007), China (Zhan et al. 2020), worldwide germplasm (Ujiie et al. 2005), wild soybean (Dwiyanti et al. 2016) will be introduced here. It is important to take note that the measurement differs between studies. Rani et al. (2007) used μg/g of oil, Carrão-Panizzi and Erhan (2007) used ppm, Ujiie et al. (2005) used mg/ 100 g seed meal whereas

Dwiyanti et al. (2016) used percentage of total tocopherol content.

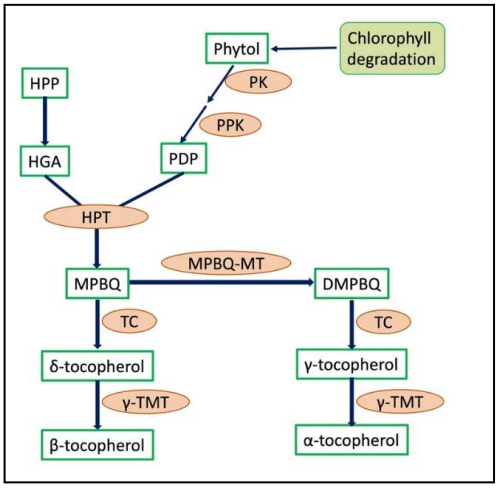


Figure 3. Tocopherol biosynthesis pathway. Substrates are shown in green boxes: HPP: *p*-hydroxyphenylpyruvic acid, PDP: phytyl diphosphate, HGA: homogentisic acid, MPBQ: 2-methyl-6-phytyl-1,4-benzo-quinol, DMPBQ: 2,3-dimethyl-6-phytyl-1,4-benzoquinone. Enzymes are shown in brown circles. PK: phytol kinase, PPK: phytyl phosphate *kinase*, HPT: homogentisate phytyltransferase, MPBQ-MT: MPBQ methyltransferase, TC: tocopherol cyclase, γ-TMT: γ-tocopherol methyltransferase.

Screening of 1,109 worldwide soybean and wild soybean accessions, Ujiie et al. (2005) identified three soybean accessions having high α-tocopherol ratio Keszthelyi Aproszemu Sarga (KAS), Dobrogeance, and Dobrudza 14 Pancevo). Two soybean cultivars used as control (Toyokomachi and Ichihime) have total tocopherol content 10 - 12 mg/ 100 g seed meal. Initial screening using seeds from genebank showed that KAS had higher α-tocopherol content, but significantly lower total tocopherol content compared with other accessions. Its α-tocopherol content was about 4.26 mg / 100 g seed meal of total tocopherol content (8 mg / 100 g seed meal), thus the α-tocopherol ratio was more than 50% (Ujiie et al. 2005). The other high α-tocopherol accessions Dobrogeance and Dobrudza had α-tocopherol content about 3.71 mg / 100 g seed meal and 3.26 mg / 100 g seed meal, but their total tocopherol content was similar to that of Toyokomachi and Ichihime (Ujiie et al. 2005). It is important to note that the screening used seeds coming from plants grown at different locations. KAS was grown in National Shikoku Agricultural Experiment Station, Kagawa; Dobrogeance and var. Dobrudza 14 Pancevo were grown at the National Agricultural Research Center for Tohoku Region, Akita; and the control cultivars were grown at Hokkaido University Agricultural Farm (Ujiie et al. 2005). Among these, Kagawa is the southernmost location. When grown in Hokkaido, the α-tocopherol ratio of KAS will be about 20% of total tocopherol content (Dwiyanti et al. 2007, Dwiyanti et al. 2011). It is known that the α-tocopherol ratio is largely affected by temperature during seed filling (Britz and Kremer 2002, Dwiyanti et al. 2016), thus the 50% α-tocopherol ratio of KAS in initial screening may be resulted from the temperature effect. In addition, screening of 600 wild soybean accessions originated from Japan and South Korea (Dwiyanti et al. 2016) identified 16 wild soybean accessions having α-tocopherol ratio more than 20%. Cultivation of these accessions in controlled temperature showed that the α-tocopherol ratio was more than 20% for 15 accessions, and 14% for 1 accession (Dwiyanti et al. 2016), indicating that the high α-tocopherol ratio in these accessions was genetically inherited. Genetic analysis showed that the genetic variations in *γ-TMT3*, one of the genes encoding γ-tocopherol methyltransferase were associated with high α-tocopherol ratio in soybean KAS and wild soybean B04009.

Analysis of 66 Indian soybean cultivars (Rani et al. 2007) showed large variability for all tocopherol isoforms as well as total tocopherol content. The α-tocopherol content of 66 Indian soybean cultivars ranged from 58 µg/g to 794 µg/g, with an overall mean value of 269 g/g of oil, whereas the total tocopherol content ranged from 422 µg/g to 3,311 µg/g of soybean oil. If the value is converted to ratio, the α/total tocopherol ratio ranged between 11 to 26%, with an average value 18%. This ratio is higher than the values reported in Ujiie et al. (2005). On the other hand, analysis of 89 Brazil soybean accessions showed the range from 11 ppm to 191 ppm for α-tocopherol, and from 561 ppm to

1,983 ppm for total tocopherols (Carrão-Panizzi and Erhan 2007). The α-tocopherol ratio range was between 1.8% to 11.7%, which is lower than the values obtained from India or Japan study. Recently, Zhan et al. (2020) investigated the tocopherol content of 180 soybean accessions from China. The populations were grown at two locations for 3 years. The range of α-tocopherol and total tocopherol content was different for each year × location but the range was between 1.4 μg/g to 98.0 μg/g for α-tocopherol content, and 155.0 μg/g to 399.30 μg/g for total tocopherol content.

2.1.3. Effect of growing condition to soybean tocopherol composition and content

High temperature and drought stress during seed filling affected α-tocopherol content (Britz and Kremer 2002). A 5°C increase in temperature from 23°C to 28°C during seed filling increase the α-tocopherol up to twice of the usual content (Britz and Kremer 2002). The same phenomenon was also observed in wild soybean. Growing 16 wild soybean genotypes having high α-tocopherol ratio in 20°C, 25°C, and 30°C during seed filling period showed that α-tocopherol ratio increase following the increment in temperature (Dwiyanti et al. 2016). Interestingly, the increase rate differed among genotypes. For example, the α-tocopherol ratio of genotype B09092 matured in 30°C was twice of the α-tocopherol ratio of the same genotype matured in 25°C. On the contrary, the α-tocopherol ratio of genotype B00092 was not significantly different between seed matured in 25°C or in 30°C (Dwiyanti et al. 2016). Difference in temperature response may be resulted from genetic variations in genes involved in tocopherol biosynthesis pathway. Park et al. (2019) investigated the expression level of three soybean genes (*γ-TMT1*, *γ-TMT2*, and *γ-TMT3*) encoding γ-tocopherol methyltransferase of developing seeds of TK780 (soybean, low α-tocopherol ratio) and B04009 (wild soybean, high α-tocopherol ratio) grown in 20°C and 30°C during seed filling. Previous studies showed that genetic variations in *γ-TMT3* are associated with high α-tocopherol ratio in KAS (Dwiyanti et al. 2011) and B04009 (Park et al. 2019). Interestingly, the gene expression analysis showed that only *γ-TMT2* expression level increased in both genotypes when temperature increased to 30°C. In contrast to expectation, *γ-TMT3* expression decreased in both genotypes when temperature increased to 30°C (Park et al. 2019). Whether this phenomenon is common to all soybean genotypes is remained to be elucidated.

Other environment effects such as seeding rate, seeding date, row spacing, and P+K fertilization were also investigated in Canada environment (Seguin et al. 2010). Among these, early seeding date resulted to higher α-tocopherol ratio up to 45% compared to that of later seeding date. It is suggested that higher temperature during seed filling in early seeding date regime affected the α-tocopherol ratio. Other treatments impacted the α-tocopherol ratio, but the results were not consistent across environments.

It is important to note that increasing only α-tocopherol in soybean seeds may not be beneficial. It has been suggested that γ-tocopherol plays important role in seed desiccation tolerance, which may be the reason of γ-tocopherol being the major isoform of tocopherols in seeds of most plants (Fritsche et al. 2017). If present as tocopherol mixture in oil, α-tocopherol will be oxidized first followed by other tocopherol isoforms (Seppanen et al. 2010). In addition, the antioxidant effect of 50% α-tocopherol : 50% γ-tocopherol mixture and natural tocopherols mixture from soybean (13% α-tocopherol, 64% γ-tocopherol and 21% δ-tocopherol) were 250 and 500 ppm, respectively (Huang et al. 1995), indicating that α-tocopherol over certain ratio can act as prooxidant.

2.2. Lutein

2.2.1. Lutein in soybean and biosynthesis regulation

Lutein is known as pro-vitamin A carotenoids and is able to prevent early, intermediate, and advanced AMD (age-related macular degeneration) (Ranard et al. 2017). Major sources of lutein are egg yolk, green leafy vegetables, and orange/yellow fruits. Lutein is often present together with zeaxanthin, another type of carotenoid, and they are both present in the macula, a part of retina where photoreceptor cells are highly concentrated. Daily intake about 3-5 mg lutein per day may reduce the

risk of all stages of AMD (Ranard et al. 2017). The maximum dose recommended for dietary intake is yet to be determined, however it is suggested that the intake up to 20 mg/day for lutein is considered safe (Ranard et al. 2017, Toti et al. 2018).

Lutein is also an important component in poultry feed. Since animals cannot produce carotenoids by their own, lutein supplementation into the poultry feed helps enrichment of lutein content in eggs. Purified lutein supplements up to 1,000 mg/kg, increased lutein concentrations by up to 10-fold in eggs (Pitargue et al. 2019). Lutein supplements for feed are mainly sourced from corn co-products, alfalfa, marigold extract, or algae (Pitargue et al. 2019). Globally, soybean meal contributed up to 69% of all protein sources for animal feeds. In the U.S., it reached up to approximately 92% of animal feed, and 48% of which was used for poultry feed (Cromwell, 2017). It has been an interest in high-lutein soybean in order to save cost used in lutein supplementation from other sources.

Lutein is the most abundant carotenoids in soybean seeds (Monma et al. 1994). Carotenoid biosynthesis pathway has been elucidated (Figure 4). Carotenoid biosynthesis starts by combining two geranylgeranyl pyrophosphate (GGPP) to form phytoene. The process is catalyzed by phytoene synthase (PSY). Four reaction steps catalyzed by phytoene desaturase, ζ-carotene desaturase, ζ-carotene isomerase and carotenoid isomerase convert phytoene to lycopene. Two processes catalyzed by lycopene ε-cyclase and lycopene β-cyclase respectively convert lycopene to α-carotene. Finally, α-carotene is converted to lutein through two pathways, zeinoxanthin or α-cryptoxanthin. Generally, immature soybean seeds contain lutein. However, these compounds degrade during seed filling, as the result, lutein is present at low concentration in mature yellow soybean seeds (Monma et al. 1994).

2.2.2. Genetic diversity of lutein content in soybean and wild soybean

Genetic diversity of lutein content in soybean was reported by Kanamaru et al. (2006) who evaluated 490 soybean and 610 wild soybean genotypes. The lutein content of soybean genotypes ranged between 0.16 to 1.48 mg/100 g meal (Kanamaru et al. 2006). Wild soybean showed higher range of lutein content, between 0.58 to 3.28 mg/100 g meal (Kanamaru et al. 2006). Of these, seven wild soybean genotypes had lutein content more than 2.1 mg/100 g meal, and the values were stable across two years planting experiment. High lutein wild soybean can be distinguished by deep yellow cotyledon. High lutein wild soybean also had high β-carotene and chlorophyll content but these compounds were found in seed coat whereas lutein was produced in cotyledon. Crossing high lutein wild soybean to low lutein soybean cultivar produced progenies with segregating lutein content (Kanamaru et al. 2008). Comparing lutein content of high lutein progenies and low lutein progenies showed that there was no difference in lutein content at immature seed state (Kanamaru et al. 2008). However, lutein content significantly decreased toward maturation in low lutein progenies compared to high lutein progenies, indicating that genetic variations in gene(s) involved in lutein degradation may be associated with high lutein content.

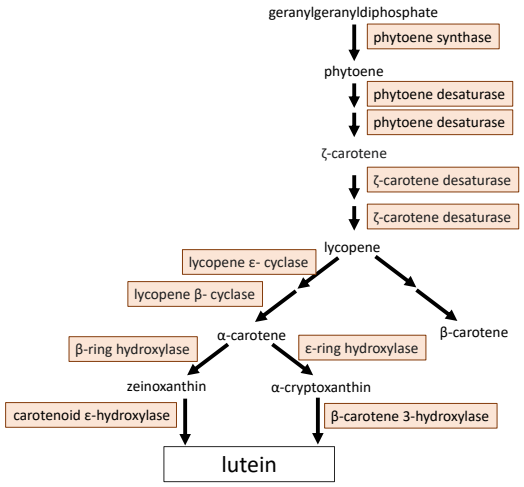


Figure 4. Lutein biosynthesis pathway (redrawn based on kyoto encyclopedia of genes and genomes (KEGG) database: http: //www.genome. jp/keggbin/show_pathway?gmx00906; last accessed July 8th, 2020).

2.2.3. Effect on crop management and growth environment on soybean lutein content

Unlike tocopherols, soybean's lutein content is more determined by the genotype and it is less affected by crop management practice and growth environment (Kanamaru et al. 2006, Lee et al. 2008, Seguin et al. 2011). However, more studies are needed to confirm the effect of planting date on lutein content, particularly in the tropical regions where there is little difference in daylight length and temperature. A genotype × environment interaction study conducted on 7 high lutein wild soybean genotypes showed that there was no large variation in between two years planting (2004 and 2005; Sapporo, latitude 43°04'N) (Kanamaru et al. 2006). The trend was confirmed by another study showed that when 15 soybean genotypes grown on farms in Portageville, US (latitude 36°44'N) (Lee et al. 2008). Lee et al. (2008) also showed that planting date affected lutein content but the response between soybean genotypes varied. However, a study conducted in Quebec, Canada showed that the planting date affected on lutein content (Seguin et al. 2011). This study only tested the planting date effect on two soybean genotypes, AC Proteina and OAC Vision, grown in two locations Ste-Anne-de-Bellevue (45°24'N) and Normandin (48°49'N). Later planting date in Ste-Anne-de-Bellevue significantly increased lutein content but the same treatment in Normandin significantly decreased lutein content. Whether temperature during seed filling affects lutein content is remained to be investigated.

Other crop management practices were assessed such as seeding rate, row spacing, P and K fertilization and there was no clear trend of between treatments and the differences were small. It was less than 8% for row spacing and P treatment, as well as there was no difference in K fertilization (Seguin et al. 2011).

