IMPROVEMENT OF PHYSICO-CHEMICAL PROPERTIES AND ANTIOXIDANT ACTIVITY OF SACHA INCHI RESIDUE BY PHYSICAL AND ENZYMATIC PROTEOLYSIS TECHNIQUES FOR DRINK PRODUCT DEVELOPMENT

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ABSTRACT

The overall objective of this study is to investigate effects of stabilization and enzymatic proteolysis techniques on improvement of physico-chemical properties and antioxidant activities of Sacha Inchi residue (SIR) obtained from screw press oil extraction process for utilization in foods and beverages. In the first part of this study, various stabilization processes of the SIR including extrusion (barrel temperature of 80, 90 and 100 °C and in-barrel moisture level of 39.5, 50.6 and 61.8 %), autoclaving (temperature of 105, 110 and 121 °C for 10, 15 and 30 min), drum-drying processes (rotation speeds 3, 5 and 7 rpm/min and drum surface temperature of 100, 120 and 140 °C), heat-moist treatment (moisture content of 15, 20 and 25% and temperatures of 90, 100 and 110 °C) and a combine process (autoclaving at 121 °C for 30, 45 and 60 min and extrusion at barrel temperature of 100, 125 and 150 °C and moisture level of 61.8%) were studied. The results show that all stabilization processes could improve the functional properties of the SIR compared to the control. It can be seen that extrusion process at barrel temperature of 100 °C and in-barrel moisture level of 61.8% resulted in a significant higher ($p < 0.05$) protein content (PC=61.33%), emulsion capacity (EC=60.0%), emulsion stability (ES=60.7%), foaming capacity (FC=13.6%), oil binding capacity (OBC=16.3%), soluble protein (SP=16.53%), water absorption index (WAI=5.63%) and water solubility index (WSI=1.47%) in comparison with those of the control sample

(PC=54.3%, EC=46.5%, ES=37.0%, FC=4.90%, OBC=2.1%, SP=5.48%, WAI=3.22% and WSI=0.39%, respectively). The sample stabilized with extrusion at lower temperature of 80 °C and 39.5% moisture level possessed highest antioxidant activities indicated by $ABTS^{\bullet+}$ (2.4 mg Trolox /g powder), FRAP (0.7 mg $FeSO_4$ /g powder), DPPH (1.5 mg Trolox /g powder) and TPC (2.4 mg gallic acid /g powder) compared to the control ($ABTS^{\bullet+}$=0.2 mg Trolox /g powder, FRAP=0.2 mg $FeSO_4$ /g powder, DPPH=0.3 mg Trolox /g powder and TPC=0.6 mg gallic acid /g powder).

A combination of autoclave (121 °C for 60 mins) and extrusion (150 °C and moisture level of 61.8%) was the most efficient stabilization process to inhibit microbial growth, antinutrients (phytic acid and tannin) and oxidation. Quality changes of stabilized samples were monitored under storage temperature of 30, 40 and 50 °C and the shelf-life was evaluated in terms of oxidation activity. The result showed that autoclaving and combined process could inhibit microbial growth and oxidation of the SIR with longest shelf life of more than 12 weeks at of 4 °C to 50 °C storage temperatures. For utilization of stabilized Sacha Inchi residue (SSIR) in food products, the impact of the SSIR on pasta making and qualities was also studied. The SSIR at levels 0-50% (w/w) was used to substitute semolina in pasta formulations. Results indicate that utilizing 30% of SSIR in the formulation successfully replaced semolina in pasta making and significantly improved the nutritional quality, textural quality, cooking weight values (187.83%) and swelling index (9.96%) of the product.

The second part of this study aims to improve the overall properties of Sacha Inchi proteins in terms of antioxidant activities, antihypertensive (ACE-inhibitor), dipeptidyl peptidase-IV inhibitor (DPP-IV), inhibition of a digestive enzyme and functional properties through protein hydrolysis process. Diffident protease enzymes (Alcalase, Neutrase, Alcalase combined Neutrase, Pepsin, Papain and Flavourzyme) were used to produce Sacha Inchi protein hydrolysate represented as SPHA, SPHN, SPHAN, SPHPe, SPHPa and SPHFl, respectively. In addition, the protein hydrolysate samples were fractionated into different peptide sizes (<1, 1-3, 3-5 and 5-10 kDa) using ultrafiltration membrane. Each peptide fraction was analyzed for its physicochemical properties, antioxidant activities, and functionalities of bioactive peptides (α-amylase, α-glucosidase, trypsin inhibition activity and pancreatic lipase inhibition). Gel electrophoresis results indicated that proteins in the SIR is composed

of several polypeptide sizes ranging from 10-200 kDa while Sacha Inchi protein isolate (SIPI) consisted mainly of 10 kDa polypeptide with a minor content of 60 kDa polypeptides. Amino acid analysis show a significant *($p < 0.05$)* higher levels of hydrophobic amino acids in the SPHPe, which was consistent with higher surface hydrophobicity when compared to the SIPI. All emulsions exhibited monomodal oil droplet size distribution. Overall results, the SPHPe sample possessed highest protein yield (34%), ACE and DPP-IV inhibitory activities (IC_{50}=0.33, 0.82 mg/mL), and antioxidant activities (hydroxyl radical scavenging assays [HRSAs], EC_{50}=2.50 mg/mL; iron chelating, EC_{50}= 0.37 mg/mL; FRAP, 0.06 mmol Fe^{2+}/g). The SPHPe was fractionated through membrane ultrafiltration to produce peptide fractions. Sacha Inchi peptide fractions were tested ACE and DPP-IV inhibitory activities and antioxidant properties through free radical scavenging abilities (DPPH and HRSAs), inhibition of metal iron (FRAP and metal chelation) and inhibition of linoleic acid peroxidation. The <1 kDa and 5-10 kDa peptide fractions showed significantly *($p<0.05$)* higher ability to scavenge DPPH (61.91%, 61.94%), HRSAs (71.87%, 72.04%). SPHPe had the highest iron chelating as 24% and FRAP as 0.167 mmol Fe^{2+}/g. While there was a progressive increase in inhibition of the membrane fraction against ACE and DPP-IV with the <1 kDa having significantly *($p < 0.05$)* higher (IC_{50}=0.51 and 0.44 mg/mL) inhibition compared to other. We concluded that Sacha Inchi protein hydrolysate and its membrane fractions possessed potent bioactive peptide. The beverage product prototype from Sacha Inchi protein hydrolysate was also developed. Three different beverage flavours were developed with various amount of SPHPe, brown rice protein isolate, pea protein isolate, stevia, cocoa, coffee or vanilla powder and analyzed for their proximate composition, functional properties (wettability, dispersibility and solubility) and in vitro protein digestion. Results revealed that vanilla formula had highest PC as 11.23% and carbohydrates contents as 82.25%. The coffee formula had the highest wettability as 146 s., solubility as 38.57% and *in vitro* protein digestibility as 87.56%. This study show a significant finding about high-valued addition to by-products from agro-industry that has a potential for uses as a new functional ingredient with outstanding biological activities. The extend utilization and production of Sacha Inchi protein hydrolysate at industrial scale should be further investigated.

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LIST OF ABBREVIATIONS

ABTS	2, 2'-Azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)
AEBSF	4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride
ALA	Alpha-linolenic acid
ANFs	Antinutritional factors
ANOVA	Analysis of variance
ANS	8-Anilino-1-naphthalenesulfonic acid
ANVISA	According to the National Agency for Sanitary Surveillance
ASLT	Accelerated shelf-life test
BSA	Bovine serum albumin
CCP	Critical control point
DASH	Dietary approaches to stop hypertension
DH	Degree of hydrolysis
DPPH	2, 2-Diphenyl-1-picrylhydrazyl
DPP-IV	Dipeptidyl peptidase-IV
EAA	Essential amino acid
EC	Emulsion capacity
EDTA	Ethylenediaminetetraacetic acid
ES	Emulsion stability
FAPGG	N-(3-[2-furyl]acryloyl)-phenylalanylglycylglycine
FC	Foaming capacity
FFA	Free fatty acids
FRAP	Ferric-reducing antioxidant power
FRSA	Free radical scavenging activity
GSH	Reduced glutathione
GSH	Reduced glutathione
H_2O_2	Hydrogen peroxide
HPLC	High performance liquid chromatography
HRSAs	Hydroxyl radical scavenging assays
MW	Molecular weight
NPD	New product development

LIST OF ABBREVIATIONS (CONT.)

$^1O_2$	singlet oxygen
OBC	Oil binding capacity
OPA	Ortho-phthalaldehyde
PC	Protein content
PV	Peroxide Value
PY	Protein yield
ROS	Reactive oxygen species
RSA	Free radical scavenging properties
SBP	Systolic blood pressure
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SI	Swelling index
SIPI	Sacha Inchi protein isolate
SIR	Sacha Inchi residue
S_o	Surface hydrophobicity
SP	Soluble protein
SPHA	Sacha Inchi protein hydrolysate produced by 1% Alcalase
SPHAN	Sacha Inchi protein hydrolysate produced by 0.5% Alcalase/0.5%Neutrase
SPHFl	Sacha Inchi protein hydrolysate produced by 1% Flavourzyme
SPHN	Sacha Inchi protein hydrolysate produced by 1% Neutrase
SPHPa	Sacha Inchi protein hydrolysate produced by 1% Papain
SPHPe	Sacha Inchi protein hydrolysate produced by 1% Pepsin
SSIR	Stabilized Sacha Inchi residue
TBARS	Thio barbituric acid reactive substance
TI	Trypsin inhibition
TNBS	2, 4, 6-trinitro-benzenesulfonic acid
TPC	Total phenolic content
TSS	Total soluble solid
WAI	Water absorption index
WSI	Water solubility index

CHAPTER I

INTRODUCTION

Background

Sacha Inchi, scientifically known as Plukenetia Volubilis, is a perennial oilseed vine belonging to the Euphorbiaceae family, native to the rainforests of South America (Fu et al., 2014). Sacha Inchi seeds contain 25-27% protein and 41-54% oil, which consists of approximately 90% unsaturated essential fatty acids, namely C18:3 ω 3 (α-Ln, cis,cis,cis-9,12,15-octadecatrienoic acid; α-linolenic) and C18:2 ω 6 (L, cis, -cis-9,12-octadecadienoic acid; α -linoleic) fatty acids and is rich in vitamins E and A (Hamaker et al., 1992). Today, Sacha Inchi is one of the most valuable agricultural commodities because of its unique chemical composition and multiple uses as food and feed. Sacha Inchi protein contains a significant amount of essential amino acids, most of which are present in amounts that closely match those required for humans and animals. Furthermore, it also contains many biologically-active components, including tocopherols (Follegatti-Romero et al., 2009), polyphenolic compounds (Fanali et al., 2011) and phytosterols (Bondioli et al., 2006). In addition, the amino acid profile of the Sacha Inchi protein fraction showed a relatively high level of cysteine, tyrosine, threonine, and tryptophan, compared to other oilseed sources (Hamaker et al., 1992). Due to its nutritional value and low cost, Sacha Inchi protein is one of the largest commercially available vegetable protein in the world at present, and it is an important alternative to existing animal-derived proteins. Sacha Inchi proteins are also of particular interest because they impart high functionality in food systems, being used to obtain better quality products.

Industrially, Sacha Inchi seeds are normally used for the extraction of oils, which comprise about half of the seed's weight. The main by-product in oil production is the seed residue, which has a very high content of protein (54.3% [w/w]). However, this nutritive and potentially bioactive byproduct is usually discarded as waste or only used as animal feed. Since Sacha Inchi seed residue also seems to be a valuable source of good quality proteins in terms of their amino acid compositions, it could potentially be used as functional food ingredients. Moreover, stabilization through thermal processing

and enzymatic hydrolysis can improve the functional and biological properties of proteins. Thermal treatment is the oldest and most frequently used method for the modification of proteins. The purposes of thermal modification for proteins are different. Stabilization of the residue through thermal treatments can reduce microbial contamination and protease inhibitor activity, eliminate lipoxygenase and volatile compounds that induce undesirable flavors and also improve product functionalities (e.g., solubility, foaming, gelling, water binding and fat binding properties) (Barac et al., 2004). At higher temperatures, some reactions may take place, such as the breakdown of disulfide (S-S) bonds with a release of H_2S, and NH_3 from amide groups, dissociation of subunits and /or breakdown of these subunits into compounds of small molecular weights, which may be responsible for the enhanced protein solubility (Shimada, & Cheftel, 1988). Heating of protein probably causes dissociation of quaternary structure, releasing smaller peptides and facilitating their solubilization (Yamagishi et al., 1983; Rangavajhyala et al., 1997).

In recent years, enzymatic hydrolysis has been employed to use proteins as ingredients that provide functional properties or act like nutritional supplements (Benitez et al., 2008). The functionality of the peptides formed after hydrolysis will depend on the molecular size, structure and specific amino acid sequence (Klompong et al., 2007). Limited enzymatic hydrolysis with a degree of hydrolysis (DH) <10% has been widely used to improve the functional properties of proteins (solubility, emulsifying power, foaming capacity) without affecting their nutritional value and widely diversifying the use in the food industry (Moure et al., 2006). Enzymatic hydrolysis becomes extensive when DH >10%. Peptides with very low molecular weight can have good solubility, high digestibility, providing high nutritional and even therapeutic value with positive effects on immunomodulatory responses, as antioxidants, antihypertensives, metal ion chelators (Megias et al., 2008), against α-amylase, α-glucosidase, pancreatic lipase, trypsin and chymotrypsin, the main enzymes responsible for carbohydrate, protein and lipid digestion within the human gastrointestinal tract (Awosika, & Aluko, 2019). Therefore, the overall objectives of this research were to study effects of various stabilization processes (extrusion, autoclaving, drum drying, heat-moisture treatment or combined process) on physico-chemical properties, antioxidant activities and shelf life extension of Sacha Inchi residue and to investigate the influence of different

protease enzymes on Sacha Inchi protein hydrolysis performance and their physico-chemical properties, antioxidant activities, inhibitory in vitro activities of angiotensin-1 converting enzyme, dipeptidyl peptidase-IV and peptide fractionation using ultrafiltration membrane technique. The output of this study has the great potential for applications in functional foods and beverages. Investigation on stabilized Sacha Inchi application in pasta making and development of Sacha Inchi protein hydrolysate based beverage products was also studied.

Hypothesis

1. The stabilization techniques through thermal treatments can improve the functional properties (solubility, water absorption, emulsification, fat adsorption and foaming) of Sacha Inchi residue. The thermal process can break disulfide bonds (native structures of proteins) with a release small molecular weights or hydrophobic group, which may be responsible for improve the functional properties and thermal causes irreversible denaturation of the enzymes and proteins, which will lead to eliminate anti- nutritional, microbial and enzyme activity.

2. Enzymatic hydrolysis of Sacha Inchi protein by different proteases enzyme can release various types of small peptides that consists of hydrophobic amino acids like Val, Pro, Met, Phe, Leu and Try. These peptides can regulate important functions through their myriad activities, including antihypertensive, antioxidant, an enzyme inhibitor and improve functional properties. The results will be beneficial to develop the food product.

Objectives

1. To study the effect of various stabilization processes (extrusion, autoclaving, drum drying, heat-moisture treatment or combined process) on physico-chemical properties, antioxidant activities and shelf life extension of Sacha Inchi residue.

2. To study the effect of protease enzymes on Sacha Inchi protein hydrolysis and their physico-chemical properties, antioxidant activities, inhibitory in vitro activities of angiotensin-1 converting enzyme, dipeptidyl peptidase-IV and peptide fractionation using ultrafiltration membrane technique.

3. To develop the food product (pasta) and beverage prototypes based on stabilized Sacha Inchi protein and its protein hydrolysate.

CHAPTER II

LITERATURE REVIEW

Sacha Inchi (Plukenetia volubilis Linneo)

Sacha Inchi also known as Inca peanut, wild peanut, Sacha peanut or mountain peanut, is an oleaginous plant that belongs to the Euphorbiaceae family show in fig 1. It grows in the lowlands of the Peruvian Amazon, having been cultivated for centuries by the indigenous population, as well as in the northern part of Thailand (Gutierrez et al., 2011). The protein content of Sacha Inchi is approximately 27% and it is rich in amino acids such as cysteine, tyrosine, threonine and tryptophan including vitamins E and A (Hamaker et al., 1992). The other bioactive compounds, such as tocopherols (Follegatti-Romero et al., 2009), carotenes (Hamaker et al., 1992), polyphenolic compounds (Fanali et al., 2011) and phytosterols, have been previously reported in SI (Bondioli et al., 2006). In addition, the amino acid profile of the Sacha Inchi protein fraction showed a relatively high level of cysteine, tyrosine, threonine and tryptophan, compared to other oilseed sources (Hamaker et al., 1992). This content is similar to those from sesame seed (approximately 25%), sunflower (24%) and peanut (23%). Except for histidine, SI has all of the essential amino acids in adequate amounts, when compared with the FAO/WHO recommended amino acid pattern (Gutierrez et al., 2011).

A wide range consumption of bioactive is important in our diet in terms of health. Thus, protein and amino acids are very important for the prevention of coronary heart disease, against renin, hypertension, and free radicals (Mundi, & Rotimi, 2014). In addition, numerous health benefits have been attributed to phytosterols, tocopherols, carotenoids and phenolic compounds. Phytosterols have been reported to reduce blood cholesterol and to decrease the risk of certain types of cancer (Lagarda et al., 2006). Tocopherols have vitamin E properties and display a strong antioxidant activity, conferring protection against lipid peroxidation in foods (Hounsome et al., 2008). Phenolic compounds are considered to promote human health, since they are responsible for critical biological functions (Huang et al., 2005).

Figure 1 Visual appearance of Sacha Inchi seeds

Source: Thomson, 2016

Chemical and mineral composition of seeds

The average composition of Sacha Inchi seed (SIS) is presented in Table 1. As seen in this table, the moisture content of SIS (3.3%) was within the range of 0-13% reported to be suitable for storage and processing without microorganism degradation of the triacylglycerols (James, 1995). As expected, SIS were rich in oil (41.1% (w/w)) and crude protein (24.7% (w/w)). The oil extraction yields obtained with hexane (42.0%) were slightly higher but not significantly different than those reached using chloroform-methanol, indicating a low content of polar lipids, such as phospholipids, in the investigated seeds (Gutiérrez et al., 2008). The SIS contains a relatively low value of total carbohydrate (30.9%), due to the high levels of crude oil and protein.