2.3. Saponins

2.3.1. Saponins in soybean and biosynthesis regulation

Soyasaponins are oleanane-type triterpenoids, widely distributed among Leguminosae species. Based on its aglycone structure, soyasaponins are categorized into soyasapogenol A and soyasapogenol B (Figure 5, Takada et al. 2013, Krishnamurthy et al. 2019). Soyasapogenol A and soyasapogenol B can be distinguished by the presence of hydroxy group on the C-21 position. In plants, sugar chain is attached to the C-3 position of soyasapogenol A, creating group A saponins. In soybean seeds, soyasapogenol B group is present as DDMP saponin. In addition to attachment of sugar chain at C-3 position, a DDMP moiety is attached to C-22 position, creating DDMP saponin. Based on type of sugars attached to C-3 or C-22 position, there are more than 30 saponins exist in soybean seeds (Table 1). When soybean seeds are crushed, extracted, or processed in cooking, DDMP saponin degrades to B saponin and E saponin. Several studies report the saponin composition or content of soybean seeds as group A and group B saponins, because when DDMP saponin is extracted from soybean seeds, it is rapidly degraded to B saponins during the processing or during analysis using high performance liquid chromatography (HPLC) (Takahashi et al. 2017).

The acetylated group A saponins cause bitter and astringent taste of soybean (Sundaramoorthy et al. 2018), however recently it is also reported that group A saponins may have anti-obesity effect (Yang et al. 2015). On the other hand, group DDMP saponins and its derivative B saponins are reported to be less bitter and possess health benefits such as radical oxygen scavenger, anti-mutagenic activities (Berhow et al. 2002), inhibitor of HIV infection in vitro (Nakashima et al. 1989), and colon cancer prevention (Tsai et al. 2010). It has been an interest to increase DDMP saponin and to decrease the amount of A saponin in soybean seeds. Group A saponins are present mainly in hypocotyl, whereas DDMP saponins are found in cotyledon (Sundaramoorthy et al. 2018).

Saponin biosynthesis pathway in soybean has not been fully elucidated (Figure 5). The first step of saponin biosynthesis is the creation of β-amyrin. The next step is the hydroxylation of C-24 position of β-amyrin to 24-hydroxyl β-amyrin. The conversion may be in several steps, but it is not elucidated yet. Hydroxylation of C-22 position creates soyasapogenol B, and hydroxylation of C-

Table 1. Soyasaponin types based on sugar-chain at C-3 and C-22 positions

C-3 position (R1)	C-22 position						
	Soyasapogenol A				Soyasapogenol B		
	Aa-series acetylXyl-	Ab-series acetylGlc-	A0-series H-	A-series *	DDMP DDMP	Group B H-	Group E O=
β-**D**-glucosyl-(1→2)-*O*-β-**D**-Gal-†	Aa	Ab	A0-αg	A-αg	αg	Ba	Bd
β-**L**-rhamnosyl-(1→2)-*O*-β-**D**-Gal-	Au	Ac	A0-βg	A-βg	βg	Bb	Be
β-**D**-Gal-	Ae	Af	A0-γg	A-γg	γg	Bb’	Be’
β-**D**-glucosyl-(1→2)-*O*-α-**L**-Ara-‡	Ax	Ad	A0-αa	A-αa	αa	Bx	Bf
β-**L**-rhamnosyl-(1→2)-*O*-α-**L**-Ara-	Ay	Az	A0-βa	A-βa	βa	Bc	Bg
α-**L**-Ara-	Ag	Ah	A0-γa	A-γa	γa	Bc’	Bg’
(δ-series) H-	Aa-δ	Ab-δ	A0-δ	A-δ	DDMP-δ	B-δ	E-δ

*A-series saponins do not contain arabinose at the C-22 position. †: galactose; ‡: arabinose. The categorization was redrawn based on Khrisnamurthy et al. (2019).

21 of soyasapogenol B creates soyasapogenol A. Additions of sugar chains and DDMP create DDMP saponins, whereas the additions of sugar chains at C-3 and C-22 positions of soyasapogenol A create group A saponins.

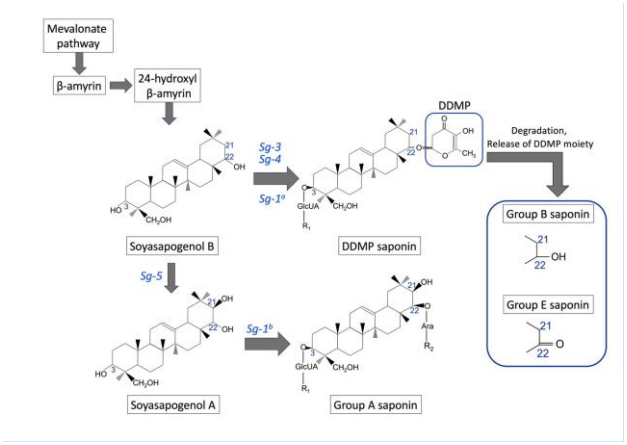


Figure 5. Putative saponin biosynthesis pathway in soybean (redrawn based on krishnamurthy et al. 2019). *Sg-1*^{a}, *Sg-1*^{b}, *Sg-3*, *Sg-4*, and *Sg-5* are loci where genes involved in saponin biosynthesis pathway are located. number 3, 21, 22 correspond to the hydroxyl position in the soyasapogenol.

There are several types of *Sg-1* locus that lead to structural diversity of sugar at C-22 position of group A-saponins (Sayama et al. 2012). *Sg-1*^{a} and *Sg-1*^{b} encode UDP-sugar–dependent glycosyltransferases, UGT73F4 and UGT73F2, which add xylose and glucose, respectively, to the arabinose residue at the C-22 position. A rare *sg-1*^{0} mutant does not have the ability to add sugar chain, and this is resulted in A0-αg type soybean. The *sg-1*^{0} mutant has been used in breeding program, led to development of ‘Kinusayaka’, a soybean cultivar having less beany and astringent flavor, and suitable for soymilk and tofu (Kato et al. 2007).

Sg-3 locus contains a gene *Glyma.10G104700* encoding a putative glycosyltransferase (UGT91H9) that adds glucose as third moiety atsugar chain at C-3 position of saponins (Yano et al. 2018). Soybean cultivar ‘Mikuriya-ao’ is a *sg-3* natural mutant that lost *Glyma.10G104700 gene, resulting in the absence of* Ab and αg saponins and accumulation of Af saponin. *Sg-4 locus contains gene Glyma.01G046300 encoding a UDP-arabinose glycosyltransferase that adds second arabinose moiety at the C-3 position of soyasaponins (Takagi et al. 2018). Sg-5 locus contains gene Glyma.15G243300 encoding* the cytochrome P450 72A69 enzyme and is responsible for conversion of soyasapogenol B to soyasapogenol A (Sundaramoorthy et al. 2018). A mutant lacking sg-5 gene and has no group A-saponins has developed into variant ‘Tohoku 152’ (Sakai et al. 2002).

Recently, rare *Sg-6* saponin types were identified from wild soybean germplasms in Japan and South Korea (Krishnamurthy et al. 2014), of which there

are additional moieties to C-29 position and hydroxy group at C-22 position, creating new type of soyasapogenols, designated as soyasapogenol-H, soyasapogenol-I, and soyasapogenol-J. Based on type of sugar chain at C-29 position, soyasapogenol-H, soyasapogenol-I, and soyasapogenol-J have -CH_2OH, -COOH, and malonyl-CH2- moiety, respectively (Krishnamurthy et al. 2014).

2.3.2. Genetic diversity of saponin content and composition in soybean and wild soybean

Three major studies analyze saponin content and composition will be introduced here. A study conducted on 3,720 Korean wild soybean accessions discovered 7 common saponin allele combination (Panneerselvam et al. 2013). The 7 combinations are *Sg-1^a/sg-4/sg-6* (phenotype: Aa), *Sg-1^b/sg-4/sg-6* (Ab), *Sg-1^a/Sg-4/sg-6* (AaBc), *Sg-1^b/Sg-4/sg-6* (AbBc), *Sg-1^a/sg-4/Sg-6* (Aa+α), *Sg-1^a/Sg-4/Sg-6* (AaBc+α) and *Sg-1^b/Sg-4/Sg-6* (AbBc+α). Interestingly, the composition of allele combinations differs between regions. Aa phenotype is the most common in east region of Korea (50%), followed by AaBc (42%). From west region, the composition is reversed with AaBc phenotype is the most dominant (60-65%) followed by Aa (30%). The wild soybean originated from South region of Korea has similar composition with the west region, but the percentage of Aa is lower (4-16%), and the third genotype AaBc+α is 2 to 16% of total genotypes. Another study on soyasaponin diversity in the Chinese wild soybean germplasm was conducted on 3,795 accessions (Takahashi et al. 2017). These wild soybeans contain combination of several saponins, with Aa and Ae being two dominant group A-saponins. 78% and 78.9% of total samples have Aa and Ae saponins, respectively. Of DDMP saponins, almost all samples have αg and βg saponins (99% and 100%, respectively). Five accessions carrying rare A0-αg type similar to Kinusayaka, and three accessions carrying both A0-αg and A0-αa saponins. The genetic basis of these components is unknown, but 7 accessions were collected from northeastern China and 1 accession from Henan Province, central China. Interestingly, an accession No.3137 showed Bd accumulation. Bd is derived from soyasapogenol E, which is thought as degradation product of DDMP saponin. Takahashi et al. (2017) claimed that Bd accumulation observed from No. 3137 was inherited through three generations. The last study came from the screening of high-density soybean mutant library (Panneerselvam et al. 2019). The mutant library was developed by treating cultivar 'Enrei' with ethyl methanosulfonate to induce genetic mutations. Thirty-five mutants having unusual saponin phenotypes were identified. Among these, six mutants deficient in total saponins, 11 mutants deficient in DDMP saponins, 3 mutants accumulating only group A saponins, and 5 mutants showing A0-type saponins will be useful for further study of saponin biosynthesis pathway (Panneerselvam et al. 2019). Interestingly, mutants lacking total saponins did not show any abnormalities in the progenies. Mutants lacking in total saponins will be also useful as donor parent in breeding new soybean cultivars.

2.3.3. Saponin content as response to environment

There are only few reports on growing environment effect on saponin content in soybean seeds (Tsukamoto et al. 1995, Seguin et al. 2014). Tsukamoto et al. (1995) particularly investigated the effect of temperature during seed development to DDMP saponin content in three soybean cultivars (Koganedaizu, Suzuyutaka, and Higomusume). There were tendency of higher αg, βg-saponin in higher temperature regime, and lower βa-saponin in three cultivars, although the result was not statistically significant. There was no difference in total DDMP saponin content between high and low temperature. Similar trend was obtained from study in Canada (Seguin et al. 2014). Seguin et al. (2014) investigated the effect of seeding dates and high temperature on soyasaponin B content of 20 early maturing soybean genotypes grown at four locations in Canada. Early seeding date was associated with higher soyasaponin B content (Seguin et al. 2014). In Canada, early seeding date is usually associated with higher temperature during seed filling. Interestingly, test on high temperature during seed filling using two soybean genotypes OAC Champion and AC Proteina showed that the effect depends on soybean genotypes. Exposure to high temperature (33/25

°C, day/night temperatures) exclusively during seed filling, total soyasaponin B content in AC Proteina increased by 28% compared to that of the control condition (23/15 °C), but not in OAC Champion. However, high temperature during all growing stages decreased the total soyasaponin B content in both cultivars. The decrease may be resulted from the heat stress experienced by the plants; therefore, this factor should be considered when growing soybean for high soyasaponin B content purpose in the tropical regions.

Another interesting report was the investigation on the effect of artificially damaged goldenrod volatiles (ADGV) to saponin content in black soybean seeds (Shiojiri et al. 2020). The idea came from plant-plant communication concept; when a plant is damaged by insects or herbivores, it will release volatiles that are received by other plants. When the other plants received such volatiles, they also release volatiles as defense response. In the study, two soybean cultivars: black soybean cultivar 'Hyokei Kuro-3' and yellow soybean cultivar 'Yumesayou' were grown together with goldenrod (*Solidago altissima*). The Ab, βa, and Bc saponin content of 'Hyokei Kuro-3' increased when exposed to ADGV, compared to non-exposed plants. For 'Yumesayou', the increase was observed for Af, Ab, αg, βg, γg, compared to non-exposed plants. It is important to note that the ADGV effect might differ depending on soybean genotypes.

III. UTILIZATION OF THE RESOURCES INTO SOYBEAN BREEDING

Most of accessions having high nutraceutical content here are landraces or wild soybean. Thus, these traits can be introduced to current leading cultivars through breeding. For efficient breeding, it is important to introgress only the genetic variations associated with high nutritional content. To do this, we need to elucidate the genetic variations associated with the beneficial traits and develop genetic markers. The availability of whole genome sequence information of soybean (Schmutz et al. 2010) and wild soybean (Xie et al. 2019) accelerates characterization of genetic variations exist in soybean and wild soybean germplasm. It is also important to further study the genotype × environment effect to the traits. Most of examples shown here based on studies in subtropics, where the daylength change greatly throughout the growing period and temperature is much lower compared to tropical countries such as Indonesia. Particularly, temperature effect should be investigated in case of α-tocopherol. Study cases shown here used temperature maximum at 28°C. Temperature higher than that may increase α-tocopherol content, but it also induces heat stress in plants. Lastly, as breeders may want to stack several beneficial traits into one cultivar, it is important to investigate whether it is possible to increase the content of α-tocopherol, lutein or DDMP saponin without decreasing the content of oil, protein, or other carotenoids that share same biosynthesis pathway.

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PRODUCT DEVELOPMENT OF LOW SUGAR READY-TO-DRINK (RTD) SOY JELLY DRINK

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ABSTRACT

Soymilk was processed into a nutritious jelly drink product with low sugar level to overcome the problems of current comercial jelly drink. The optimum concentration of iota-carrageenan (0.1-0.3%) was determined based on sensory evaluation and texture analysis. The preferable sweetness level (2.5-7.5%) was determined based on sensory evaluation. The optimum combination of stevia and sucrose (g/l, 0.211:45, 0.27:45, 0.579:45, 0:75) was determined based on sensory evaluation. The effect of the heating time (30-60 min) and the effect of storage temperature (4 and 25 ^{o}C) were evaluated by using product characteristic and microbial activity. Results presented in this work suggest that iota-carrageenan was more suitable to be used to produce RTD soy jelly drink than kappa-carrageenan, with 2% addition as the optimum concentration. The combination of 4.5% sucrose and 0.0579% stevia could produce less sugar RTD soy jelly drink with good sensory acceptance. Standard heating process (30 minutes) could hold the microbial activity below the maximum limit up to 21 day at 4 °C storage condition, but only has 6 hours at 25 °C storage condition. The additional heating time could hold up to about 11 hours. The heating process could increase the texture value of product, and the texture characteristic product would be changing during storage at 4 ^{o}C.

Keywords: *Carrageenan, jelly drink, ready-to-drink, soybean, stevia*

ABSTRAK

Susu kedelai diproses untuk menjadi minuman jelly yang bernutrisi dan rendah gula untuk menyelesaikan masalah produk-produk komersial yang sudah ada. Konsentrasi iota-karagenan optimal (0,1-0,3%) didapatkan berdasarkan analisis evaluasi sensori dan analisis tekstur. Tingkat rasa manis yang disukai (2,5-7,5%) ditetapkan berdasarkan evaluasi sensori. Kombinasi optimal penambahan stevia:sukrosa (g/l, 0,211:45, 0,27:45, 0,579:45, 0:75) didapatkan berdasarkan analisis evaluasi sensori. Efek waktu pemanasan (30-60 menit) dan suhu penyimpanan (4 dan 25 ^{o}C) dievaluasi berdasarkan parameter karakteristik produk dan aktivitas mikroba. Hasil penelitian ini menunjukan bahwa iota-karagenan lebih cocok digunakan untuk produksi RTD soy jelly drink dibandingkan dengan kappa-karagenan, dengan konsentrasi optimal adalah 2%. Kombinasi 4,5% sukrosa dan 0,0579% stevia dapat menghasilkan RTD soy jelly drink yang rendah gula namun tetap memiliki penerimaan rasa dan penerimaan umum yang baik. Pemanasan standar (30 menit) mampu menahan nilai aktivitas mikroba tetap dibawah batas maksimal sampai 21 hari pada penyimpanan 4 °C. namun pada penyimpanan 25 °C hanya selama 6 jam. Waktu pemanasan tambahan mampu menahan selama sekitar 11 jam. Proses pemanasan dapat menyebabkan peningkatan nilai tekstur produk, dan karakteristik tekstur produk juga dapat berubah bila produk disimpan di suhu 4 ^{o}C.