The mineral content of SIS is presented in Table 1. The data show that SIS contains significant amounts of dietary essential minerals, such as magnesium (3210 mg/kg), calcium (2406 mg/kg), and zinc (49 mg/kg), and suggest that SIS could be used in the human diet for supplying these elements. Potassium was the most abundant mineral found in SIS (5563.5 mg/kg), while minor amounts of iron (103.5 mg/kg), sodium (15.4 mg/kg) and copper (12.9 mg/kg) were also presents (Gutierrez, Rosada, & Jimenez, 2011). Although the soil composition is a factor influencing the mineral composition of seeds, when comparing with other seed oils such as cottonseed, linseed, peanut and safflower (Blackwood, 2007), SIS show the highest concentration of Zn, and the lowest content of Na, Cu and Fe. The levels of calcium were similar to those reported by Smith (2007) for safflower seeds (2406 vs. 2140 mg/kg).

Table 1 Chemical composition of the Sacha Inchi seed

Component Value	Seeds
Moisture (%)	3.3±0.3
Fat (%)	42.0±1.1
Protein (%)	24.7±0.5
Ash (%)	4.0±0.7
Total carbohydrate (%)	30.9±0.6
Potassium (mg/kg)	5563.5±6.4
magnesium (mg/kg)	3210.0±21.2
Calcium (mg/kg)	2406.0±7.1
Iron (mg/kg)	103.5±8.9
Zinc (mg/kg)	49.0±1.1
Sodium (mg/kg)	15.4±0.5
Cooper (mg/kg)	12.9±0.3

Source: Gutierrez, Rosada, & Jimenez, 2011

Fatty acid composition of Sacha Inchi oil (SIO)

The fatty acid composition of SIO extracted with hexane is presented in Table 2. No significant differences were found with the fatty acid composition of SIO obtained using chloroform methanol. As it can be appreciated in Table 2, SIO has a unique fatty acid profile, being very rich in a-linolenic (50.8%) and linoleic (33.4%) acids, with low levels of oleic (9.1%), palmitic (4.4%) and stearic (2.4%) acids (Gutierrez, Rosada, & Jimenez, 2011). The fatty acid profile of SIO was relatively similar to that of the seabuckthorn seed oil (Gutierrez et al., 2008), except for the content of a-linolenic acid, which was higher in SIO. Taking into account that the optimal dietary intake of a-linolenic has been suggested to be about 2 g/day in order to achieve essential amounts and cardiovascular benefits (Gebauer et al., 2006), SIO could be used as a source of this essential fatty acid, and be considered in the elaboration of food supplements (Gutierrez, Rosada, & Jimenez, 2011).

Table 2 Fatty acid composition in crude and in lipid fractions of Sacha Inchi oil

Fatty acid	Crude oil	Neutral lipids	Free fatty acids	Phospholipids
Fractions (%)	100	97.2±0.90[a]	1.2±0.70[b]	0.8±0.40[c]
C16:0 (Palmitic)	4.4±0.02[a]	4.4±0.02[a]	5.2±0.20[b]	26.9±0.50[c]
C18:0 (Stearic)	2.4±0.02[a]	2.3±0.15[a]	3.0±0.10[b]	11.7±0.30[c]
C18:1n-9 (Oleic)	9.1±0.01[a]	9.1±0.04[a]	9.4±0.08[b]	9.6±1.50[c]
C18:2 (Linoleic)	33.4±0.04[a]	32.9±0.02[b]	32.3±0.10[c]	40.3±2.70[d]
C:18:3 (a-Linolenic)	50.8±0.03[a]	51.2±0.10[b]	50.1±0.10[c]	11.5±1.30[d]

Note: [a] Values are means ± standard deviations of duplicate determinations. [b] means in the same row followed by the same letter are not significantly different by LSD test.

Source: Gutierrez, Rosada, & Jimenez, 2011

Electrophoretic characterization of Sacha Inchi protein

The soluble seed proteins were characterized by polypeptides in the 10-70 kDa range. The soluble seed flour proteins are mainly composed of two molecular species (32-35 kDa and ~60-62 kDa). Of the total soluble proteins, true albumin, globulin, and glutelin were the major fractions in the seed flour soluble proteins with prolamin contributing in a small proportion. Typically, over 90 % of the seed flour proteins could be solubilized by the aqueous solvents used for protein fractionation. Prolamin fraction was mostly composed of four polypeptides in the molecular weight range 6-12 kDa see Figure 2 (Sathe, Kshirsagar, & Sharma, 2012).

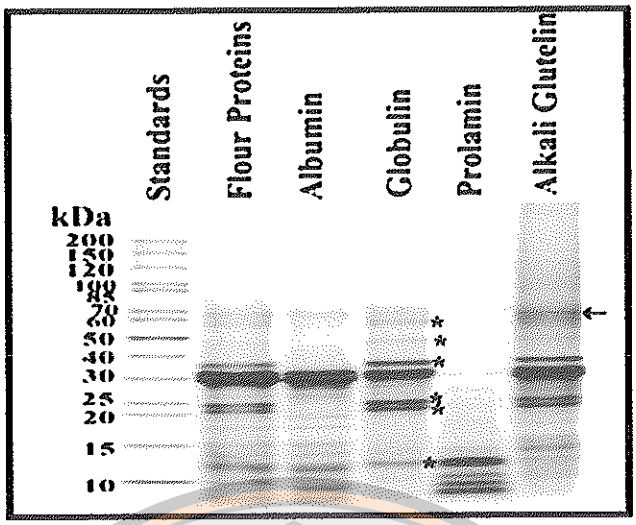


Figure 2 SDS-PAGE (8-25% linear monomer acrylamide gradient) in the presence of a reducing agent for Sacha Inchi protein

Source: Sathe, Kshirsagar, & Sharma, 2012

Amino acid profile of Sacha Inchi protein

Sacha Inchi oil is an important source for the prevention of coronary heart disease and hypertension showing a hypocholesterolemic effect when used as supplements and functional foods. From elsewhere, the high protein content of Sacha Inchi is comparable to, and in some respects better than that of other oilseeds. Hamaker et al. (1992) determined high levels of amino acid profile of Sacha Inchi includes phenylalanine and tyrosine (79 mg/g), leucine (64 mg/g), tyrosine (55 mg/g), isoleucine (50 mg/g), lysine (43 mg/g), cysteine (25 mg/g), threonine (43 mg/g), sulfur amino acids (methionine + cysteine), 37 mg/g, and valine (40 mg/g). Table 3 shows the amino acid profile of Sacha Inchi protein compared to other oilseed. Sacha Inchi protein flour (powder) is a vegetable protein of high quality with excellent composition of essential and non-essential amino acids easily digestible for children and adults in adequate quantity when compared with the patterns of amino acids recommended by the FAO/WHO (JOINT FAO/WHO Expert consultation, 1990). A recent study identified a water-soluble storage protein (albumin) from Sacha Inchi seeds as the first plant protein to date that contains all the essential amino acids required by humans. This

storage albumin accounted for approximately 25% (w/w) of defatted seed flour weight, representing 31% of the total seed protein. Sacha Inchi albumin is a 3S storage protein composed of two glycosylated polypeptides, with estimated molecular weights of 32,800 and 34,800 Da, respectively and a sugar content of 5%. The water-soluble storage albumin is a basic protein and contains all of the essential amino acids in adequate quantity when compared with the patterns of recommendation of FAO/WHO for a human adults. Albumin SIS is a highly digestible protein in vitro, when it is heat denaturation. Sacha Inchi protein seed is a complete, highly digestible, and high quality non-animal protein with the amino acid requirement for adults suitable for vegetarians and vegans that can be used for protein concentrates to build muscle, beverages, yogurt, supplements, vegetarian and vegan diets, vega energizing smoothie among others (Sathe, Kshirsagar, & Sharma, 2012).

Table 3 Amino acid profile of Sacha Inchi protein compared to other oilseed protein

Amino acid	Seeds [a,b]					FAO/WHO /UNU Scoring pattern[c]
	Sacha Inchi	Soybean	Peanut	Cottonseed	Sunflower	
Total Protein, %	27	28	23	23	24	
Essential						
HIS	26	25	24	27	23	19
ILE	50	45	34	33	43	28
LEU	64	78	64	59	64	66
LYS	43	64	35	44	36	58
MET	12	13	12	13	19	
CYS	25	13	13	16	15	
MET+CYS	37	26	25	29	34	25
PHE	24	49	50	52	45	
TYR	55	31	39	29	19	
PHE+TYR	79	80	89	81	64	63
THR	43	39	26	33	37	34
TRP	29	13	10	13	14	11
VAL	40	48	42	46	51	35

Table 3 (cont.)