Kata kunci: *Jelly drink, karagenan, kedelai, ready-to-drink, stevia*

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INTRODUCTION

Jelly drink is a ready to drink beverage that was firstly created as a snack to delay hunger that contains water, sweeteners, carrageenan (that creates jelly texture), artificial flavoring, sweetener, and coloring that create an end product with some unique characteristics such as chewy and sippable by straw (Agustin and Putri, 2014; Nuraeni *et al.*, 2019). However, this product is lacking nutritional value.

People nowadays are getting busier with their work and in need of something practical yet nutritious to temporarily replace their meal with something that is able to delay hunger without disturbing their work time. People also tend to be concerned about the sugar level contained in a product (Sardarodiyan and Hakimzadeh, 2016). By developing the common jelly drink to be more nutritious and less sugar content but still practical, it will surely overcome those problems.

Soy milk extracted from soya bean or *Glycine max L.* is known to be good for human's general nutrition as the protein source (Shurtleff and Aoyagi, 2016). Many studies have reported the beneficial impacts of the addition of soya bean's derivatives to other food products, such as reducing the risk of coronary heart disease and treatment for iron deficiency. The nutritional content that has the role on the beneficial of soymilk are iron, protein, and amino acids (Baba *et al*., 2005; Hugelshofer, 1986; Setyaningrum *et al*., 2017). Regarding that nutritional content, soymilk could be processed into a nutritious jelly drink product.

The combination of sugar with stevia has a potential to produce soy milk jelly drink products with less sugar content but still high in sensory acceptance (Gasmalla *et al*., 2014; Saniah and Samsiah, 2012; Takaichi and Hatai, 1998). Formulation the proper combination ratio of sugar with stevia is important to be known to achieve that condition.

To make a jelly drink product, carrageenan can be used because of its ability as a gel-forming agent (Kaya *et al*., 2015). Jelly drink texture is different from normal jelly products because it should be firm enough to form a jelly but soft enough to be sipped using straw with a little amount of syneresis presence (Hartati and Djauhari, 2017). It is important to know the optimum concentration of carrageenan addition to produce soy milk jelly drink products with good texture characteristics.

Microbes tend to grow rapidly in soybean-based products, and soy milk jelly drink has a similar problem. Heating treatment could kill bacteria and prolong the shelf-life of a soy milk product (Kohli *et al*., 2017). Heating process also takes place in the production of soy milk jelly drink to activate the gelling properties of carrageenan, and producing jelly texture. The observation of the effect of the heating process to the texture characteristic, microbial number, and stability during storage of RTD soy jelly drink product is important.

In this context, the objectives of this work were to determine the optimum concentration of carrageenan addition, the optimum combination ratio of sugar with stevia, and to evaluate the effect of heating process to the texture characteristic, microbial activity, and stability during storage. The parameters used were sippable and microscopic observations. The optimum concentration of iota carrageenan was obtained from analysis of sensory evaluation and texture analysis parameters. The preferable sweetness level of RTD soy jelly drink was determined from analysis of sensory evaluation parameter. The optimum combination of stevia and sucrose to produce less sugar-RTD soy jelly drink was obtained from the analysis of sensory evaluation parameters.

MATERIALS AND METHOD

1. Materials

Materials to produce soy milk were soybean (Cap BOLAM), Baking Soda (NaHCO3, Koepoe Koepoe), and mineral water. While the materials to produce low sugar Ready to Drink soy jelly drink were soy milk, sucrose (Gulaku), stevia (Steviol), iota-carrageenan (technical grade), kappa-carrageenan (technical grade), and aluminum laminated pouch.

2. Method

Soy milk production

The selected soybeans were soaked in NaHCO3 solution (0.5% w/w) for 8 h minimum in room temperature. The soaked soybeans were drained and washed using clean water. The soybeans skins were separated from its kernel. The soybeans kernels were collected and blanched in boiling water for 30 min. One hundred g of blanched soybeans were grinded with 600 g of hot water (80-90 °C) for 2 min at high speed. The grinded soy milk slurry was filtered through a strainer and clean kitchen towel (Nirmagustina and Rani, 2013).

Jelly drink production and preliminary analytical stage

Soy milk, carrageenan, sugar, and stevia were mixed and heated at 90 °C for 30 min. This process also aimed to be the pasteurization process of the product. Specifically to evaluate the effect of the heating process, 2 additional heating times were conducted (15 and 30 min). After the product was heated, the product was poured into cups (for analysis) or packed and sealed into pouches directly after to prevent microbial contamination. The products were chilled in the refrigerator for 4-6 h minimum to let the gelling process complete. At the preliminary analytical stage, the production used two types of carrageenan (iota-carrageenan and kappa-carrageenan), in four concentrations of carrageenan addition (0.3, 0.4, 0.5, 0.6 % w/v), and used 5% of sugar concentration. Products from all combinations were tested for sippable test and microscopic observation to determine the carrageenan type and concentration used for further analytical stages.

Sippable test

The products from two types of carrageenan in preliminary analytical stages were tested by using the straw of commercial jelly drink. The samples were sucked through the straw to check whether the samples were able to be sipped or not.

Microscopic observation

The product from two types of carrageenan were observed by using stereo microscope with objective lens magnification: 4.5X, and ocular lens magnification: 16X. The analysis was used to determine the suitable carrageenan type to be used for RTD soy jelly drink production.

Texture analysis

Three different iota-carrageenan concentrations (0.1%, 0.2%, 0.3%) on day of production (day 0 at 4 °C) were measured by Penetrometer and statistically analyzed with ANOVA. The result was used to determine the Optimum Carrageenan Concentration.

Texture of chosen carrageenan concentration was analyzed by Penetrometer. The analysis was done after the heating process, and during 3 weeks storage, at 4 °C and 25 °C of storage condition. Then the result was statistically analyzed with non-parametric analysis. The analysis was done to evaluate the effect of the heating process to the texture characteristic, microbial activity, and stability during storage.

Sensory evaluation

The hedonic test was used to determine the optimum concentration of carrageenan addition (sample: 0.1%, 0.2%, 0.3%), evaluation of preferred sweetness level of RTD soy jelly drink (sample: 2.5%, 5%, 7.5%), and the optimum combination ratio of sugar with stevia (stevia:sucrose, g/l, 0.211:45, 0.27:45, 0.579:45, 0:75). The panelists involved in this work were 30 students and laboratory staff from Swiss German University Faculty of Life Science and Technology that were familiar with commercialized jelly drink products. Sensory evaluations using Hedonic tests were statistically analyzed with Friedmann's test and Wilcoxon test with 0.05 as the probability level based on overall likeness of the product using 9-points hedonic test where 1 represents "like extremely" and 9 represents "dislike extremely".

PH and brix value

The pH analysis was done by Digital pH meter and Brix value analysis was done by Refractometer. The analysis was applied to evaluate the stability of the product during 3 weeks storage time, at 4 °C and 25 °C of storage condition.

Microbial activity analysis (SNI 2897:2008)

The microbial activity that was represented by Total Plate Count (TPC) Analysis, and compared to Indonesia National Standard of TPC parameter for jelly drink (104 colony/g). Media used for Total Plate Count Analysis was Plate Count Agar using the Pour Plate method. 25 g of sample was diluted to 225 ml 0.1% Buffered Peptone Water (BPW) and homogenized. Then 1 ml of the solution was diluted into 9 ml of BPW as dilution factor 10-1. The diluted sample was again diluted until dilution factor 10-5 was obtained. 1 ml sample from each dilution was poured into a sterilized petri dish. About 12-15 ml of sterilized Plate Count Agar (PCA) was poured into each petri dish and 8-figured motion was done to homogenize the sample.

After the agar was set, the petri dish was turned upside down, sealed with Parafilm M and incubated in 37±1°C for 48 h. Colonies of microbes from range 25–250 were counted as colony form units (CFU)/ml, and converted to colony/g. The microbial analysis was applied to evaluate the effect of heating process and storage.

RESULTS AND DISCUSSION

Preliminary analytical stage

The result shown on Table 1, that the texture characteristic of kappa-carrageenan product at all concentrations caused the product could not be sipped. For the iota-carrageenan product, 0.3% of iota-carrageenan concentration was the only one that sippable. It means the maximum concentration of iota-carrageenan used for the further analysis was 0.3%. Jelly drink production from other raw materials, such as *Averrhoa blimbi* L. (Agustin and Putri, 2014) and soursop fruit (Gani *et al*.) could produce good texture characteristics by using both kappa or iota-carrageenan.

Table 1. Sipping Capaability Evaluation of Soy Jelly Drink Using Iota- and Kappa-Carrageenan in Several Concentration

Concentration	Iota-Carrageenan	Kappa-Carrageenan	Conclusion
0.3%	Able to be sipped	Not able to be sipped	maximum concentration 0.3%, range: 0.1%, 0.2%, 0.3%
0.4%	Not able to be sipped	Not able to be sipped	
0.5%	Not able to be sipped	Not able to be sipped	
0.6%	Not able to be sipped	Not able to be sipped	

Regarding its molecular structure in the normal condition, gel characteristic of iota-carrageenan is less-rigid texture than kappa-carrageenan (Median-Torres, 2003). Mineral content in the food added with carrageenan could give effect to the gel characteristic of carrageenan.

The potassium ions in soy milk would interact with kappa-carrageenan and produce firmer texture that was hard to sipped. The calcium ions in soymilk could interact with iota-carrageenan and produce soft elastic gel that is sippable (Popescu *et al*., 2007).

Iota type carrageenan was chosen because it was jelly-like, sippable by straw and there was a presence of syneresis. While the kappa-carrageenan was sticky, too hard to sip, and starchy. The texture

appearance from microscopic observation can be seen on Figure 1.

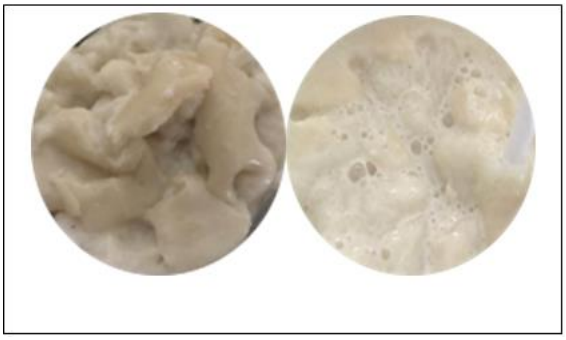

Figure 1. Microscopic visual character of soy jelly drink using (a) kappa-carrageenan, (b) iota-carrageenan

Determination of the optimum iota-carrageenan concentration

Table 2 shows that the increase of Iota-carrageenan Concentration could increase the texture value of products exponentially (R2 = 0.9922). The texture value would be highly increasing at the iota-carrageenan concentration more than 13%, which led to lower sippability of product. The data also shows that the maximum texture value for the sippable product is 15.00 ± 0.37 N. Iota-carrageenan tends to set after the heating process at 40 - 70 °C, the gel-strength ability of the carrageenan increases as the heating process takes place (Thomas, 1997).

The sensory hedonic test was observed only based on the texture profile of the product. Table 2 also shows that 0.2 % of iota-carrageenan is highly preferred by the consumers as jelly drink products much higher than 0.1 and 0.3 % carrageenan concentrations. The product with 0.3 % iota-carrageenan was too firm and hard to be sipped, though still sippable. The product with 0.1 % iota-carrageenan was too thin in texture, and it was not firm enough to be categorized as a jelly drink.

It can be concluded that RTD soy jelly drink product with texture value 7.0 ± 0.35 N was highly accepted by the consumers because of its characteristic that was solid enough yet still be able to be sipped using a straw. Those texture characteristics could be achieved by the addition of 0.2 % iota-carrageenan.

The optimum combination ratio of sugar with stevia

According to BPOM regulation (BPOM No. 13:2016 – "Pengawasan Klaim Pada Label dan Iklan Pangan Olahan"), a product can be claimed as "Less Sugar Product" if the product consists of less than 2.5 % sugar in liquid form or less than 5 % in solid form. While the jelly drink is a semi-solid form product, it should contain less than 5 %

of sugar to be claimed as a less sugar product (BPOM, 2016).

Table 2. Firmness value and hedonic score for texture of soy jelly drink using iota-carrageenan with concentration

Iota-carrageenan (%)	Firmness value (N)	Hedonic score for texture attribute
0.1	4.0 ± 0.32^{a}	4.77 ± 2.19^{a}
0.2	7.0 ± 0.35^{b}	7.30 ± 1.39^{b}
0.3	15.0 ± 0.37^{c}	4.83 ± 2.41^{c}

Table 3 shows that the concentration of sucrose 5.0 and 7.5 % gave the preferable sweet taste in the same level, but 2.0 % sucrose gave an un-preferable sweet taste. Each concentration of sucrose also gave different texture to the product as answered by 30 out of 30 panelists stated that

higher concentration of sucrose gave firmer texture. Since the sweetness profile of 5.0 and 7.5 % of sucrose were similar, all panelists preferred to consume the product with 7.5% sucrose. Therefore, 7.5 % sucrose concentration was chosen as the most desired RTD soy jelly drink sweetness profile in this stage, and chosen as the sweetness level target on the optimization of combination ratio of sugar with stevia.

Table 3. Hedonic score for taste attribute of soy jelly drink using several concentration of sucrose

Sucrose concentration (%)	Hedonic score for taste attribute
2.5	4.43 ± 2.06^{a}
5.0	6.51 ± 1.87^{b}
7.5	7.11 ± 2.22^{b}

Since 5 % is the limitation of less sugar product, 4.5 % of sucrose was used as the maximum level of sucrose concentration in sweetener combination with stevia. The three combinations of stevia and sucrose (g/l) respectively were 0.211:45 (Gasmalla, Yang and Hua, 2014), 0.579:45 (Saniah and Samsiah, 2012), 0.27:45 (Takaichi and Hatai, 1998), where all of them were categorized a less-sugar product.

Table 4 shows that the combination ratio stevia:sucrose 0.579:45 (g/l) and standard had the similar taste and overall acceptance, even though the aroma and texture were different. This result also shows that 3% of sucrose in the product could be substituted by 0.0579% of stevia addition. Since the amount of sugar used in the final product is less than 5%, the product could be claimed as less sugar product. Based on data, the desired composition to RTD soy jelly drink was 0.2 % iota-carrageenan with combination of 4.5 % sucrose and 0.0579 % stevia.