Amino acid	Seeds [a,b]					FAO/WHO /UNU Scoring pattern[c]
	Sacha Inchi	Soybean	Peanut	Cottonseed	Sunflower	
Non Essential						
ALA	36	43	39	41	42	
ARG	55	72	112	112	80	
ASP	111	117	114	94	93	
GLU	133	187	183	200	218	
GLY	118	42	56	42	54	
PRO	48	55	44	38	45	
SER	64	51	48	44	43	
TEAAd	411	418	349	365	366	
TAA[e]	976	985	945	936	94	
TEAA as percent of TAA	54	42	37	39	39	

Note: [a] Values for soybean, peanut, cottonseed, and sunflower were taken from Bodwell, & Hopkins, 1985

[b] Values shown are mg/g of protein, unless otherwise noted (N X 6.25)

[c] Recommended level for children of preschool age (2-5 years), although recently recommended for evaluation of dietary protein quality for all age groups except infants (Joint FAO/WHO Expert Consultation, 1990)

[d] TEAA = total essential amino acids.

[e] TAA = total amino acids.

Source: Hamaker et al., 1992

Thermal processing of food

The use of high temperatures to preserve and ensure the safety of food is based on the effect of microbial destruction. Thermal processing is one of the most widely used unit operations employed in the food industry and is frequently determined as a Critical Control Point (CCP). The basic purpose for the thermal processing of foods is to reduce or destroy microbial activity, reduce or destroy enzyme activity and to produce physical or chemical changes to make the food meet a certain quality standard e.g. denaturation of proteins to produce edible food. There are a number of types of heat processing employed by the food industry. Sterilization is the destruction of all bacteria including their spores. Heat treatment of such products must be severe enough to inactivate/kill the most heat resistant bacterial microorganisms, which are the spores of Bacillus and Clostridium. The efficiency of the sterilization process depends on two major factors. One of them is the thermal death time, i.e. the time microbes must be exposed to at a particular temperature before they are all dead. The second factor is the thermal death point or temperature at which all microbes in a sample are killed (Adler et al., 1998).

1. Autoclaves

Of all the methods available for sterilization, moist heat in the form of saturated steam under pressure is the most widely used and the most dependable. The basic principle of steam sterilization, as accomplished in an autoclave, is to expose each item to direct steam contact at the required temperature and pressure for the specified time. Thus, there are four parameters of steam sterilization: steam, pressure, temperature, and time. Pressure serves as a means to obtain the high temperatures necessary to quickly kill microorganisms. Specific temperatures must be obtained to ensure the microbicidal activity. The two common steam-sterilizing temperatures are 30 minutes at 121°C (250°F) or 4 minutes at 132°C (270°F) (Agalloco et al., 1998).

2. Extrusion

Extrusion is a thermal processing that involves the application of high heat, high pressure, and shear forces to an uncooked mass, such as cereal foods to obtain a wide range of products including snacks, cereals, confectioneries and crisp breads (Kim, 2006). The shear forces created by the rotating action of the screws, together with frictional, compressive and pressure forces provide the necessary

environment for rapidly cooking and transforming the food into visco-elastic melt (Firibu, 2011). Extrusion cooking may be defined as a thermomechanical process in which heat transfer, mass transfer, pressure changes and shear are combined to produce effects such as cooking, sterilization, drying, melting, texturizing, conveying, puffing, mixing, kneading, conching, forming etc. In addition to the usual benefits of these processes, extrusion offers the possibility of modifying the functional properties of food ingredients and/or of texturizing them. Like other processes for heat treatment of food, extrusion cooking may have both beneficial and undesirable effects on nutritional value. Beneficial effects include destruction of antinutritional factors, increased soluble dietary fibres, reduction of lipid oxidation and contaminating microorganisms and retains natural colours and flavours of foods. In addition, the extrusion process denatures undesirable enzymes and sterilises the finished product (Bjiirck, 1983; Singh, 2007).

3. Drum drying

In the food industry, drum-dryers are typically employed for the production of food powders from viscous slurries. Food powders are used in a wide range of applications in the food industry, from beverage powders, instant soups, spices or flours and flavours (Almena et al., 2019). Drum drying is constrained by the fact that it is only compatible with viscous and sticky products, but is quite energy efficient due to its use of contact drying with steam instead of convective hot air drying. It consists of two large steel cylinders that turn toward each other and are heated from the inside by steam. The concentrated product is applied to the hot drum in a thin sheet that dries during less than one revolution and is scraped from the drum by a steel blade (Courtois, 2013).

4. Heat-moisture treatment

Heat-moisture-treated have important applications in the food industry, especially due to their improved thermal stability (Adebowale et al., 2005). It is physical modifications that change the physicochemical properties of sample without destroying its granular structure. Currently, the growing popularity for physical modifications and are also referred to be safer than chemical possess. The physical method of heat-moisture method is particularly favorable for the food applications with novel functional properties (Maache-Rezzoug et al., 2008). The significance of

this impact was related to the moisture levels during heat treatment and source. These two related processes both require that the source-to-moisture ratio, temperature, and heating time are controlled (Chung et al., 2009b). However, these treatments differ in the amount of water and in the temperature used. HMT is carried out under restricted moisture content (10-30%) and higher temperatures (90-120 ◦C) for a period of time ranging from 15 min to 16 h (Maache-Rezzoug et al., 2008).

Effect of thermal processing on the functional, antinutritional and antioxidant properties

Thermal treatment may also have marked impacts on product functionality (e.g., solubility, foaming, gelling, water binding and fat binding properties). Functional properties affect processing applications, food quality and acceptance, and how ingredients are used in foods and in food formulations (Mahajan, & Dua, 2002). Generally, these properties are contributed by the protein components of foods and are affected by composition, structure, conformation, interactions with other food components, and the environment (Kinsella, & Melachouris, 1976). Protein denaturation occurs during thermal treatment, and the nature and type of the proteins as well as the degree to which they are denatured are important factors which can influence the functionality of pulse flours (Wu, & Inglett, 1974). In common with other food constituents, proteins contribute significantly to the physical properties of foodstuffs, especially through their ability to build or stabilize gels, foams, doughs, emulsions and fibrillar structures. Table 4-5 shows some typical examples of functional properties of proteins in relation to important food systems. Foaming, gelling, and emulsifying properties will be discussed in more detail.

Table 4 Typical functional properties caused by proteins in food systems

Functional property	Mode of action	Food system
Solubility	Protein solvation, pH-dependent	Beverages
Water absorption and binding	Hydrogen bonding of water, entrapment of water (no drip)	Meat, sausages, bread, cakes
Viscosity	Thickening, water binding	Soups, gravies
Gelation	Protein matrix formation and setting	Meat, curds, cheese
Cohesion–adhesion	Protein acts as adhesive material	Meat, sausages, baked goods, pasta products
Elasticity	Hydrophobic bonding in gluten, disulfide bridges in gels (deformable)	Meat, bakery
Emulsification	Formation and stabilization of fat emulsions	Sausages (e.g., Bologna), soup, cakes
Fat adsorption	Binding of free fat	Meat, sausages, doughnuts
Flavor binding	Adsorption, entrapment, release	Simulated meat, bakery, etc.
Foaming	Formation of stable films to entrap gas	Whipped toppings, chiffon desserts, angel cakes

Source: Weder, & Belitz, 2003

The effects of processing a given product may vary considerably with different processing procedures or conditions. An ideal thermal processing procedure should sufficiently inactivate antinutritional factors while simultaneously maintaining the bioavailability of essential amino acids in the product (Van Barneveld, 1993) see table 5. The optimization of efficient thermal processing depends on the combination of temperature, time (duration), moisture content and particle size (Melcion, & van der Poel, 1993). One of the problems associated with most of the seed plants are presence

of antinutritional factors such as trypsin inhibitor, protease inhibitor, phytic acid, saponin, tannins, polyphenol and lectins. Some other antinutritional factors such as polyphenols, phytates, enzyme inhibitors (trypsin, chymotrypsin, α-amylase) and hemagglutinins also responsible to limit its use (Adebowale et al., 2005). These antinutritional factors limit the nutritional properties and affect the digestibility of certain nutrients (Ramakrishna et al., 2006). The loss of nutrients occurs during food preparation and processing, however the processor should limit these losses in order to enhance nutritional quality of food. Different processing techniques are required to inactivate or remove of antinutritional factors, thus enhancing the nutritional quality of seed plants. Heat process is widely used for food preparation, the heat processing has both beneficial and detrimental effects on food products. A good example is that the protein found in legumes became more digestible by inactivating trypsin inhibitor (Arif et al., 2012). Heat treatments improved the nutritional value and quality of legume grains so that the heat treatments are used to enhance the protein quality of legume grains by inactivating the antinutrients factors, mainly the trypsin inhibitor and hemagglutinins (Sathe et al., 1984).

Antioxidant properties of seed plants will be affected by processing temperature during food thermal processing (Dewanto et al., 2002). Food processing involves changes in structural integrity of the plant material and this produces both negative and positive effects on their antioxidant activity in table 5. The antioxidant activity is diminished owing to inactivation of antioxidant compounds caused by different chemical reactions enhanced by the effect of heat. The positive effects of food processing include in some cases transformation of antioxidants into more active compounds, such as the deglycosylation of onion quercetin, as well as an increase in the antioxidant activity owing to inhibition of enzymes (Wim, 2002). Thermal processing of tomatoes enhances their nutritional value by increasing the bioaccessible lycopene content as well as the total antioxidant activity (Kerkhofs, 2005). The effect of heating on antioxidant effectiveness and the chemical composition of basil, cinnamon, clove, nutmeg, oregano and thyme essential oils was studied on scavenging of DPPH radical.

Table 5 Examples showing the effect of thermal process on physico-chemical properties

Raw materials	Process conditions	properties		Reference
		Increase	Decrease	
Corn gluten meal	Extrusion processing (feed moisture content; 15% w.b., barrel temperature;100, 120,150°C)	1. protein digestibility 2. water holding capacity 3. protein solubility		Bhattacharya, & Hanna, 1988
Australian sweet lupin seed coat	Extrusion (feed moisture content; 25% w.b., barrel temperature; 100, 110, 120°C)	1. soluble dietary fiber 2. water solubility 3. protein solubility	1. insoluble dietary fibre 2. water binding capacity	Zhong et al., 2019
Sorghum-soya blends	Extrusion (moisture content; 12-20% w.b., barrel temperature; 110, 125, 150°C)		1. phytic acid 2. tannin 3. trypsin inhibitor activity	Kumar et al., 2018
Purple-flesh sweet potato flours	Extrusion (moisture contents; 10%, 13%, 16% and screw speeds; 250, 325, 400 rpm.)	1. water holding capacity 2. water solubility indices 3. total phenolic content 4. antioxidant activities (DPPH and ABTS)		Soison et al., 2014

Table 5 (cont.)

Raw materials	Process conditions	properties		Reference
		Increase	Decrease	
Chick pea	Autoclaving (pressure; 121°C for 15, 30 min)		1. phytic acid 2. tannin 3. trypsin inhibitor activity	Sharma et al., 2016
Soy bean	Moist heat treatment (feed moisture content 20% w.b., temperature of 60°C)	1. protein solubility		Zhang et al., 2017
Soy bean	autoclaving (pressure;121°C for 10 min)	1. protein concentration 2. protein solubility	1. microorganisms	Zhang et al., 2018
Rice berry flour	Drum drying (temperature;110, 120,130 °C)	1. water absorption index 2. swelling power	1. water solubility index 2. total phenolic content 3. antioxidant activity	Wiriyawattana et al., 2018
Purple-flesh sweet Potato flours	Drum drying (temperature;120, 130, 140°C)	1. total phenolic content 2. antioxidant activities (DPPH and ABTS)		Soison et al., 2014

Shelf-life of a product determined

The shelf-life, literally the "life of the product on the shelf" is the period of time during which the product maintains its qualities under normal conditions of storage and use. The shelf life is closely related to how long the food will last, which can be expressed either as a "use by date" or a "best before date". These two phrases may seem similar but in fact they have very different meanings. Indeed, when the shelf life is preceded by the phrase "use by date" it is, by law, a strict expiration date beyond which the product is no longer safe and does not maintain its distinctive properties. While when the shelf life is preceded by the phrase "best before date" it is a less restrictive expiration date, a rough indication of the date of minimum durability for the product (Claessens, 2015). A best before date is applicable to food where deterioration affects consumer acceptance without impacting on health and safety. Many product changes will affect consumer acceptance, including: rancidity, enzymatic browning or microbial spoilage (Roberts et al., 1996). The end point can be indicated from relevant food legislation, guidelines provided by government or professional organizations, or the use of acceptable industry practices. Often acceptability limits are chosen based on self-determined end points. For the most part, the food industry relies on sensory perception as an indicator of product failure. Product acceptability may be determined when there is a significant difference in the aging sample compared to a fresh sample by using discrimination testing. Descriptive analysis with expert panelists describes the change in sensory attributes (e.g. odor, taste, appearance, and texture) and can indicate consumer rejection. Lengthy real-time studies have led food processors to seek methods that accelerate shelf-life testing. One of the most common methods to accelerate oxidative reactions is to store product at elevated temperature. For simple systems, such as fat and oil, there is a direct relationship between oxidation rate and temperature. This mathematical equation can be used only if there is a correlation between the storage behavior under normal conditions and under accelerated conditions. In reality, foods are more complex and reactions may occur that would not proceed at normal temperature storage. Increasing storage temperature may lead to changes that affect the deterioration process such as increased water activity, denaturation of proteins, and decreased solubility of gases. Relative humidity may also affect reaction rate (Zweep, 2018).

Production of protein enriched pasta

Pasta is cheap to produce and very convenient to prepare, which makes it one of the most popular high-carbohydrate food products, which can be bad for you when consumed in large amounts (Wood, 2009). It also contains gluten, a type of protein that causes issues for those who are gluten-sensitive. On the other hand, pasta can provide some nutrients that are important to health. Pasta is a type of noodle that's traditionally made from durum wheat, water or eggs, contains carbohydrates from 74% to 77% and protein from 11% to 15% (Shewry, 2007). It is formed into different noodle shapes and then cooked in boiling water. Most people prefer refined pasta, meaning that the wheat kernel has been stripped of the germ and bran along with many of the nutrients it contains. Refined pasta is higher in calories and lower in fiber. This may result in decreased feelings of fullness after you eat it, compared to eating high-fiber, whole-grain pasta. However, components that increase nutritional value or exert a beneficial effect on health can be used in its production (Sun-Waterhouse, & Wadhwa, 2014).

Due to high nutritional value, Sacha Inchi meal obtained from them may be considered a valuable protein additive in the production of food. They contain significant amounts of protein content (54%).

Protein hydrolysates

Today, we have four ways to get amino acids into the bloodstream: 1) whole food proteins: 2) intact protein supplements, 3) free form amino acids, and 4) protein hydrolysates. Protein can be hydrolyzed, producing small chains of amino acids called peptides. This process mimics our own digestive actions thus making it an ideal way to process protein (Manninen, 2002). Protein hydrolysates are produced from purified protein sources by heating with acid or preferably, addition of proteolytic enzymes, followed by purification procedures (Bucci, & Unlu, 2000). Protein digestion to produce hydrolysates or bioactive peptides can be carried out via enzymes, acid or alkali. Acid and alkali hydrolysis (both termed as chemical hydrolysis) tend to be difficult processes to control and yield products with reduced nutritional qualities. For example, acidic treatment destroys tryptophan, glutamine, and asparagine, while alkali treatment destroys cysteine, serine, threonine and produce toxic by-products (e.g. lysine-alanine

and D-amino acids) (Clemente, 2000). On the other hand, enzymatic hydrolysis enables a more efficient tailoring of peptide products without the formation of toxic products or destruction of amino acids. It is also the most common and preferred method because of its moderate cost, high quality of the end products and also due to the fact that the overall amino acid composition of enzymatic protein hydrolysates is similar to that of the starting material (Aluko, 2008; Udenigwe, & Aluko, 2012). Enzymatic hydrolysis of the parent protein is performed using either single or multiple proteases to release peptides of interest (Udenigwe, & Aluko, 2012). Proteolytic enzymes hydrolyze the peptide linkage between amino acids to yield a mixture of peptides with different molecular sizes (Li-Chan, 2015). Proteolytic enzymes are classified into 2 groups according to their mechanism of hydrolysis: exoproteases and endoproteases. Endoproteases hydrolyze the peptide bonds within protein molecules to produce relatively large peptides, while exoproteases systematically remove amino acids from either the N-terminus or the C-terminus by hydrolyzing the terminal peptide bonds (Gofferje et al., 2015). Other digestive enzymes and different enzyme combinations of proteinases such as Alcalase, Chymotrypsin, Pancreatin, Thermolysin as well as enzymes from bacterial and fungal sources have also been used for the generation of these bioactive peptides (Korhonen, & Pihlanto, 2006). Enzymatic hydrolysis works without destructing amino acids and by avoiding the extreme temperatures and pH levels required for chemical hydrolysis, the nutritional properties of the protein hydrolysates remain largely unaffected (Celus, 2007). Production of protein hydrolysates in the food industry involves the use of digestive proteolytic enzymes from animals including chymotrypsin, trypsin and pepsin, or food grade enzymes obtained from plants and microorganisms which are regarded safe for human nutrition. The first category consists of fractions with a high amino acid content. The second category consists of bioactive peptides with an amino acid sequence which is inactive in the intact protein molecule but becomes active in the hydrolysate following exposure to digestive and/or proteolytic enzymes. Protein hydrolysates have been defined as "mixtures of polypeptides, oligopeptides and amino acids that are manufactured from protein sources using partial hydrolysis" (Schaafsma, 2009). The considerably greater absorption rate of amino acids from the dipeptide than from the amino acid mixture appears to be the result of uptake by a system that has a greater transport capacity than

amino acid carrier system, thus minimizing competition among its substrates (Di Pasquale, 1997). The effect of bioactive peptides on major body systems. It has emerged that protein hydrolysates have many uses in human nutrition; ingredients in energy drinks, weight-control and sports nutrition products (Frokjaer, 1994), sources of nutrition for elderly and immuno-compromised patients (Nagodawithana, 2010). Protein hydrolysates can have a bitter taste and the elimination or reduction of this bitterness is essential to make the hydrolysates acceptable to consumers. The bitterness of protein hydrolysates is attribuatable to their hydrophobic amino acid content (Schaafsma, 2009) and the release of these amino acids by exoproteases can reduce bitterness (Pedersen, 1994).