Table 4. Hedonic evaluation of soy jelly drink using several combination ratio of stevia and sucrose

Sensory attribute evaluated	Hedonic score in soy jellydrink that is using stevia and sucrose with combination ratio (g/l)			
	0.211 : 45	**0.27 : 45**	**0.579 : 45**	**0 : 75 (standard)**
Aroma	6.73 ± 1.34^{a}	6.83 ± 1.33^{b}	6.90 ± 1.51^{c}	6.90 ± 1.45^{d}
Taste	5.93 ± 1.48^{a}	5.97 ± 1.48^{a}	7.20 ± 1.45^{b}	7.43 ± 1.60^{b}
Texture	6.77 ± 1.79^{a}	6.63 ± 1.82^{b}	6.90 ± 1.66^{c}	6.90 ± 1.96^{d}
Overall	6.13 ± 1.67^{a}	5.77 ± 1.84^{a}	6.87 ± 1.70^{b}	7.23 ± 1.73^{b}

Effect of heating process

Table 5 shows that the standard heating time (30 min) was able to kill the microbial activity in the products. The maximum of microbial contamination based on SNI about "Jelly Agar" products is 104 colony/g (Standar Nasional Indonesia, 1994). From the result, it also could be concluded that heating time on 30 min created an edible and safe-to-consume soy milk jelly drink product. Table 5 also shows that the standard

heating time could produce RTD soy jelly drink with shelf life at 4 °C storage temperature up to 21 days. As on day 28, the presence of moulds were detected.

Table 5. Total microbial count (colony/g) of soy jelly drink with 30, 45, and 60 min heating treatment during 28 days storage at 4 °C

Storage time (day)	Total microbial count of soy jellly drink that receive …… heating treatment		
	30 min	45 min	60 min
0	<900	<900	<900
7	<900	<900	<900
14	<900	<900	<900
21	<900	<900	<900
28	TNTC**	-	-

NOTE:

900 -> no microbes appeared; Accept Standard

** Mould appeared

- no data

Figure 2 shows that even though the heating process could produce RTD soy jelly drink with no microbes appearing on microbial activity tests, the microbes would be growing rapidly if the product was stored at 25 °C. The shelf life was only 6 h. The additional 15 min of heating time could prolong the shelf life up to about 11 h, but the more heating time addition (30 min) could not give an additional impact to the shelf life.A study showed preservation by high-pressure processing treatment was more effective to kill aerobic and anaerobic bacteria in soy milk product (Kohli *et al*., 2017). It might be caused by the insufficient heat temperature treated to the product so the spores of microbes from the original sample or the packaging itself tend to grow rapidly at 25 °C storage temperature. Another method of the production process should be considered to get a longer shelf life of RTD soy jelly drink products that are able to be stored at 25 °C.

Table 6 shows that the increase of heating time could increase the texture value of the product, while the result of pH and Brix value were not significantly changed. The strength of carrageenan gel is increasing as the increase of temperature, and will be decreasing when the temperature of heating

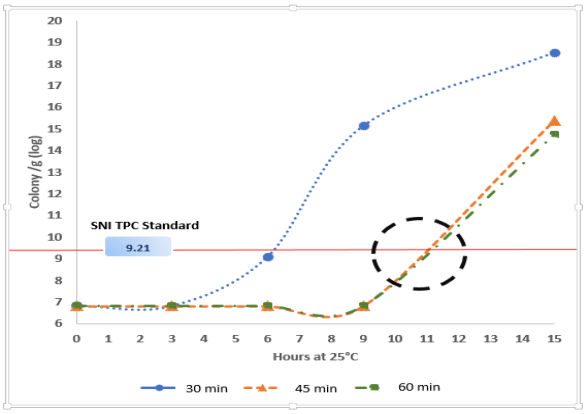

Figure 2. Total microbial count (log colony/g) of soy jelly drink with 30, 45, and 60 min heating treatment during 16 h Storage at 25 °C

has reached the temperature around 110 °C (Tziboula and Horne, 2000). The heat will alter the networks chain of gel, and at the certain heating condition the gel strength will be lost due to the loss of networks chain of gel (Ainsworth and Blanshard, 1879) . In this work, the temperature used for production was 90 °C, and it was still under 100 °C as the maximum temperature of carrageenan heat treatment. This heating condition still gave the positive impact to the gel strength of the product. The increase of texture value has a

potential to change the sensory acceptance and sippability of the product. Unfortunately, this work has no data regarding the sensory evaluation of products on different heating times.

Table 6. Firmness, acidity, total solid, and microbial count of soy jelly drink with 30, 45, and 60 min heating treatment

Heating Time (min)	Firmness Value (N)	Acidity (pH value)	Total Solid (% Brix)	Total microbial count (colony/g)
30 (standard)	8.0 ± 0.69[a]	7.3 ± 0.05[a]	3.5 ± 0.00	<900
45	9.8 ± 0.39[b]	7.0 ± 0.11[b]	3.5 ± 0.00	<900
60	12.1 ± 0.65[c]	7.1 ± 0.121[c]	3.5 ± 0.00	<900

Figure 3 A and B show that the texture characteristics of products with standard heating processes during storage at 4 °C were changing. Based on data, the texture of products was still under the maximum acceptability limit (15.0 N) until day 21, even though the texture was becoming harder. This changing characteristic was caused by the condition that water inside gel form-carrageenan was moved out from the solid matrix, producing a harder gel form and higher syneresis value of product.

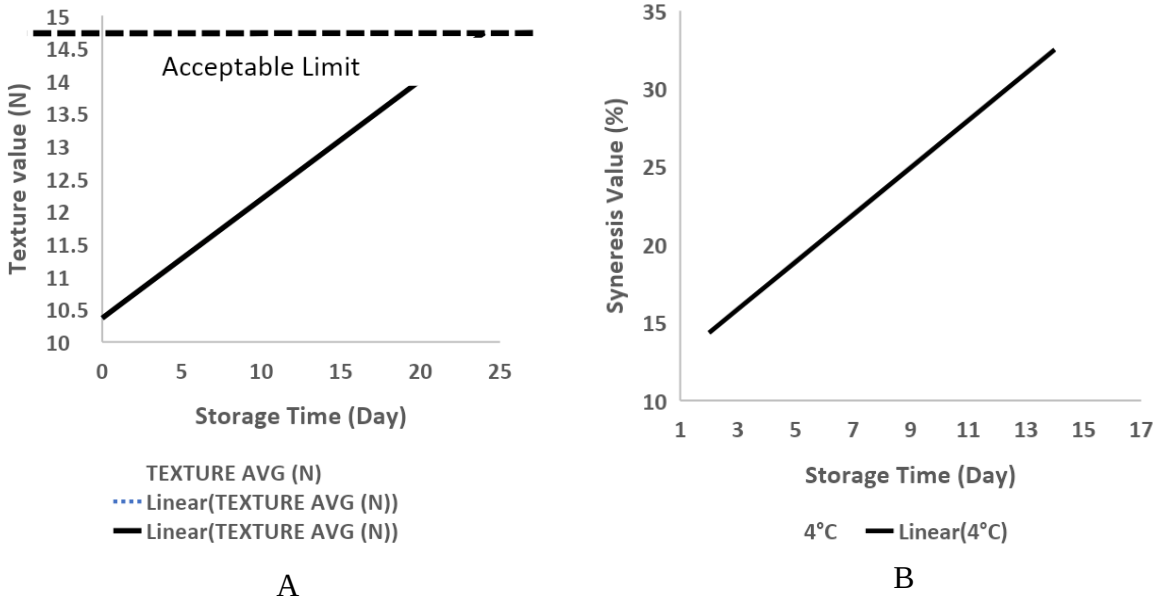

Figure 3. Trend lines of (A) texture stability and (B) syneresis; during storage at 4 °C

CONCLUSION

Results presented in this work suggest that iota-carrageenan was more suitable to be used to produce RTD soy jelly drink than kappa-carrageenan, with 2 % addition as the optimum concentration. The combination of 4.5 % sucrose and 0.0579 % stevia could produce less sugar RTD soy jelly drink with good sensory acceptance. Standard heating process could hold the microbial activity below the maximum limit up to 21 day at 4 °C storage condition, but only has 6 h at 25 °C

storage condition. Additional heating time could hold the microbial activity below the maximum limit only up to about 11 h at 25 °C storage condition. The heating process could increase the texture value of the product, and the texture characteristic product would be changing during storage at 4 °C.

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ENCAPSULATION OF CANDLENUT OIL BY FREEZE-DRYING METHOD

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ABSTRACT

Candlenut oil is a potential source of omega fatty acids that can be used as a food supplement or nutrient for food fortification. It contains high amount of omega fatty acids and also available in high quantity, especially in Indonesia. However, due to its off-odor and its thermal-sensitivity which makes candlenut oil prone to oxidation, the application into food products still needs more improvement. Encapsulation is one of the techniques that is used to protect the candlenut oil from oxidation. This research aimed to find the best encapsulating agent to protect the omega content from candlenut oil against oxidation through freeze-drying method. Factors such as encapsulating agent (whey protein isolate, sodium caseinate, β-cyclodextrin, gum Arabic) and ratios of encapsulating agent to oil (3:2, 1:1 and 2:3) were investigated to find out the most appropriate microcapsule and conditions to ensure there will be no change of the candlenut oil characteristics. Moisture content, microencapsulation efficiency (ME), and peroxide value (PV) were analyzed as the product parameter. The highest encapsulation efficiency was obtained by using sodium caseinate (43.22 ± 0.9 %) with the ratio of encapsulating agent-oil was 3:2. The second stage of candlenut oil encapsulation was carried out to improve the efficiency of microcapsule, and the result showed that the efficiency of encapsulated oil with sodium caseinate as encapsulating agent was increased to 64.86%.

Keywords: *Candlenut oil, encapsulation, freeze-drying, omega fatty acid, oxidative stability*

ABSTRAK

Minyak kemiri merupakan sumber asam lemak omega yang potensial untuk digunakan sebagai suplemen atau nutrisi tambahan pada fortifikasi makanan. Minyak kemiri memiliki kandungan asam lemak omega yang tinggi serta kelimpahan yang cukup besar di Indonesia. Namun, minyak kemiri yang memiliki sifat sensitif terhadap panas menjadi mudah untuk teroksidasi. Hal ini menyebabkan diperlukannya pengembangan produk sebelum mengaplikasikan minyak kemiri tersebut ke dalam produk makanan. Enkapsulasi merupakan salah satu teknik yang digunakan untuk melindungi minyak kemiri dari reaksi oksidasi. Penelitian ini bertujuan untuk menentukan bahan penyalut (*encapsulating agent*) terbaik untuk melindungi kandungan asam lemak omega dari minyak kemiri dengan menggunakan metode *freeze drying*. Variasi bahan penyalut (*whey protein isolate, sodium caseinate, β-cyclodextrin, gum Arabic*) dan rasio bahan penyalut terhadap minyak (3:2, 1:1 dan 2:3) dilakukan untuk mendapatkan mikrokapsul minyak kemiri dengan kualitas terbaik. Kadar air, efisiensi mikroenkapsulasi dan bilangan peroksida dianalisis sebagai parameter produk hasil enkapsulasi. Efisiensi enkapsulasi terbaik didapatkan dengan dengan menggunakan *sodium caseinate* (43,22 ± 0,9 %) dan rasio bahan penyalut terhadap minyak 3:2. Optimasi enkapsulasi minyak kemiri pada tahap ke dua menunjukkan peningkatan efiensi mikroenkapsulasi minyak kemiri dengan *sodium caseinate* sebagai bahan penyalut hingga 64,86%.

Kata kunci: *Asam lemak omega, enkapsulasi, freeze-drying, minyak kemiri, stabilitas oksidasi*

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INTRODUCTION

Candlenut (*Aleurites moluccanus*) is widely known as culinary spices and herbs which can be easily found in Indonesia and South East Asia. Currently, candlenut is only being utilized as a spice, an ingredient for cooking, and for hair growth. In fact, the oil content from cold-press candlenut is relatively high, and it contains mostly polyunsaturated fatty acids (PUFAs) (Wijaya, 2008). Ander, Dupasquier, Prociuk, & Pierce (2003) stated that polyunsaturated fatty acids have more than one double bond in their backbone, hence they can provide health benefits. Moreover, omega-3 fatty acid can help to regulate the lipid circulation in the body, by increasing the high-density lipoprotein (HDL) and lowering the low-density lipoprotein (LDL) cholesterol and reducing atherosclerotic development (Ander, Dupasquier, Prociuk, & Pierce, 2003). PUFAs are essential oil, means that they are not produced in the human body.

Due to the fact that omega fatty acids can cause numerous health issues, there is an increasing interest from Indonesian consumers in foods which have been enriched or fortified with omega fatty acid. Omega fatty acid sources in Indonesia are still limited and usually they are relatively expensive. In this case, candlenut oil can be an alternative source since it contains high PUFAs and available abundantly, especially in Indonesia. However, due to its off-odor and its thermal-sensitivity which makes candlenut oil prone to oxidation, the application into food product still needs more improvement. Recent studies about food fortification with omega-3 from candlenut oil showed that, when the candlenut oil is added to the product, the omega fatty acid content is decreased (Kamil, 2016). The omega fatty acid in candlenut oil is also susceptible to promote oxidative degradation in oil when the product is exposed to oxygen and heat (Tjhin, 2014).

Microencapsulation is one of the techniques that is used to protect candlenut oil from oxidation (Kaushik, Dowling, Barrow, & Adhikari, 2015). In microencapsulation process, droplets or tiny particles are protected or surrounded by wall matrix or encapsulating agent, thus forming very small capsules (Wang, Tian, & Chen, 2011; Raybaudi-Massilia & Mosqueda-Melgar, 2012. Selecting the encapsulating agent is important in microencapsulation process. The encapsulating agent must fulfill certain criteria. They must have high stability, high water solubility, emulsifying properties, a tendency to form a network during drying, and low viscosity for spray-drying (Calvo, Castaño, Lozano, & González-Gómez, 2012). According to Calvo et al. (2012), protein-based encapsulating agent was more effective in preserving the quality of microencapsulated extra-virgin olive oil. In the previous study about the encapsulation of candlenut oil with hydroxypropyl methylcellulose (HPMC), gum Arabic (GA), β-cyclodextrin (β-CD), and alginate by using spray-drying method, the best performance in microencapsulation efficiency was showed by β-cyclodextrin. However, gum arabic showed the best performance in terms of peroxide value (Verianto, 2018). It still cannot be concluded what is the best encapsulating agent for encapsulating candlenut oil.

In this research, sodium-caseinate (SC) and whey protein isolate (WPI) will be used as the alternative encapsulating agents with freeze-drying method. The result of this alternative encapsulating agent will be compared to the previous encapsulating agent used for candlenut oil, which are β-cyclodextrin and gum arabic, to know which type of encapsulating agent and formulation that could optimize the encapsulation of candlenut oil.

MATERIALS AND METHOD

Materials and equipments

Candlenut kernels were obtained from a local market in Tangerang. β- cyclodextrin, gum Arabic, sodium caseinate and whey protein isolate, maltodextrin, and lecithin were obtained from Richest Group Ltd., China. For analysis of microencapsulation, n-hexane for analysis, chloroform for analysis, acetic acid glacial 100%, saturated potassium iodide for analysis, starch, and sodium thiosulfate for analysis were obtained from Merck.