Enzymatic hydrolysis is the process of breaking down the peptide bonds of the parent protein using proteases, whereas the degree of hydrolysis is the ratio of the number of peptides cleaved that occur during hydrolysis to the number of peptide bonds contained in the protein mass (Nasri et al., 2013). Various enzymes are used for protein hydrolysis, among which, Alcalase is generally used due to the high degree of hydrolysis that can be obtained in a relatively short time under moderate pH conditions compared to other neutral or acidic enzymes (Ovissipour et al., 2009). The degree of hydrolysis (DH) is generally used as a proteolysis monitoring parameter and is the most widely used as an indicator for comparison among different protein hydrolysates (Gimenez et al., 2009). According to Li et al. (2013), the degree of hydrolysis could affect the functional properties of the hydrolysates produced

Sacha Inchi protein denaturation and associated molecular interactions

Sacha Inchi protein structure can be modified by different treatments to improve specific functional properties. It has been demonstrated that the functionality depends on the degree of dissociation or denaturation. Protein denaturation is any change of original native structure without alternation in sequence of amino acids (Adler-Nissen, 1976). Denaturation occurs because the bonding interactions responsible for the secondary structure and tertiary structure are disrupted; these include electrostatic, hydrophobic interactions and hydrogen bonding, covalent bonds and ionic bonds (Cramp, 2007).

1. Electrostatic bonds

Electrostatic interactions are known to play a crucial role in protein structure and function (Roy, & Taraphder, 2007). Electrostatic interactions are collectively known as Van der Waals forces and include dipole-dipole interactions, London dispersion forces, and hydrogen-bonding. These forces are less than 1/6 as strong as covalent or ionic bonds (Cramp, 2007). Estimation of electrostatic bonds is the heart of any theoretical modeling of proteins. The electrostatic interaction between proteins or peptides can be reduced by addition of 0.3M NaCl; the counter- ions of NaCl interrupt electrostatic interactions leading to the breakdown of electrostatic bonds (Damianou, & Kiosseoglou, 2006; Zhong et al., 2006).

2. Ionic bonds

Ionic bonds are very strong and occur in proteins when salts are present. These are created by the attraction between the positive and negative charges on salts (Na^+, Cl, K^+ and Ca^{2+}). The charges created by salts can effect on functional properties of soy protein (Cramp, 2007).

3. Covalent bonds

Covalent linkages of amino acids in protein are largely limited to the peptide bonds. The most common exception to this rule is the disulfide bond, S-S chemical bond that results from an oxidative process that links nonadjacent (in most cases) cysteines of a protein (Kadokura et al., 2003). Disulfide bonds are covalent bonds that may break and form under appropriate thermal conditions, such as the disulfide bonds between 11S acidic and basic subunits in soy protein (Cramp, 2007). Identification of covalent (disulfide) bonds can be doneby dispersing protein samples in solvent containing reagent of 0.2M 2-Mercaptoethanol, which reduces disulfide bonds to sulfhydryl groups (Zhong et al., 2006).

4. Hydrophobic bonds

Hydrophobicity is defined as the molecular driving force behind many important biological processes, such as protein folding (Li et al., 2007). Hydrophobic interactions are due to repulsion of water by hydrophobic molecules (Cramp, 2007). Identification of hydrophobic bonds can be done by dispersing protein samples in solvent containing 8M urea. Zou et al. (1998) reported that urea binds to amide groups

through hydrogen bonds, decreasing the hydrophobic effect through dehydration of protein molecules.

Properties of Sacha Inchi proteins in food systems

The main functional properties of Sacha Inchi proteins are solubility, emulsification, and foaming. Sacha Inchi proteins in their various forms have functional properties that make them useful in food systems.

1. Protein solubility

Solubility is the most important and generally the first techno-functional property examined during the development of new protein ingredients due to its considerable effect on other techno-functional properties (Vojdani, 1996). It has been proposed that reduction of the secondary structure of a protein and the enzymatic release of smaller polypeptide units are responsible for the increased solubility of hydrolysates compared to the original intact protein (Adler-Nissen, 1986). Protein solubility is probably its most important property in foods, not only because soy ingredients must form stable dispersions when incorporated into beverages and other food systems, but also because other functionalities, such as gelling, emulsifying and foaming, are closely associated with solubility (Wang, & Johnson, 2001). Solubility is the amount of a solute that can be dissolved in solvent. Thermodynamically, the protein solubility is the protein concentration in the solvent in a sample or two-phase system in balance state. Mathematically, the solubility degree of a protein is the amount of protein present in liquid phase in relation to the total amount of protein in liquid and solid phases (Hall, 1996). The protein solubility also can be defined as a certain quantity of the protein retained in the supernatant after the dispersion has been centrifuged for a certain time period (Pelegrine, & Gasparetto, 2005). Knowing the solubility profile of soy proteins in various environmental conditions is important to the industry in evaluating other physicochemical and functional properties in order to screen them for potential applications. Solubilities of Sacha Inchi products are highly dependent on the physicochemical states of protein molecules, which are either favourably or adversely affected by heating, drying, and other processing treatments during their manufacture and storage. This property is therefore one of the most widely used characteristics of protein products. The solubility characteristics of Sacha

Inchi protein products can be influenced by a wide range of environmental conditions, including pH, ionic strength, and temperature (Lee et al., 2003).

2. Emulsifying ability

Commercial preparations of protein may cause physical and chemical changes that in turn affect the proteins functional properties. Consequently, different commercial varies widely in their emulsifying properties reflecting their difference in composition, conformation, net charge and structure. An emulsion is formed from one immiscible phase distributed as small droplets in the matrix of a second phase by means of an emulsifying agent. The dispersed phase is the discontinuous phase and the dispersion medium is the continuous phase. Emulsions are characterized by the presence of at least one polar hydrophilic liquid and at least one lipophilic liquid (Al-Malah et al., 2000). The two basic types of emulsions are dispersions of a lipophilic or oil phase in a hydrophilic or watery phase or versa. With oil and water being the most common liquids for the preparation of food emulsions, these basic types of emulsions are referred to as oil-in-water (O/W) emulsions and water-in-oil (W/O) emulsions, respectively. More complex types consist of three or more phases, which can be achieved by e.g. dispersing a W/O emulsion into a second watery phase, leading to a water in-oil-in-water (W/O/W) (Schubert et al., 2006). However, many factors in the surrounding environment such as pH, ionic strength, temperature and presence of other components affect functional behaviour, making it very difficult to predict protein emulsifying ability in a given system (Elizalde et al., 1996). Food emulsion properties depend on several factors such as temperature, homogenization pressure, food compositions, type and concentration and of emulsifier and/or stabilizer (Peamprasart, & Chiewchan, 2006). The ability of proteins to interact with lipids and form stable emulsions is essential to yield a stable food product. Rapeseed protein hydrolysates have higher emulsifying activity (at least 20% greater) and stability than rapeseed protein isolates. It is generally accepted that limited hydrolysis improves the emulsification properties of proteins by exposing hydrophobic amino acid residues (which may interact with oil), while the hydrophilic residues interact with water (Vioque et al., 2000). Similarly, an increase in hydrophilicity as a result of acid modification has been shown to increase the emulsification activity index (EAI) of okara protein isolates (Chan, & Ma, 1999).

The formation of a food emulsion is dynamic, yet thermodynamically unfavourable, process due to the increase in interfacial area following emulsification. Hence, after enough time, any emulsion will collapse as the two phases attempt to minimize contact area. There are five main mechanisms, which can contribute to emulsion instability: 1) creaming; 2) flocculation; 3) Ostwald ripening; 4) (partial) coalescence; and 5) phase inversion. Creaming (or settling) is due to density differences between two phases under the influence of gravity, which leads to phase separation. Flocculation is best described as the aggregation of particles due to weak attractive forces between colloids (Figure 3). Ostwald ripening is the growth of larger droplets at the expense of smaller ones and is related to the solubility gradient found between small and large droplets. Partial coalescence is two colliding droplets which form single large droplet (Figure 32). Phase inversion is where an oil-in-water (O/W) emulsion becomes water-in-oil (W/O) emulsion, as during the churning of butter (Rousseau, 2000).

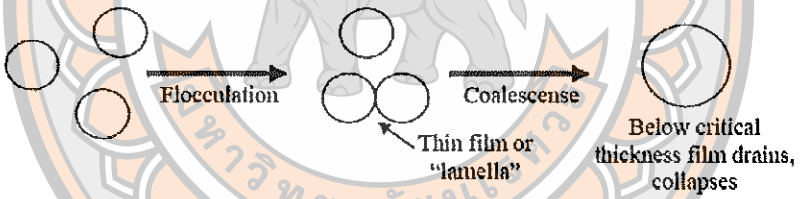


Figure 3 Schematic presentations of flocculation and coalescence

Source: Cramp, 2007

3. Foaming

Foaming is of special interest in the food industry as it provides a desirable and unique texture to a range of aerated foods and beverages. It is essential that food foams are stable for consumer acceptability, since consumer perception of quality is influenced by appearance. The absence of large protein components, which function to stabilise the foam, may contribute to the observed lack of foam stability (Claver, & Zhou, 2005). The foam was also most stable at DH of 5%, with 40% of foam volume sustained after 60 minutes. There was an inverse relationship between DH and foam

stability, with stability in the order of DH 5% > DH 10% > DH 15%. Similar to suggestions by Claver, & Zhou (2005), the stability of the foams was attributed to the presence of larger component proteins and a partial hydrolysis, whereas a higher DH increases the number of polypeptide chains which do not have the ability to stabilise foams (Kong, 2007). Regarding the effect of pH on foaming, barley hydrolysates had greater foam stability at basic pH values and very low stability at acidic pH (Yalcın, 2008).