The equipment used for candlenut oil extraction was customized cold press expeller. For the candlenut oil microencapsulation, homogenizer (IKA Labortechnik RW 20N) was used for homogenizing the emulsion. Freeze dryer (Operon FDB – 5502) was used to dry the samples. Moisture content analyzer (Satorius MA 35) was used to determine the moisture content. Rotary evaporator (IKA HB 10) was used to evaporate n-hexane in microencapsulation efficiency analysis. The omega fatty acid content was analyzed at PT Saraswanti Indo Genetech by using gas chromatography with a flame ionization detector (GC-FID). The GC analysis was carried out by using a polyethylene glycol (PEG) capillary column (30m x 0.25mm ID x 0.25µm film thickness). Nitrogen was used as the carrier gas, with a flow rate of 1 mL/min. The injector and detector temperatures were set at 260 ^{o}C. The oven temperature was programmed from 120 ^{o}C to 240 ^{o}C at a rate of 4 ^{o}C/min.

RESEARCH METHODOLOGY

Extraction of candlenut oil.

Candlenut kernels were extracted by using cold-pressed expeller and then centrifuged at 6000 rpm and 15 ^{o}C for 20 minutes to obtain pure candlenut oil. The omega content of the centrifuged candlenut oil was analyzed by using GC-MS. Emulsification of the candlenut oil using four different types of encapsulating agents (Sodium caseinate, Whey Protein Isolate, β-cyclodextrin and Gum Arabic) with maltodextrin as the secondary encapsulating agent were prepared with the ratio 3:2, 1:1 and 2:3 of encapsulating agent to candlenut oil. The limited amount of water was added into the mixture. Homogenization process was conducted at room temperature for 5 minutes. Emulsion was frozen prior drying process. The drying process was done for 48 hours at –60 ^{o}C. Microencapsulation efficiency, moisture content analysis, and peroxide value analysis were conducted to examine the most appropriate material for encapsulating candlenut oil.

Microencapsulation efficiency analysis.

Method for microencapsulation analysis is adapted from Calvo et al. (2012) with modification. Five grams of powder was mixed with 20 ml of hexane and then vortexed for 1 minute to extract free oil. The mixture was then filtered with a Whatman No. 41 filter paper. The washed powder on the filter paper was rinsed using 50 ml of distilled water. The hexane was evaporated using rotary evaporator at 60 ^{o}C and the internal oil was scaled. The oil was weighed and then the efficiency was measured. ME was calculated by using the following formula:

$$ME = \frac{Total\ Oil - Surface\ Oil}{Total\ Oil} X100\%$$

Peroxide value analysis.

Peroxide value was conducted by following the method from Toure (2007) with modification. Chloroform (6 mL) and glacial acetic acid (9 mL) were mixed together. Then, 1 gram of powder sample was dissolved in the solution. One mL of saturated solution of potassium iodide (KI) was added into the sample solution. The solution mixture was manually shaken for 30 seconds and stored in the dark for 30 minutes. Starch indicator solution (1%, 0.5 mL) was added into the mixture until the color of the solution changed from yellow to dark blue. Then 0.01 N of standardized sodium thiosulphate solution was used for titration against the sample solution until the the blue color almost disappeared. Blank titration was conducted under the same condition. To determine the POV value, the following equation was used:

$$POV\left(\frac{meq}{kg}\right) = \frac{Cx(V - V_0)x1000}{m}$$

Where,

C = concentration of sodium thiosulphate (mol/L)

V = volume of sodium thiosulphate for the sample titration (mL)

V_0 = volume of sodium thiosulphate for the blank titration (mL)

m = mass of candlenut oil (g)

Moisture content analysis.

The moisture content was measured by using moisture content analyzer Satorius MA35. Around 1 gram of powder sample was put in the analyzer for one hour at 105 °C. As a result of continuous heating, the moisture content would evaporate. The experiment was terminated once the sample mass had reached the constant value. For the moisture content after drying process, to minimize the time used, the moisture content was measured manually by using Memmert oven. The samples were weighed for about 1 gram and then heated at 105 ^{o}C for 24 hours, and then the weight was measured. The analysis stopped when constant weight was achieved.

RESULTS AND DISCUSSION

Candlenut oil extraction

The extraction of the oil from the candlenut kernels was carried out by using cold press expeller machine due to the sensitivity of the oil to heat. The friction between candlenut kernels and the expeller machine induced the increment of the temperature which leads to the degradation of omega content of the oil. Thus, the usage of cold press expeller becomes the critical factor to protect the oil from degradation. As much as 16 % of yield of candlenut oil was obtained from this extraction process.

The unsaturated fatty acids and omega content of the extracted candlenut oil were analyzed by using GC. The result of the analysis is shown in Table 1.

According to the GC data in Table 1, it is shown that oil obtained from candlenut is high in unsaturated fatty acid, which was 90.89%. The unsaturated fatty acid content obtained by cold-press extraction is higher than the previous study from Wijaya (2008), which was only 79.65% of unsaturated fatty acids. The difference in omega content might be caused by the different source of candlenut kernels. Extraction method also affects the omega content of the oil. Oil which was extracted using common extraction has lower omega content compared with oil which was extracted by using cold-press expeller. This is because heat degrade the omega content of the oil through thermal oxidation. Wijaya (2008) stated that the unsaturated fatty acid content of candlenut oil extracted by cold expeller was higher (79,65%) than normal expeller (71.12 %). Comparing with another unsaturated fatty acid oil, unsaturated content of candlenut oil is still higher than olive oil (about 77%) which was studied by Susilo (2012) and fish oil (about 75%) which was studied by Aditia *et al.,* (2014). Based on the data, the highest omega content contained in the candlenut oil is omega-6 fatty acids, followed by omega-3 and omega-9. In this research, it is proved that extraction by using cold-press expeller can maintain the high content of omega fatty acids in the oil.

Table 1. Omega fatty acid content of pure candlenut oil extract

Parameter	Result (%)
Unsaturated fats	90.89 ± 0.06
Omega 3 fatty acids	26.31 ± 0.06
Omega 6 fatty acids	40.10 ± 0.00
Omega 9 fatty acids	24.30 ± 0.00

Encapsulation of candlenut oil

The efficiency of microencapsulation was analyzed to determine the amount of surface oil presents on the particles' surface and degree in which the encapsulating agent can prevent external or internal extraction of the oil. According to the result in Table 2, the highest efficiency was obtained by using sodium caseinate as the encapsulating agent with the ratio 3:2 of encapsulating agent to oil. In this case, sodium caseinate has an emulsification property and amphiphilic character, where they possess both hydrophilic and lipophilic properties. Because of the emulsifying properties, the stability of the emulsion by using sodium caseinate was higher than the other encapsulating agent hence the result showed that the efficiency of encapsulated oil with sodium caseinate was the highest.

However, there was no significant difference between each encapsulating agent (P = 0.22) but there was a significant difference between each ratio (P = 0.0032). This indicates that the efficiency

was influenced by the formulation's ratio. When the amount of oil load was higher than the encapsulating agent, the solid particle was insufficient to give a full protection to the droplets of oil.

Table 2. Microencapsulation efficiency (me), peroxide value, and moisture content of the encapsulated candlenut oil

Encapsulating agent	Me (%)	Pov (meq/kg)	Moisture content (%)
B1	40.94 ± 2.0	1.99 ± 0.00	5.6 ± 0.35
B2	37.70 ± 1.6	1.49 ± 0.70	4.55 ± 0.82
B3	30.79 ± 4.8	2.73 ± 0.34	6.57 ± 0.37
W1	42.24 ± 0.5	0.99 ± 0.002	3.09 ± 0.54
W2	35.58 ± 1.8	1.24 ± 0.34	1.61 ± 0.09
W3	27.35 ± 6.1	2.97 ± 2.8	2.80 ± 0.55
S1	43.22 ± 0.9*	1.24 ± 0.35	2.07 ± 0.66
S2	36.22 ± 3.7	1.73 ± 0.35	2.42 ± 0.02
S3	28.40 ± 10.7	2.73 ± 0.34	2.45 ± 1.01
G1	38.37 ± 4.3	0.99 ± 0.0003	2.86 ± 0.62
G2	26.76 ± 13.0	1.24 ± 0.34	2.88 ± 0.38
G3	22.56 ± 8.8	1.74 ± 1.05	3.47 ± 2.40

B=β-cyclodextrin; W=whey protein isolate; S=sodium caseinate; G=gum Arabic. 1,2,3=formulation ratio, 1= 3:2 encapsulating agent to oil; 2= 1:1 encapsulating agent to oil; 3= 2:3 encapsulating agent to oil , * = highest microencapsulation efficiency.

The peroxide value of encapsulated candlenut oil was conducted to measure the oxidation occured in the candlenut oil. Unsaturated fatty acids are prone to oxidation because of the double-bond presence in their structure. The presence of heat and oxygen promotes the oxidation. When unsaturated fatty acids are in contact with oxygen or exposed to heat, hydroperoxide is formed. This analysis measures hydroperoxide formed during oxidation. It is expected that by using freeze-dry method, could minimize the peroxide number of the product. The result of encapsulated candlenut oil in peroxide value is showed in Table 2.

The lowest peroxide number was found by using whey protein isolate and gum Arabic as the encapsulating agent. This is probably because gum Arabic has an antioxidant activity to prevent the formation of hydroperoxide during oxidation of the oil due to the presence of hydroxyl group which can scavenge the free radicals (Chew, Tan, & Nyam, 2018). However, there was no significant difference between each encapsulating agent (P = 0.55), the ANOVA result showed that there was a significant difference between each ratio (P = 0.037).

The higher oil loading showed higher peroxide value. This result corresponded with the efficiency of the microencapsulation process. Since the surface oil was higher as the oil loading increase, the peroxide value was also increased. It is probably due to the oxidation of the un-encapsulated oil surface, thus it was not completely protected.

As can be seen in Table 2, the lowest moisture content after drying process through freeze-drying method was obtained by using whey protein isolate with ratio 1:1, where the highest moisture content was obtained by using β-cyclodextrin with the ratio 2:3. According to the data, the moisture content is affected by the ratio of the encapsulating agent to the oil. There was a significant difference between each encapsulating agent (P = 0.001). The post-hoc analysis showed that β-cyclodextrin was significantly different with whey protein isolate (P = 0.0017), sodium caseinate (P = 0.0013) and gum Arabic (P = 0.0049). The greater amount of solid particle in a system increases the volatile retention depending on the type of the solid and the type and amount of the volatile. It was suspected because the volatile compound was entrapped in the solid matrix system after the formation of the droplets. Higher solid content can reduce the circulatory system on the emulsion. This theory answers the result of β-cyclodextrin, as the higher amount of solid content has higher moisture content.

Screening of encapsulating agents for candlenut oil

Re-formulation of the total solid content and water used was conducted in order to achieve higher efficiency. At first, the utilization of water was limited in order to minimize the drying time, however, the limitation of water was responsible for the low efficiency of microencapsulation, besides the homogenization process. The screening of encapsulating agents for candlenut oil encapsulation was carried out by increasing the water content up to 50% and ratio encapsulating agent to oil 3:2. As a result, the efficiency of microencapsulated candlenut oil was increasing, as can be seen in Table 3.

Table 3. Microencapsulation efficiency (me), peroxide value, and mositure content of the encapsulated candlenut oil

Encapsulating agent	Me (%)	Pov (meq/kg)
B1	49.64	2.49 ± 0.50
W1	57.48	0.99 ± 0.00
S1	64.86	0.49 ± 0.76
G1	47.38	0.99 ± 1.40

By increasing the amount of water, the stability of the emulsion was increased and it was possibly because the size of oil droplets was also decreasing, so the encapsulating agent were more effectively in protecting the oil droplets. Based on the data, the highest microencapsulation efficiency was obtained by using sodium caseinate. Sodium caseinate is one of the best encapsulating agent for lipophilic compound because of its intrinsic stability to form protein-ligand complexes and casein micelles. In addition, the amphiphilic structure of caseins promote a rapid adhesion to the surface of oil droplets to form a thick layer that protects the newly formed droplets against flocculation and coalescence (Mujica-Álvarez, Gil-Castell, Barra, Ribes-Greus, Bustos, Faccini, & Matiacevich, 2020). The microencapsulation of the candlenut oil with sodium caseinate increased significantly from 43.22 to 64.86%. The lowest peroxide value of encapsulated candlenut oil also obtained by using sodium caseinate as encapsulating agent. The peroxide value decreased from 1.24 to 0.49 meq/kg.

To analyze the stability of the microcapsules, heating process at 170 ^{o}C was conducted for 1.5 hours for the new formulation of encapsulated oil and also the un-encapsulated candlenut oil under the same treatment. During the exposure to the heat, it is noticeably that oxidation will occur. However, to evaluate the fluctuation of the peroxide value, the stability test was conducted. The result was explained in Table 4.

Table 4. Peroxide value of heated encapsulated candlenut oil with new formulation

Encapsulating agent	Pov (meq/kg) Day 0	Pov (meq/kg) day 5
Oil	23.75	24.60
B1	21.90	22.38
W1	20.36	22.43
S1	19.88	20.37
G1	24	>24

Un-encapsulated candlenut oil has the highest peroxide value after the heating process was done (at day 0). Sodium caseinate exhibited the lowest peroxide value at day-0, followed by whey protein and β-cyclodextrin. After five days of storage, peroxide value of all encapsulated oil and un-encapsulated oil were increasing. The auto-oxidation of the encapsulated and un-encapsulated oil was occurred during the heating process. However, both on day-0 and day-5, all the encapsulated oil showed lower peroxide value compared with the un-encapsulated candlenut oil. It was suspected that the peroxide value was increased due to the oxidation from the surface oil, suggesting the inside oil was well protected inside the matrix of encapsulating agent (Wang *et al.*, 2011). However, for gum Arabic, the peroxide value after being heated was increased significantly. Carneiro et al. (2013) stated that the peroxide oxidative stability was affected by the type of encapsulating agent used. A study about encapsulation of flaxseed oil by using spray drying and a combination of encapsulating agent between maltodextrin and gum Arabic, also showed the

poorest oxidative stability compared with whey protein concentrate. It can be concluded that gum Arabic exhibit inadequate protection against oxidation after heat was applied.

After the encapsulation process was carried out and the best encapsulating agent (in terms of efficiency) was obtained, the omega content of the product was analyzed to check the decrement of the omega content. However, the omega fatty acid content of encapsulated oil by using sodium caseinate showed a decrement in all omega content as seen in Table 5.

Table 5. Omega content comparison between pure candlenut oil, encapsulated candlenut oil and commercial product

Parameter	Result (%)		
	Pure candlenut oil	Encapsulated candlenut oil	Commercial product
Unsaturated fats	90.89	50.75	36
Omega 3 fatty acids	26.31	14.31	5
Omega 6 fatty acids	40.10	22.01	21
Omega 9 fatty acids	24.30	14.21	10

This decrement corresponded with the low microencapsulation efficiency of the freeze-dried product. The purpose of this study was to retain the omega content through encapsulation process. However, the result of the first stage formulation showed that the surface oil was too high, which means that the oil was not completely encapsulated. Therefore, the un-encapsulated oil or surface oil was not well protected inside the encapsulating agent and hence the omega oil content was decreasing. Heinzelmann et al. (2000) explained that during emulsification and homogenization process, oil may degraded and oxidized with the presence of oxygen. Also, after the encapsulation process, during storage, the un-encapsulated oil or surface oil may be oxidized as well since there was no encapsulating agent that coated the droplets of oil (Kaushik et al. 2015). Thus, the omega content was decreasing significantly. However, the omega fatty acid content of encapsulated candlenut oil through freeze-drying method was higher compared to the commercial fat powder from MCT Lipids Life which was encapsulated with sodium caseinate through spray-drying method.