Hydrophobicity of protein

Hydrophobicity is usually understood as measuring the relative tendency of a protein to prefer a non-aqueous to an aqueous environment. It is also defined as a measure of the tendency of proteins to aggregate in solution. Both measures express some kind of phobia of the analytes towards the aqueous medium. Hydrophobicity is one the most important structure-related factors influencing functional properties of proteins and surface hydrophobicity is significantly correlated with protein gelation properties. The fluoresceient probe (with 1-anilino 8- naphthalene sulfonate "ANS") method is widely adopted in researches to determine the surface hydrophobicity due to simplicity and high sensitivity. Wanger et al. (2000) observed that the lower the solubility, the lower the surface hydrophobicity exposed by the proteins. This could be explained in two ways: (a) the protein species undergoing aggregation are more hydrophobic, so that only the hydrophilic ones remain soluble; and (b) as the proteins aggregate they hide or occlude the hydrophobic zones, leaving part of the proteins as soluble aggregates of low surface hydrophobicity. Hydrophobicity is a major factor that controls protein solubility. Nakai (1983) observed an increase in the hydrophobicity upon heating of proteins, indicating unfolding of the molecules.

Bioactive peptides

Dietary proteins are a rich source of energy and amino acids, which are essential for growth and maintenance; they are also known to carry a wide range of nutritional, functional and biological properties (Korhonen, & Pihlanto, 2006). Bioactive peptides can be defined as "specific protein fragments that have a positive impact on body functions or conditions and may ultimately influence health" (Kitts, & Weiler, 2003).

These peptides influence health in a number of ways and are known to have multifunctional properties (Meisel, & FitzGerald, 2003). The bioactivity of a peptide is dependent primarily on its structural properties (e.g. chain length) and physicochemical characteristics of the amino acid residues (e.g., hydrophobicity, molecular charge) (Udenigwe, & Aluko, 2012). Bioactive peptides can be used in the formulation of therapeutic products and have been identified as health-promoting agents against various human health and disease conditions (Aluko, 2008; Udenigwe, & Aluko, 2012). Research shows that application of bioactive peptides are preferred as therapeutic ingredients or health-promoting agents because they do not have the toxic or severe negative side effects that are associated with therapeutic drugs, even though they may have less physiological effectiveness (Aluko, 2008). The use of bioactive peptides in the formulation of diets or therapeutic products have advantages such as their low cost, safety and the additional nutritional benefits they provide (Sarmadi, & Ismail, 2010). However, the function of these bioactive peptides is dependent on their structure as well as their absorption and bioavailability in the target tissues (Udenigwe, & Aluko, 2012). Peptides are beneficial because they can cross the digestive epithelial barriers and travel to the site at which they have an impact (Li et al., 2006). Protein absorption in the form of short chain peptides is considered a more efficient method of amino acid absorption compared with an equivalent amount of free amino acids. This is due to the availability of peptide-specific transport systems and the fact that peptides are less hypertonic than free amino acids mixtures, enabling good absorption of other dietary components and eliminating osmotic problems (Clemente, 2000; Penas et al., 2006).

Bioactive peptides are encrypted within the primary structure of proteins and thus have to be released for them to become active, as evidence suggests that protein hydrolysates and peptides have a higher bioactivity in comparison to their parent protein (Aluko, 2008; Udenigwe, & Aluko, 2012). Bioactive peptides remain inactive as long as they remain within the primary structure of their parent protein; they only become active when liberated. Bioactive peptides are produced or released from their parent protein via various methods such as microbial fermentation, food processing and enzyme-catalyzed proteolysis invitro or within the gastrointestinal tract after human consumption (Aluko, 2008; Udenigwe, & Aluko, 2012). Most bioactive peptides are produced via hydrolysis (by enzyme, alkali or acid treatment) (Sarmadi, & Ismail,

2010; Singh et al., 2014). Studies that investigate health effects of bioactive peptides apply them in 2 different forms either as hydrolysates of precursor proteins or as bioactive peptides (Sarmadi, & Ismail, 2010). Hydrolysate is a mixture that is mainly composed of peptides and amino acids which are produced via protein hydrolysis; on the other hand, bioactive peptides are several linked amino acids purified from hydrolysates (Sarmadi, & Ismail, 2010). In cases where protein hydrolysis is induced by endogenous proteases, the term "autolysate" is usually used rather than hydrolysate (Sarmadi, & Ismail, 2010). Enzymatic protein hydrolysates contain short-chain peptides with characteristic amino acid composition and defined molecular size. They are composed of free amino acids, short peptides (di and tripeptides) and longer peptides that normally contain between 2-20 amino acid residues (molecular mass ≈ 1500-6000 Da) (Singh et al., 2014; Capriotti et al., 2015). Protein hydrolysates show technological advantages such as improved solubility, heat stability and relatively high resistance to precipitation by many agents such as pH or metal ions (Alashi et al., 2013; Tavano, 2013).

Functions of bioactive peptides

The interest of bioactive peptides in foods lies in their potential pharmaceutical and/or nutraceutical benefits (Maestri et al., 2016). Bioactive peptides have been documented to possess activities and/or functions that are beneficial to human health (Lafarga, & Hayes, 2014); thus, they have attracted substantial interest from researchers. The health benefits attributed to bioactive peptides are based on their inherent amino acid composition and sequence as well as the size of active sequences, which vary from 2-20 amino acid residues (Korhonen, & Pihlanto, 2006). They have been shown to affect body systems by possessing potent biological activities such as antioxidant, antihypertensive, antithrombotic, immunomodulatory, anticancer, antimicrobial, mineral binding, opiate-like and lipid-lowering activities (Aoife et al., 2013; Moughan et al., 2014; Lafarga, & Hayes, 2014) (see in Table 6).

1. Reactive oxygen species, oxidative stress and antioxidants in human health

An inevitable consequence of everyday metabolic processes is the production of reactive oxygen species (ROS) such as the non-radical hydrogen peroxide (H_2O_2) or singlet oxygen ($^1O_2$) as well as free radicals like superoxide anion

($O_2^{\cdot-}$) or hydroxyl radicals ($^{\cdot}OH$) (Sharma et al., 2012; Udenigwe et al., 2017). ROS are also generated when growth factors and cytokines act to facilitate intracellular signalling (Storz, 2011) from exogenous sources such as diet (Cardoso et al., 2013) as well as during cellular response to xenobiotics and stressful or adverse environmental conditions like metal toxicity, UV-B radiation, extreme temperatures and microbial pathogen attack (Ray et al., 2012; Sharma et al., 2012). For instance, alcohol consumption is known to promote ROS generation (Wu, & Cederbaum, 2003) while high fat diets and the accompanying steatosis are often associated with increased ROS production (Cardoso et al., 2013). When produced either in excess or in considerable amounts for prolonged periods, ROS can lead to oxidative stress and overwhelm the body's endogenous antioxidant defence system causing structural and function-related damages to macromolecules like DNA, lipids and proteins, and impairing physiological processes such as membrane fluidity, enzyme activity, ion transport, protein synthesis and protein cross-linking (Sharma et al., 2012; Storz, 2011). The generation of excess ROS in cells has the potential to upset intracellular redox homeostasis and often marks the onset of oxidative stress, thus orchestrating a cascade of mostly aberrant cellular events including cellular and organismal aging, senescence and ultimately, apoptosis (Ray et al., 2012; Storz, 2011). As a result of ROS-mediated damage of biological macromolecules, oxidative stress is often linked with the onset and progression of diseases, conditions and processes like diabetes, atherosclerosis, carcinogenesis, aging, inflammation and chronic neurodegeneration (Lobo et al., 2010; Ray et al., 2012; Wu, & Cederbaum, 2003). In fact, oxidative stress is believed to play key roles in neurological disorders (muscular dystrophy, Alzheimer's disease, Parkinson's disease), inflammatory diseases (glomerulonephritis, arthritis, vasculitis), ischemic diseases (stroke, heart diseases), as well as gastric ulcers, AIDS, emphysema and hemochromatosis among others (Lobo et al., 2010).

The human body is equipped with a robust antioxidant defence system to counteract the effects of ROS produced during normal physiological processes as well as in extreme metabolic events. This system includes a battery of antioxidant enzymes such as catalase, superoxide dismutases and enzymes of the glutathione system (glutathione peroxidases, glutathione reductase, glutathione S-transferases), in addition to non-enzymatic antioxidants like ascorbic acid, vitamin E, glutathione and melatonin

(Gorlach et al., 2015; Lobo et al., 2010; Storz, 2011). the food industry, food products have been supplemented with synthetic antioxidants such as propyl gallate, butylated hydroxyanisole, butylated hydroxytoluene and tert-butyl hydroquinone in order to enhance their antioxidative potential and as preservatives to curtail oxidation-mediated rancidity (Ni et al., 2000). The protection against oxidative stress provided by dietary components typically found in fruits and vegetables such as such as polyphenols, ascorbic acid and carotenoids, as a result of their antioxidant properties is well documented (Crowe et al., 2011; Fiedor, & Burda, 2014; Genkinger et al., 2004; Padayatty et al., 2003; Pandey, & Rizvi, 2009). More recently, bioactive peptides derived from food proteins have received significant research attention as a result of their antioxidant function (Ahmed et al., 2015; Irshad et al., 2015; Mechmeche et al., 2017; Aloglu, & Oner, 2011). These food protein-derived antioxidative peptides are obtained following enzymatic hydrolysis, gastrointestinal digestion, food processing and/or microbial fermentation, and are different from the antioxidant peptides naturally present in foods.