CONCLUSION

Four different encapsulating agents from carbohydrate and protein groups, i.e whey protein isolate, sodium caseinate, β-cyclodextrin, and gum Arabic, were used for candlenut oil encapsulation. Both carbohydrate and protein based encapsulating agents were potentially protecting the oil against oxidation process from the aspects of microencapsulation efficiency, peroxide value (PoV), and moisture content. In terms of efficiency, the highest efficiency was obtained by using sodium caseinate with ratio 3:2. The screening of encapsulating agents for candlenut oil was conducted by using new formulation with higher moisture content and ratio encapsulating agent to oil 3:2. The result showed that sodium caseinate was the most suitable encapsulating agent to protect the candlenut oil from oxidation. The efficiency of encapsulated oil with sodium caseinate as encapsulating agent was increased from 43.22 to 64.86% and the peroxide value was decreased from 1.24 to 0.49 meq/kg.

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THE ANTI HYPERTENSIVE NUTRACEUTICALS OF *VIGNA* SP BEAN PROTEIN HYDROLYZED BY ALCALASE AND FLAVOURZYME

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ABSTRACT

Peptide with hydrophobic amino acids had been studied for their inhibitory activity against angiotensin-I converting enzyme (ACE-1) transformation into ACE-2 and prevention of hypertension. The active peptides may come from alcalase and flavourzyme hydrolysis of bean protein. This study aimed to measure ACE-1 inhibitory of protein hydrolysates from *Vigna* sp. bean (mung bean and cowpea) that grew in Indonesia, and its solubility. The bean protein (22.9 - 23.6 %) was extracted using isoelectric precipitation at pH 4-4.6. The extracts were hydrolyzed at pH 8 for alcalase and pH 7 for flavourzyme, followed with inactivation at 80-85 ^{o}C. ACE-1 inhibitory activity was calculated based on the amount of hippuric acid (HA) formed by the hydrolysis of *Hippuryl-His-Leu* (HHL), in spectrophotometry detection method (228 nm). Ultra-chromatography evaluation showed that the protein hydrolysates of mungbean contained higher hydrophobic amino acids (382 mg/g protein) compared to those of cowpea (329 mg/g protein). Protein hydrolysates of both beans from alcalase hydrolysis have higher ACE-1 inhibitory activity rather than those from flavourzyme. Protein hydrolysate from *Vigna* spp bean protein hydrolysis by alcalase, contained small molecular weight peptides (3.9-4.63 kDa) and high ACE-1 inhibition ability (80-93 %), and therefore suggested as antihypertensive nutraceuticals. Highest solubility of protein hydrolysates resulted from alcalase hydrolysis of both beans were observed at pH 8, while those resulted from flavorzyme hydrolysis were at pH 7, respectively.

Keywords: *ACE-1 inhibitory ability, alcalase, anti-hypertensive, flavourzyme, vigna sp*

ABSTRAK

Peptida dengan asam amino hidrofobik telah diidentifikasi berkemampuan menghambat aktivitas angiotensin-I converting enzyme (ACE-I) menjadi ACE-2 dan mencegah hipertensi. Senyawa peptide tersebut dapat berasal dari hidrolisis protein kacang-kacangan oleh alkalase dan flavorzim. Studi ini bertujuan menilai aktivitas penghambatan ACE-1 oleh hidrolisat protein dari kacang-kacangan *Vigna* sp (kacang hijau dan kacang tunggak) yang tumbuh di Indonesia, dan juga kelarutannya. Protein kacang (22,9 -23,6%) diekstrak menggunakan metoda presipitasi sesuai titik isoelektrik pada pH 4-4,6. Ekstrak dihasilkan dari hidrolisis alkalase pada pH 8 dan flavourzyme pada pH 7, diikuti inaktivasi pada suhu 80-85 ^{o}C. Aktivitas penghambatan ACE-1 diukur berdasarkan jumlah asam hipurat (HA) yang terbentuk dari hidrolisis Hippuryl-His-Leu (HHL), dan pembacaan spektroskopi (228 nm). Evaluasi ultra kromatografi menunjukkan bahwa asam amino hidrofobik dalam protein hidrolisat dari kacang hijau (382 mg/g protein) lebih tinggi dibandingkan yang berasal dari kacang tunggak (329 mg/g protein). Aktivitas penghambatan ACE-1 lebih tinggi pada protein hidrolisat kacang *Vigna* sp yang dihidrolisis oleh alkalase daripada flavorzim. Protein hidrolisat kacang genus *Vigna* spp yang dihidrolisis oleh alkalase menghasilkan peptide molekul kecil (3,9-4,63 kDa) dan aktivitas penghambatan ACE-1 tinggi (80-93%), dan dengan demikian direkomendasikan sebagai nutrasetikal untuk antihipertensif. Kelaruratan terbaik hidrolisat protein kedua kacang dari hidrolisis alkalase adalah pada pH 8 sedangkan dari hidrolisis flavorzim pada pH 7.

Kata kunci: *Aktivitas penghambatan ACE-I, alkalase, antihipertensif, flavorzim, vigna sp*

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INTRODUCTION

Mung beans (*Vigna radiata* (L) R. Wilzeck) and cowpea (*V. unguiculata* (L) Walph) are protein rich vegetables with more than 20% protein content. The hydrolyzed protein of the bean grown in Afrika (Adeyemi., et al, 2012), including Nigeria (Aremu et al., 2017), and also those in China (Li et al., 2005) and Mexico (Seguro-Campos et al., 2011) were found to have inhibitory activity against angiotensin-1 converting enzyme (ACE-1) that play roles in hypertension prevention. The bean protein hydrolysate was able to inhibit the ACE-I action in becoming ACE-2, that stimulated aldosterone secretion and consequently, increased blood pressure. Such beans were also grown in Indonesia, and though not yet studied before, protein hydrolysates coming from Indonesian beans may also be a potential source of natural functional ingredients or nutraceuticals with ACE-1 inhibitor or antihypertensive activities.

The angiotensin-1 converting enzyme (ACE-1) inhibitory ability arises from the action of protein hydrolysates that containing a mixture of short-chain peptides (2-15 amino acid residues) with hydrophobic amino acids at their end sites, such as Arg-Lys, Val-Ala-Pro, Phe-Val-Ala-Pro, and Try-Phe-Trp-Leu. Previous studies shown that the peptide composition in bean protein hydrolysates also consist of hydrophobic amino acids at the end side of its chain, that are leusine (Leu), phenylalanine (Phe), valine (Val) and isoleucine (Ileu). Previous studies have shown that the alcalase and flavourzyme,exogenous proteases, hydrolyzed the bean protein yielding the small peptides fragment with low molecular weight with high ACE-I inhibitory ability (Forghani et al., 2012; Hernandez-Ledesma dan Hsieh, 2013; Iwaniak et al., 2014). Various studies have shown that bean protein hydrolysates antihypertensive activity were correlated to their performance in inhibiting ACE-1 activity (Arihara et al., 2000; Daskaya-Dikem et al., 2017; Nakamura et al., 1995; Torruco-Uco et al., 2009).

Cowpea contains high protein (23-32%) (Diouf, 2011) that consist hydrophobic amino acids, namely Ala, Gly, Leu, Val, Ileu, Phe, Pro, Cys, and Met; making this bean potential yielding ACE-1 inhibitor peptides (Segura-Campos et al., 2013). Mungbean contain high protein (23.7%) (Brishti et al., 2017) and high amount of hydrophobic amino acids (Li et al, 2005) as well. Objectives of the study was to evaluate the ACE-1 inhibitory ability of alcalase and flavourzyme hydrolyzed protein from two kinds of V*igna* genus bean that are mung bean (*V. radiata* (L) R. Wilzeck) and cowpea (*V. unguiculata* (L) Walph; and therefore their potencies as antihypertensive nutraceuticals.

MATERIALS AND METHOD

Materials and instruments

The main ingredients used in the study were mung beans (*Vigna radiata* (L) R. Wilzeck) and cowpea (*V. unguiculata* (L) Walph) obtained from Tanjung Market, Jember, East Java. The enzymes used were alcalase 2.4L (2.4 U/g) (Sigma P 4860); flavourzyme 500L (500 U/g) (P6110 Sigma), ACE-I (angiotensin-I converting enzyme) from rabbit lungs (2.0 units/mg protein) (A6778 Sigma). The chemicals used were HHL (Hippuryl-L-Histidyl-L-Leucine) (H1635 Sigma), n-hexane (Merck), NaOH (Merck); 1 M HCl (Mediss); K_2SO_4; $CuSO_4$ H_2SO_4; MM-MB (MBC) Mediss); boric acid; trichloroacetic acid (TCA) (Merck); bovine serum albumin (BSA) (Sigma); Lowry A (Folin-Ciocalteau (Merck) (phosphotungstat-phosphomolybdat acid solution) with aquades 1: 1); Lowry B (Na_2CO_3 2% in 1N NaOH; 1% $CuSO_4.5H_2O$; sodium potassium tartaric (2%); sodium borate buffer pH 8.3; buffer solution pH 7 (Merck), and aquades.

Specific instruments used were a freeze dryer (CHRIST Alpha 1-2 LD plus); shaking waterbath (StuartSBS40); centrifuge (Tomy MRX-150 and Hitachi CR21GIII); pH meter (Horiba F-51); LAF (Laminar Air Flow)(Nuaire); spectrophotometer (Hitachi type U-2900 UV-Vis); kjeldahl flask (BUTCHI); destilator (BUTCHI K-355)

METHODS

Mung bean and cowpea protein extraction

The bean were cleaned from contaminants, then grinded and sieved with an 80 mesh size. The flour was then defatted according to the method of

Viernes et al. (2012), using n-hexane with a ratio of flour to solvent of 1:10 (w/v) for 1 hour in a cold room (4 ^{o}C) with constant stirring. The solvent was removed by decantation, while the remaining solvent in flour precipitate was evaporated at room temperature. The dried flour was then stored in a 4 ^{o}C storage room until being used for further processing.

Protein extraction was performed by the isoelectric precipitation method, following the method of Lie et al. (2005) for mung bean, and the methods of Salcedo-Chavez et al. (2002) for cowpea. The material in the form of flour was suspended by adding distilled water at a ratio of 1:10 (w/v) for mung bean 1:6 (w/v) for cowpea. The highest solubility pH was then adjusted to pH 9 for mung bean and pH 11 for cowpea by adding 1 dan 2 M NaOH. Constant stirring was applied during pH adjustment using a magnetic stirrer until the pH was stable, and then left for 1 -2 hour with constant stirring at room temperature. Furthermore, centrifugation process was conducted to separate the dissolved protein from the material. On the mung bean flour this was carried out for 10 minutes at 5500 rpm at 20 ^{o}C, while for the cowpea flour it was conducted for 20 minutes at 10, 000 rpm and at 4 ^{o}C. The supernatant was separated from the precipitate for subsequent precipitation by an isoelectric pH setting of 4.5 and 4.6 for the bean using 1 N HCl., respectively. Constant stirring was applied during pH adjustment using a magnetic stirrer until the pH was stable, and then left for 30 minutes to allow the protein to be completely deposited. The suspension was then centrifuged again with the same time, speed and temperature as before to separate the protein and the remaining dissolved materials. The precipitate centrifugation results were separated from the supernatant, followed by a process of washing using distilled water by means of centrifugation, as previously. The protein precipitate were separated from the supernatant and dried using a freeze dryer.

Enzymatic hydrolysis of the bean protein

The enzymatic hydrolysis process was conducted using alcalase and flavourzyme enzymes, based on the procedure followed by Li et al. (2005) for the mung bean protein material and that of (Segura-Campos et al., 2013) for cowpea protein material. The protein extract was suspended in distilled water at a ratio of 4/100 (w/v), accompanied by stirring using a stirrer. The ratio of the enzyme/substrate concentration of the mung bean protein extract was 20 µL/g protein (alcalase 0.3 AU/g; flavourzyme 50 LAP U/g). Suspension of the mung bean and cowpea protein extract was performed by adjusting to pH 8 for alcalase and pH 7 for flavourzyme, respectively, by adding 1 N NaOH with constant stirring. Furthermore, the suspension was subjected to enzymatic processes at 55 ^{o}C for 120 minutes to mung bean protein extract, and at 50 ^{o}C for 90 minutes to cowpea extract protein. The hydrolysis process was performed by a shaking water bath. The hydrolysis was terminated by enzyme inactivation using heating for 10 minutes at 85 ^{o}C, followed by a centrifugation process to separate the supernatant from the precipitate. Centrifugation was carried out at 4 ^{o}C at a speed of 10,000 rpm for 20 minutes for the mung bean and cowpea protein extract material. The resulting supernatant was a protein hydrolysate containing a mixture of peptides and amino acids. The results were then dried using a freeze dryer and stored at -20 ^{o}C until being used for further processing.

Analysis of the bean protein content (AOAC, 2001)

One gram of each mung bean and cowpea was placed into a Kjeldahl flask, followed by the addition of 7 g of K_2SO_4, 0.8 g of $CuSO_4$ and 12 ml of concentrated H_2SO_4. The mixture was then warmed in a laminar hood for ± 6 hours, followed by cooling for 10-20 minutes. After it had cooled, distilled water was added to make a total volume of 80 ml. A 50 ml 50% NaOH (w/v) was then added, which was then distillated until 150 ml of distillate was obtained. The resulting distillate was put into an Erlenmeyer flask with 30 ml of 1% H_2BO_3 (w/v) which had been dropped by 2 drops MM-MB indicator. The obtained distillate was titrated with a standard solution of 0.1 M HCl to light purple. The same treatment was performed using distilled water as a blank. The percentage of total protein content can be obtained with the following calculation: % N = ((ml sample HCl-ml HCl blank) x M HCl x

14.01) / (sample weight x 1000) x 100% % total protein =% N x conversion factor (6.25).

Composition amino acid analysis (Genetech, 2017)

The composition of amino acids in the bean protein hydrolysate was determined using Waters Acquity UPLC H Class and H Class Bio Amino Acid Analysis System Guide year 2012. Sample was prepared using the following procedure: A 100 mg of each mung bean and cowpea protein hydrolysate was added by 5 ml HCl 6N, homogenized, then the mixture was hydrolyzed at 110ºC for 22 hours. Furthermore, the cool hydrolyzed mixture was diluted by adding aquabidest up to 50 mL, and then was filtered out with a 0.45 µm filter. A 500 µl of the filtrate was added with 40 µm AABA and 460 µl aquabidest. A 10 µl of sample solution was added 70 µl AccQ-flour Borate, and vortexed. Then, the homogenous solution was added with 20 µl reagent flour A, homogenized, hushed up for one second, and incubated for 10 second at 55 ºC. *Standard solution was prepared using the following procedure:* 40 µl amino acids mix standard was added with 40 µl AABA internal standard and 920 µl aquabidest, and homogenized subsequently. A 10 µl of the standard solution was added with 70 µl AccQ-fluor Borate and vortexed. Then, the homogenous solution was added with 20 µl reagent flour A, homogenized, hushed up for one second, and incubated for 10 second at 55 ºC. The volume of one µl of sample solution and standard solution was injected to UPLC using ACCQ-Tag Ultra C18 column at 0.7 mL per minute and 49 ºC, and detector PDA 260 nm. The amount of amino acids content can be obtained with the following calculation: Amino acid content (mg/kg) = (area standard/AABA standard x ml Final Volume x fpx x C standard) / Area sample/AABA x gr sample.

Degree of hydrolysis (Silvester et al., 2013)

A 500 µl of the bean protein was added with 500 µl of 20% TCA and then homogenized and incubated at 4 ºC for 30 minutes. The mixture was centrifuged at 6500 rpm for 20 minutes and the supernatant analyzed for dissolved protein content based on the Lowry method (Purwanto, 2014). Bovin Serum Albumin (BSA) was used as a protein standard. The percentage degree of hydrolysis was calculated using following calculation: DH (%) = (10% dissolved protein TCA (mg)) / (total protein content (mg)) x 100%

Protein solubility analysis (Muhamyankaka et al., 2013)

The bean protein hydrolysate was dissolved in distilled water (1% w/v) and the pH was adjusted (to 3, 5, 7, 8, 9, 10 and 11) by adding 1N HCl and 1N NaOH. Constant stirring was then carried out using a magnetic stirrer for 30 minutes at room temperature (25 ºC). Subsequently, the suspension was centrifuged for 15 minutes at 5000 rpm. The total protein in the hydrolyzate was analyzed by dissolving the hydrolyzate in 0.2 M NaOH (1% w/v). The content of the dissolved protein in the supernatant and suspension as a whole was measured using the Lowry method (Purwanto, 2014). The percentage of protein hydrolyzate solubility was calculated using the following formula: % Protein solubility = (Soluble protein sample) / (Total soluble protein) x 100%

Analysis of angiotensin-i converting enzyme (ACE-1) inhibitory ability (Li et al., 2005)

Evaluation of ACE-I inhibitory ability was conducted using the principle of the formation of Hippuric Acid (HA) from the reaction of Hippuryl-His-Leu (HHL) with water (H_2O). A sample of bean protein hydrolysate of 40 µl (10 mg/ml) was added with 100 µl of 5mM HHL in 0.1 M borate buffer pH 8.3 containing 300 mM NaCl, and the mixture incubated at 37 ºC for 5 minutes. A 10 µl ACE-I was then added at a concentration of 100 mU/ml and incubated for 60 minutes at 37 ºC. The reaction was stopped by adding 150 µl of HCl 1N and vortexed until homogeneous. Furthermore, 1.5 ml of ethyl acetate was added to dissolve the HA released from the HHL by ACE. The solution was then centrifuged for 10 minutes at 4000 rpm, and 1 ml of supernatant containing HA taken to evaporate the ethyl acetate. The HAobtained was further diluted by adding 3 ml of distilled water and then vortexed. The solution formed was evaluated in the spectrophotometer for its absorbance at a wavelength of 228 nm. ACE

inhibitory activity (%) was calculated by the following formula:

ACE inhibitory activity (%) = ((B - A)) / ((B - C)) X 100%

Where: A = absorbance value with the addition of ACE and sample, B = value of control absorbance (buffer replacing sample), C = blank absorbance value (HCl added before ACE)

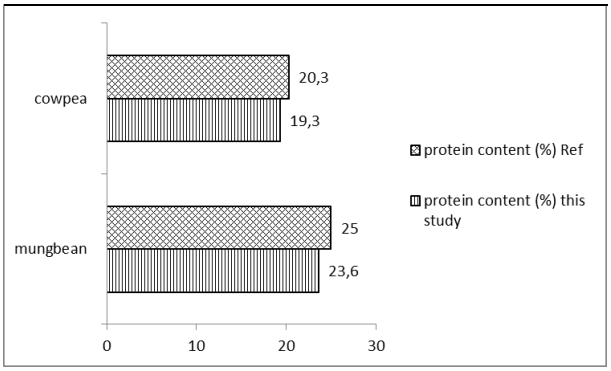

Figure 1. Comparison of protein content in mung bean and cowpea in this study and various references (Butt and Batool, 2010; Mune et al, 2013)

The levels of mung bean protein in this study were almost the same as those of Butt and Batool (2010) (23.9 percent protein content), but lower than those shown by Mubarak (2005) (25.1 percent protein content), respectively. The similarity of mung bean protein level between this study and that of Butt and Batool (2010), might be correlated to the fact that the mung bean used in the two studies were grown in Indonesia. On the other hand, Mubarak (2005) obtained higher protein content values using mung beans grown in Saudi Arabia. Carerra et al. (2011) statement, in agreement to the fact, stated that different types of climate and soil where a bean grows and develops lead to nutritional variation, including the protein content.

Total protein content of cowpea evaluated in this study (19.3 %, Figure 1) had different values compared to 20.3% protein content observed in Mune et al. (2013) and Khalid and Elharradallou (2013). The cowpea used in this research was grown in Indonesia while the other studies mentioned before used beans grown in Sudan, and Kamerun. Differences of cultivars may also play role in the nutritional variation (Habibullah et al.,2007).

Protein and hydrophobic amino acids content of mung bean and cowpea protein extract

The mung bean and cowpea protein content from the protein extract were 72.0 and 64.2 %, respectively. These results were higher (for mung bean) but lower (for cowpea) in comparison to previous studies. Comparison data of the protein content in bean protein extract and in protein hydrolysates from the results of this study and or those of others can be seen in Figure 2. Mungbean protein extract contained higher protein content value than cowpea in the results of this research. The result is in conjunction with previous studies.

The protein content of the mung bean protein extract from the results of this research were lower than previous studies (Bristhi et al.,2017; Sibt-e-Abbas et al., 2016; Butt and Batool, 2010). On the other hand, cowpea protein extract in this research consisted of protein value of (62.5 %) higher than previous studies (Mwasaru, et al.,1999; Adeyemi et al.,2012; Frota, et al., 2017). While differences in climate and soil type where the bean grown might play a role in the variation, differences in protein extraction methods might also have an influence.

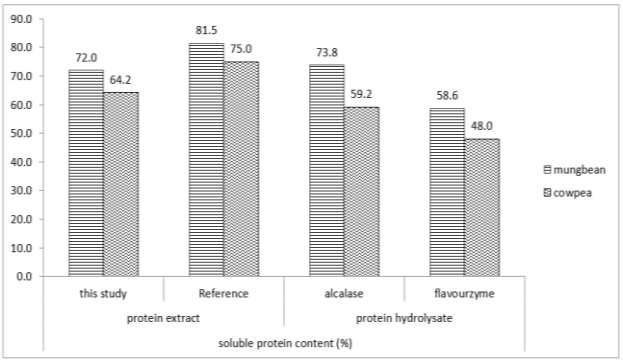

Figure 2. Comparison of protein content in extract protein and its protein hydrolysates of mung bean and cowpea in this study and other references (Bristhi et al., 2017; Khalid and Elharadallau, 2013)

The mung bean and cowpea hydrolysate protein in this study had lower protein values than hydrolysate protein found by other researchers. A comparison of the protein content in bean protein hydrolysate can be seen in Figure 2. The data in the figure showed that alcalase hydrolyzed protein both in the mung bean and cowpea had a higher protein content than those in flavourzyme hydrolyzed protein The protein hydrolyzate content of mung bean protein and cowpea hydrolyzed alcalase were higher than those of flavourzyme because alcalase enzymes hydrolyzes peptides with broad specificity, releasing hydrophobic peptide bonds such as Phe, Tyr, Trp, Leu, Ile, Val and Met (Doucet et al., 2003), which have the potential to be ACE-I inhibitors (Li et al., 2005). In addition, the enzyme flavourzyme contains protease complex endoproteinase and exopeptidase, with greater exopeptidase activity. This enzyme is only specific in breaking the peptide bonds in the leucine amino acids located in the amine group (Fonsseca et al., 2016).

With regard to amino acid, its amount and character or composition in a peptide determine the quality and physiological role of protein. Many essential amino acids are hydrophobic, but only little of those are hydrophilic. Peptides are produced from the alcalase hydrolyzed process of extract protein that is composed of hydrophobic amino acids at the end site of its chain, which is known to have ACE-1 inhibiting ability. Mung bean extract protein hydrolyzed alcalase produced five peptides with following order Lys-Asp-Try-Arg-Leu,Phe or Val-Thr-Pro-Ala-Leu-Arg and Lys-Leu-Pro-Ala-Gly-Thr (Li et al., 2006). Data in Table 1 showed that the amount of two amino acids groups in this research were lower compared to other research findings. Furthermore, the hydrophobic amino acids content in mung bean and cowpea extract protein was lower than that of hydrophilic amino acids, similar to the reference findings. This study analysis data showed that mung bean extract protein contained higher hydrophobic amino acids than that of in cowpea (Table 1), Potentially, alcalase hydrolyzed mung bean protein produced high amount peptides composed of hydrophobic amino acids that having high ACE-1 inhibitory ability.

Table 1. Comparison of amino acids in mungbean and cowpea protein extract in this research and other studies

Amino acids group	Kind of amino acids	Content (mg/g protein)			
		[1]This Study		[2] Ref	[3] Ref
		Mungbean	Cowpea	Mungbean	Cowpea
Hydrophobic	*Isoleucine (Ile)	38.48±0.18	35.64±0.13	64.5	44.9
	*Leusine (Leu)	73.45±0.34	66.95±0.27	69.1	94.5
	*Methionine(Met)	10.87±0.03	10.47±0.04	130.0	2.2
	*Cystein (Cys)	0.88±0.01	1.10±0.01	43.0	12.4
	*Phenilalanin (Phe)	67.62±0.33	46.77±0.17	53.1	55.8
	*Trypthophan (Tryp)	5.02±0.12	6.08±0.01	2.7	2.7
	*Tyrosine (Tyr)	30.05±0.13	23.51±0.08	26.6	21.3
	*Valin (Val)	43.98±0.06	39.22±0.13	32.4	54.6
	Serin (Ser)	45.31±0.18	39.76±0.13	53.2	66.5
	Prolin (Pro)	34.28±0.07	30.77±0.01	43.0	49.1
	Alanin (Ala)	32.33±0.13	28.73±0.10	29.7	66.3
		382.24	***328.97***	***547.3***	***470.3***
Hydrophylic	Arginin (Arg)	57.19±0.22	47.24±0.14	46.0	36.5
	Aspartat (Asp)	92.67±0.29	75.07±0.29	98.0	130.3
	Glutamat (Glu)	159.20±0.85	136.38±0.46	203.0	155.6
	Glysin (Gly)	30.73±0.08	26.63±0.06	28.7	66.3
	Histidine (His)	27.02±0.21	23.47±0.07	37.5	34.5
	*Lysine (Lys)	73.14±0.33	71.33±0.25	140.2	65.0
	*Threonin (Thr)	26.52±0.13	27.25±0.05	48.8	42.5
		466.45	***407.36***	***602.2***	***530.7***

* essential amino acids [1] this study result [2] Ref: Mune et al., (2013) [3] Ref: Bristhi et al., (2017)

Protein hydrolysis degree and the solubility of mung bean and cowpea protein hydrolysates

The degree of protein hydrolysis can be expressed as an indicator of the success of the hydrolysis process. The higher the percentage, the better the hydrolysis. Figure 3 shows that the values of the degree of hydrolysis in mung bean and cowpea protein hydrolyzed by alcalase were higher than with the use of flavourzyme (43>23% and 49>34%). The difference was due to the enzyme specificity of the substrate. Mung bean protein is rich in protein sources of hydrophobic amino acids such as Phe, Tyr, Trp, Leu, Ile, Val and Met. Different types of enzymes used in the hydrolysis process will produce different degrees of hydrolysis. Alcalase is an alkaline protease that is able to produce bioactive peptides with ACE-I inhibitory activity.

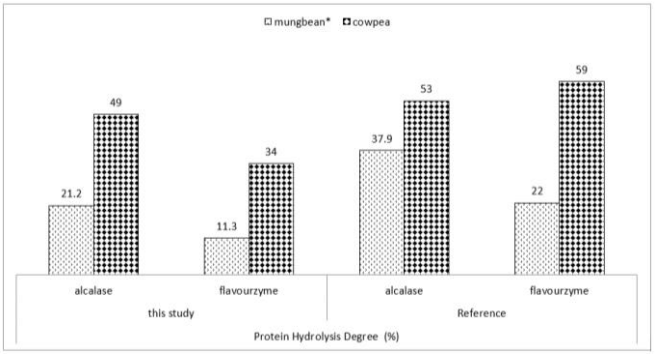

Figure 3. The degree hydrolysis values of mung bean and cowpea protein hydrolysed by alcalase and flavourzyme

Alcalase enzymes hydrolyses peptides with broad specificity, releasing bonds of hydrophobic peptides such as Phe, Tyr, Trp, Leu, Ile, Val and Met (Doucet et al., 2003), which have the potential to be ACE-inhibitors (Li et al., 2005). On the other hand, the enzyme flavourzyme contains protease complex endoproteinase and exopeptidase, with greater exopeptidase activity. This enzyme is only specific in breaking the peptide bonds in the leucine amino acids located in the amine group (Fonsseca et al., 2016). It can therefore be concluded that mung bean and cowpea were good substrates for alcalase enzymes to produce antihypertensive peptides. Solubilities of mung bean and cowpea protein hydrolyzed by alcalase were highest at pH 8. While the solubilities of those hydrolyzed by flavourzyme were highest at pH 7 (Figure 4).

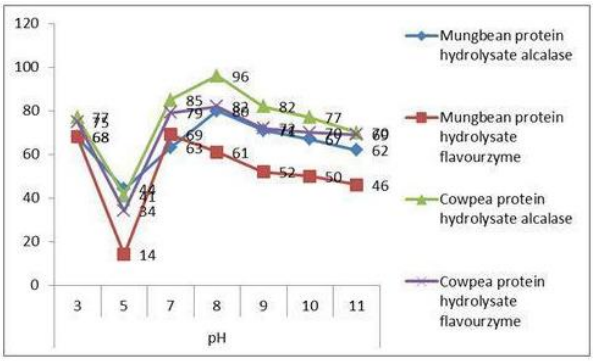

Figure 4. The solubility of mungbean and cowpea protein hydrolyzed by alcalase and flavourzyme

The SDS-PAGE electrophoresis of mungbean and cowpea protein hydrolysates showed that the fractions or peptides molecular weight ranged in between 22.6-46.3kDa. However, after alcalase hydrolyzed, the mung bean and cowpea protein hydrolysates fractions or peptides were having smaller molecular weight ranged in between 3.0-8.7 kDa (Figure 5).

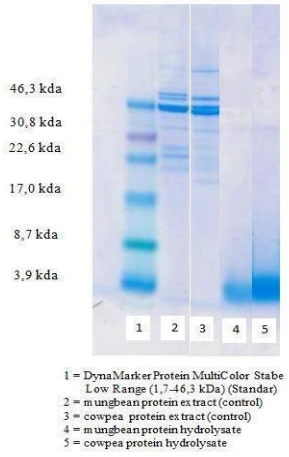

Figure 5. Fractination of mung bean and cowpea protein hydrolysed by alcalase on the molecular weight basis

ACE-I inhibiting ability

Angiotensin-I converting enzyme (ACE-I), a peptidyl peptide hydrolase enzyme plays a role in increasing blood pressure in the body. ACE-I inhibitory activity determines the potential of an ingredient utilization as an antihypertensive agent. The ACE-I enzyme used in this study was isolated from rabbit lungs and commercialized for research purposes may be used in testing the antihypertensive potential of an inhibitor in vitro. ACE-I inhibitory activity analysis was performed by reacting the protein hydrolyzate with Hippuryl-His-Leu (HHL) and involved an inhibitor; in this case the inhibitor used was in the form of protein hydrolyzate in order to obtain a percentage value of the ACE-I inhibition of an inhibitor. Comparison of the ACE-I inhibitory activity of mung bean protein and cowpea protein in this research and other studies can be seen in Table 2.

Table 2. Comparison of the ACE-I inhibitory activity of mung beanand cowpea protein in this research and other studies

Bean hydrolysates	ACE-1 inhibitory (%)		Source
	Alcalase	Flavorzyme	
Mungbean (*Vigna radiata* L. R. Wilczek) (VIMA-1)	91	72	this study
Mungbean *(Vigna radiata)*	79	-	Li et al. (2005)
Cowpea (*Vigna unguiculata* L. Walph)	80	77	this study

From the data in Table 2, the inhibition values of ACE-I mung bean protein hydrolyzed by alcalase and flavourzyme were 91 and 72 percent, respectively. While, the ACE-I inhibition value of cowpea protein hydrolyzed by alcalase and flavourzyme were 80 and 77 percent, respectively. The ACE-I inhibition of mung bean and that of cowpea protein hydrolyzed by alcalase and flavourzyme in this study were higher than previous studies. The evaluation in this study confirmed that the protein of mung bean and cowpea grown in Indonesia, and hydrolyzed by both enzymes had considerably high ACE-I inhibitory ability, since the values were above 70 percent.

The high ACE-I inhibitory activity of mung bean and cowpea hydrolyzate protein may be due to the presence of peptide content with short peptide chains (2-5 amino acid) and C-terminal proline or hydroxyproline residues, peptide chain that have stronger inhibitory effect, since theybind to ACE-I more strongly. Proline, lysine and arginine are the preferred C-terminal substrates for ACE-I, contributing greatly to the inhibition of ACE-I (Erdmann et al., 2008). These enzymatic hydrolyzed peptides have a strong affinity with the active side of the ACE-I enzyme and can interfere with its catalytic activity in hydrolyzing the hippuril-histidyl-leucine (HHL) substrate in in vitro tests, giving high percentage inhibition (Ryan et al., 2011).

Mung bean and cowpea protein hydrolyzed by alcalase expressed higher inhibitory values than those hydrolyzed by flavourzyme. This might due to the fact that enzymatic hydrolysis of proteins using alcalases tends to produce peptides with C-terminal amino acids in aromatic and aliphatic side chains, such as Ile, Leu, Val, Met, Phe, Try and Trp (Doucet et al., 2003). The alcalase enzyme is a group of endopeptidase enzymes that can cut peptide bonds in the middle of the chain of hydrophobic amino acids. In addition, the enzyme flavourzyme also has the ability of both enzyme endopeptidase and exopeptidase, which can cut peptide bonds in the middle or at the end of the chain of combined amino acids, especially in leucine amino acids (Leu) (Fonsseca et al., 2016).

CONCLUSION

The alcalase and flavourzyme hydrolysed protein of mungbean and cowpea grown in Indonesia, were

able to inhibit the activity of angiotensin-I converting enzyme (ACE-I) with inhibitory values greater than 70 percent. The amount of hydrophobic amino acids in mungbean protein extract that hydrolysed by alcalase, was higher than that of in cowpea protein extract. This might play a role in the higher ability of ACE-1 inhibitory by mung bean protein hydrolysate (91%) than that of cowpea protein extract (80%). The solubilities of alcalase hydrolyzed mung bean and cowpea protein were highest at pH 8. While the solubilities of those hydrolyzed by flavourzyme were highest at pH 7. The ACE-I inhibitory activity of these two bean protein hydrolysates were considerably high, and may be potential for further exploration as antihypertensive nutraceuticals.

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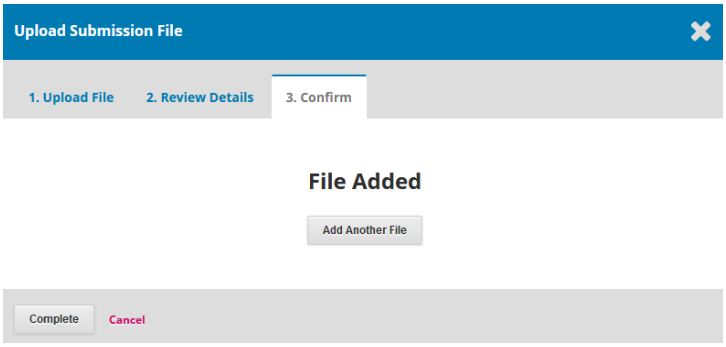

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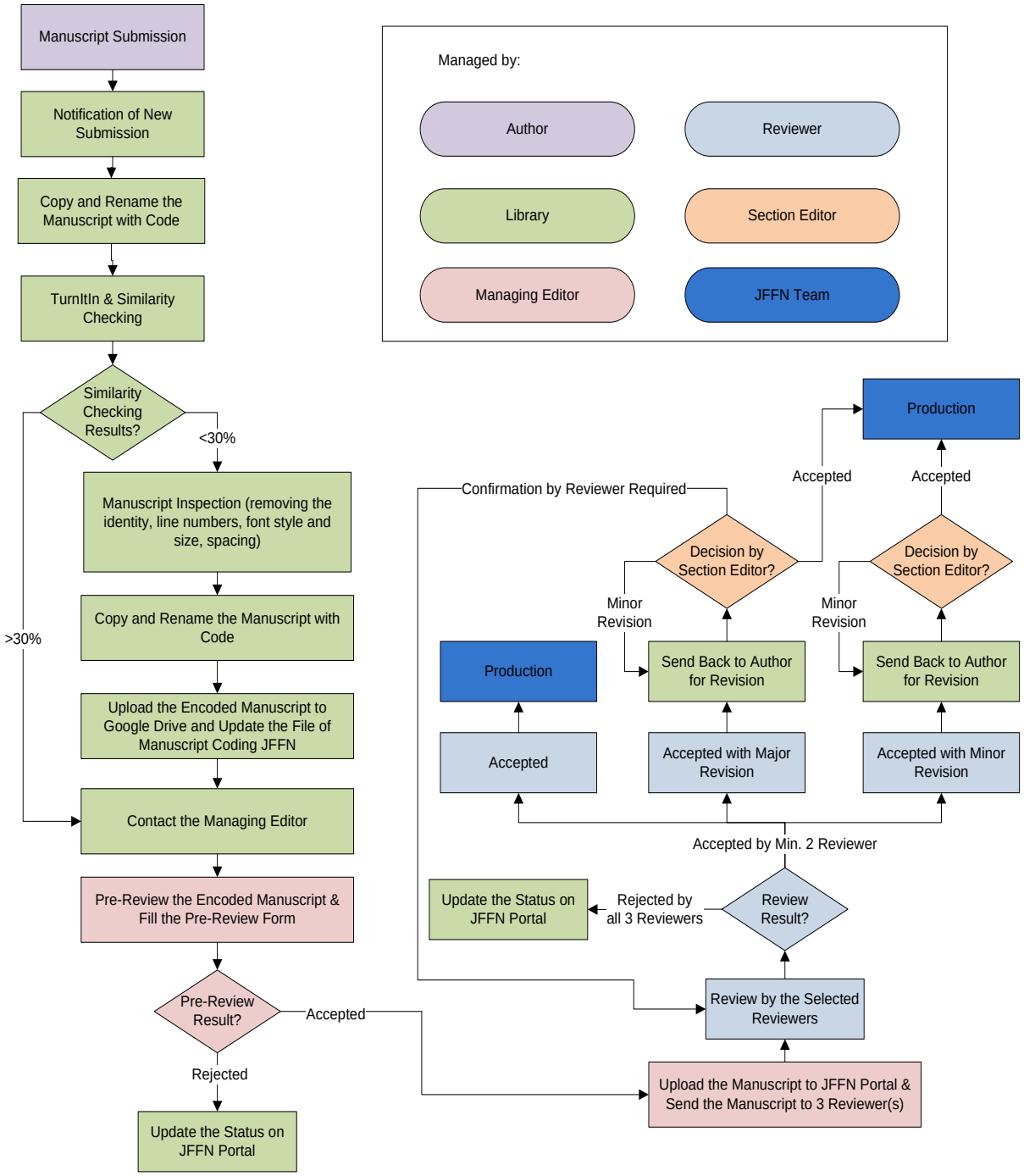

Guideline for Authors

NOTE: Please read the instructions carefully and strictly follow them to ensure smooth submission process. Papers that do not adhere to the guidelines will be rejected, hence full cooperation from the authors is highly appreciated.

- **Type of Papers**

There are three types of paper which are accepted by Journal of Functional Food and Nutraceutical: Research Papers, Review Articles, and Short Communication and Notes. Please note that the papers have not been and will not be published elsewhere, the Chief Editor reserves the right to change the paper into Short Note. The Author(s) shall retain all copyright rights held by the Author in the Manuscript.

a. **Research Papers**
   Original full-length research papers that have not been published previously, except in a preliminary form, and should not excess 7,500 words from introduction to conclusion (not including references). Research paper should not contain more than 40 references.

b. **Review Articles**
   By invitation only.

c. **Short Communications and Notes**
   Short communications of up to 3000 words from introduction to conclusion (optional), not including references, describing work that may be of a preliminary nature but merits publication. These papers should not contain more than 40 references.

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Manuscripts are accepted either in English or Indonesian language. For content written by Indonesian author/s, the author should provide title and abstract in both Indonesian and English.
Author is asked to provide manuscripts as electronic files and should be prepare using common word processor software (e.g. Microsoft-Word®) in following formats: DOC, DOCX, or RTF.
For *Research Papers* and *Short Communications and Notes* please refer to the **Template** to prepare the manuscript accordingly. Please note that the paper should be uploaded in a **SINGLE** file where title page information, manuscript, figure(s) and/or table (s) are included.

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The manuscript that submitted must be an original work. Authors should refer to the Code of Ethics to ensure its originality. Ensure the manuscript has not been previously published, nor is it before another journal for consideration (including published in different language).

It is recommended to check the manuscript for any possible plagiarism by using any program such as TurnitIn or any other software before submitting the manuscripts. Authors are responsible for the integrity of the work as a whole (including method, analysis, calculation, or other details), from inception to published article.

- **Publication Fee**

Submission Fee
Journal of Functional Food and Nutraceutical **will be charged IDR 500.000 per article** at the time of manuscript submission. Submission fee exemption can be applied with term and condition.

Membership Fee
For P3FNI and Swiss German University members will receive 50% discount for the submission fee.

- **Submission Preparation Checklist**

As part of the submission process, authors are required to check off their submission's compliance with all of the following items, and submissions may be returned to authors that do not adhere to these guidelines.

- The originality of the manuscript and ensure the manuscript has not been published previously – Please see "Originality"
- Manuscript text is prepared in accordance with the author guidelines and given template (for *Research Papers* and *Short Communications and Notes*).
- Ensure that some of the provisions are checked and present – **Please see file Check List:** http://bit.ly/checklistjffn.

- **Article Submission**

Online submission via *Open Journal System* (https://journal.sgu.ac.id/jffn). Please refer to the User Account Registration Guideline for submission process. After the submission, author who submit the manuscript will get a confirmation email and able to track their submission status by logging in to the system. The submission tracking includes a status of manuscript review and editorial process. If authors have any problems with the online submission, please contact JFFN admin at the following email: jffn@sgu.ac.id

Reviewer Guideline

Interested to become a reviewer?

The JFFN Editorial Team will send the manuscripts to the relevant reviewers according to the expertise of respective reviewers. If you are interested in becoming a reviewer of JFFN, please fill out the reviewer application form: http://bit.ly/revregform along with a brief summary of your expertise and your CV. Send all the documents to jffn@sgu.ac.id. The reviewers who pass the selection will obtain many benefits. All review process will be processed through JFFN online system.

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   The reviewers will receive an email invitation that will be sent by the JFFN system. Use the links to accept or reject the invitation. If you decide to accept the invitation as the reviewer, you will be responsible to input the review result/s to the JFFN journal editor as the requirement whether the manuscript is appropriate to be published in JFFN.

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Here are the generic questions to the reviewer. Please evaluate the submission based on the general scientific journal guideline. Please download the review form from: http://bit.ly/revformjffn.

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- Accept with major revision
- Decline

Thank you to our reviewers

Peer-review is an important step to maintain the high quality of a journal. Reviewers provide scientific critiques based on their expertise that assist editors to make acceptance decision professionally. Therefore, the Editors would like to acknowledge our reviewers listed below who have contributed their valuable time for maintaining the quality of Journal of Functional Food and Nutraceutical.

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Registrasi anggota P3FNI

Perhimpunan Penggiat Pangan Fungsional dan Nutrasetikal Indonesia (P3FNI) juga mengembangkan kontribusinya di kancah internasional bersama dengan International Society for Nutraceutical and Functional Food (ISNFF). Secara internasional terdapat klaster ISNFF seperti di Korea dan China. Untuk kepentingan percaturan internasional P3FNI menggunakan nama Indonesian Society for Functional Food and Nutraceutical (ISFFN).

Keterlibatan P3FNI atau ISFFN ini memberi manfaat anggotanya untuk memberikan kontribusi ilmu pengetahuan dan teknologi serta mengikuti pemutakhiran pangan fungsional dan nutrasetikal. Bersosialisasi dan berkesempatan dalam pertemuan ilmiah bersama penggiat pangan fungsional dan nutrasetikal dari berbagai negara untuk terus-menerus mengarah pada optimalisasi penggunaan pangan untuk kesejahteraan manusia secara bijaksana dan menjaga kelestariannya.

Siapa Yang Perlu Menjadi Anggota?

1. Akademisi dan peneliti yang terus-menerus mengembangkan dan memajukan ide alternatif dan kreatif untuk menuju kemajuan dan kesejahteraan manusia dengan menggunakan data basis ilmiah.
2. Praktisi kesehatan maupun industri yang menerapkan pangan fungsional dan nutrasetikal.
3. Mahasiswa sebagai penerus masa depan untuk melestarikan praktik-praktik pengadaan dan penggunaan pangan fungsional dan nutrasetikal secara bijaksana dan berkelanjutan.
4. Memberi advokasi dalam kasus-kasus pangan dan kesehatan.
5. Individu yang memiliki perhatian dalam pangan fungsional dan nutrasetikal.

Fasilitas Anggota P3FNI

1. Link Internasional untuk kegiatan atau program riset dan pembelajaran pangan fungsional dan nutrasetikal meliputi direktori laboratorium dan professor, lembaga kesehatan, LSM/NGO, skim hibah, dan internship/magang di industri.
2. Biaya partisipasi dalam pertemuan ilmiah yang diselenggarakan P3FNI.
3. Informasi tentang pangan fungsional dan nutrasetikal Indonesia dan isu internasional terbaru (international current issues).

Iuran Keanggotaan P3FNI

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Akademisi, Peneliti non-komersial	Rp 400.000
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