2. Antioxidant peptides and hydrolysates from food proteins

Over the years, studies have reported the isolation of antioxidant peptides and proteins from multiple and diverse food protein sources. These include chickpea, rice endosperm, peanut kernels, soy, whey, algae, alfalfa leaf, grass carp muscle and sunflower protein. To generate these antioxidant peptides, proteases as varied as Pepsin, Pancreatin, Alcalase, Collagenase, Flavourzyme, trypsin, chymotrypsin, papain, Neutrase, Thermolysin, Esperase, Flavorase and proteinase K have been used (Samaranayaka, & Li-Chan, 2011). In addition, antioxidative peptides have also been generated following (simulated) gastrointestinal digestion, fermentation or food processing. The digestion of hempseed proteins using pepsin and pancreatin, the resultant crude protein hydrolysates were found to exhibit substantial metal chelation activity (>70%), while there were notable increases in the ferric reducing power, 2,2- diphenyl-1-picrylhydrazyl and hydroxyl radical scavenging activities of the peptide fractions obtained after ultrafiltration of the hydrolysates (Girgih et al., 2011b). It has also been reported that a novel tetradecapeptide obtained from the simulated gastrointestinal digestion of mussel protein significantly inhibited lipid peroxidation more than two other antioxidants viz ascorbic acid and α-tocopherol (Jung et al., 2007). It has been suggested that the exact

contribution of individual amino acid residues to the antioxidative property of a peptide is primarily determined by the nature of the ROS the peptide is exposed to and the reaction medium involved, although the issue of the contribution of such discrete amino acid residues to the antioxidant property of the kind of peptide mixtures typically present in food protein hydrolysates remains unsettled. Since amino acid residue mixtures used in simulating the amino acid composition of antioxidant protein hydrolysates have been reported to not be as potent as peptide antioxidants, it is likely that the synergistic effects of peptide amino acids and their order of arrangement on the peptide chain are more powerful than the individual contributions of similar but free amino acids (Aluko, 2012). According to Elias et al. (2008), antioxidant activity of proteins has been related to their amino acid composition. However, such property is limited by the tertiary structure, because many amino acids with antioxidant potential can be buried within the protein core where they are inaccessible to prooxidants. Therefore, enzymatic hydrolysis favors the exposure of antioxidant amino acids in proteins, increasing antioxidant activity of peptides. Several factors affect the antioxidant properties of protein hydrolysates, and depend on: type of protein and enzyme, degree of hydrolysis and pretreatment of the substrate (Polanco-Lugo et al., 2014). Some authors have observed antioxidant activity in hydrolyzed soybean proteins due to the exposure of amino acids, resulting in high free radical scavenging activity, reducing power, inhibition of lipid oxidation and metal chelation capacity (Sanjukta et al., 2015). Nevertheless, the total antioxidant activities of seed cannot be evaluated by any single method, due to the complex nature of samples. Two or more methods should always be employed in order to evaluate the total antioxidant effects of seeds (Georgetti et al., 2006). The capacity of antioxidant compound for scavenging free radicals should be assessed by two factors, that is, rate of scavenging radicals and number of radicals each antioxidant molecule can scavenge, which are determined inherently by the chemical structure of the antioxidant compound and also the free radicals. These two parameters can be measured by following the reaction with stable reference free radical such DPPH and ABTS, negatively and positively charged free radicals, respectively. In addition, a potent radical scavenging antioxidant often acts as a potent reductant (Re et al., 1999). The FRAP method measure the ability of antioxidant to reduce ferric Fe^{3+} to their respective lower valency state (Benzie, & Strain, 1996).

3. Mechanisms of action of food protein-derived antioxidant peptides

Although specific mechanisms of action of bioactive peptides are yet to be clearly elucidated, their antioxidant properties have been demonstrated using a collection of in vitro and in vivo antioxidant assays. Such tests include free radical scavenging assays, inhibition of oxidative semicarbazide-sensitive amine oxidases, and inhibition of ROS-induced oxidation of biomolecules like nucleic acids, proteins and lipids (Udenigwe et al., 2017). Thus, it has been suggested that in general, the mechanisms of action of bioactive peptides could involve one or more of the following: (i) quenching ROS by participating in hydrogen atom transfer (HAT) or single electron transfer (SET) reactions, (ii) enhancing the antioxidative function of components of the endogenous antioxidant defence system including catalase, vitamin C, glutathione and superoxide dismutase, (iii) preventing ROS generation by inhibiting the activity of certain prooxidant enzymes, and (iv) chelating metal ions involved in free radical formation (Dai, & Mumper, 2010; Girgih, 2013; Huang et al., 2005). Although the notion is widely accepted that peptides are more effective antioxidants than their individual amino acids (since the former contain electron-donating bonds), certain reports have also suggested that the metal ion chelation, ferric reducing power and free radical scavenging capacity of some free amino acids could be greater than those of their precursor peptides (Erdmann et al., 2008; Kitts, & Weiler, 2003; Tang et al., 2009). A number of other factors including degree of hydrolysis, hydrolysis temperature, hydrolyzing enzyme specificity, peptide chain length, amino acid composition and hydrophobicity are all known to influence the antioxidant bioactivity of peptides and protein hydrolysates (Pihlanto, 2006; Udenigwe et al., 2017). For instance, apart from their contribution to the endogenous antioxidant defence system as a result of being used for synthesis of the principal intracellular antioxidant and redox buffer compound (glutathione), cysteine-containing peptides are also known to be potent antioxidants due to the presence of the thiol functional group in sulfur-containing amino acids (Meisel, 2005). It has been suggested that the electronrich aromatic rings of tyrosine, tryptophan and phenylalanine residues in peptides can facilitate the chelation of pro-oxidant metal ions by such peptides, with the aromatic ring of phenylalanine in particular being effective in the scavenging of hydroxyl radicals for the formation of more stable para-, meta- or ortho-substituted hydroxylated

derivatives thus raising the possibility for a role in the attenuation of post-ischemic dysfunction (Aluko, 2012; Sun et al., 1993). Additionally, the hydrophobicity of non-polar amino acid residues such as histidine, tryptophan and tyrosine are thought to contribute to the antioxidant potential of hydrophobic peptides by increasing the accessibility of such peptides to hydrophobic cellular targets such as the unsaturated fatty acids in biological membranes (Aluko, 2012; Erdmann et al., 2008).

4. Hypertension, antihypertensive peptides

The term hypertension or high blood pressure is used to describe a medical condition involving protracted elevated blood pressure during which blood is constantly pumped through the blood vessels with excessive force (WHO, 2011). In general, a hypertension diagnosis is made if during a period of sustained elevated arterial blood pressure, a systolic blood pressure (SBP) measurement ≥140 mmHg and/or a diastolic blood pressure reading ≥90 mmHg is obtained (Pickering et al., 2005). High blood pressure is also linked to mild cognitive impairment, cognitive decline and dementia, including Alzheimer's disease (Iadecola, 2014). Although environmental factors such as sleep quality are associated with hypertension (Liu, et al., 2015), the gains observed with dietary modification such as in DASH (Dietary Approaches to Stop Hypertension) studies suggest that nutritional strategies could be highly effective in preventing and controlling high blood pressure (Sacks et al., 1999), while underlining the status of diet as the strongest environmental factor affecting blood pressure (Ralston et al., 2011). Given that blood pressure is modulated by the renin-angiotensin system, which is known to be targeted by both pharmacologic agents such as ACE inhibitors and dietary components like food derived bioactive peptides (Majumder, & Wu, 2015), an extensive discussion of that pathway now follows.

5. The renin-angiotensin system (RAS)

Although blood pressure can be modulated via alternate pathways (Nurminen et al., 2000), the RAS, which is also known as the renin-angiotensin-aldosterone system, (RAAS), plays a central role in blood pressure regulation as well as in physiological fluid and electrolyte balance (Majumder, & Wu, 2015). Two key enzymes, renin and ACE are critical in the RAS pathway. The rate-limiting step in the cascade is catalyzed by the aspartyl protease, renin, which converts the zymogen angiotensinogen to angiotensin-I (AT-I) by cleaving the peptide bond between Leu10

and Val11 in the pathway's first reaction (Rao, 2010). AT-I, an inactive decapeptide, is subsequently converted to angiotensin-II (AT-II), a potent vasoconstrictor, by the action of ACE. In the classic RAS, the vasoconstrictive octapeptide AT-II executes its blood pressure-regulating function by binding to two major receptors namely angiotensin type 1 (AT-I) and type 2 (AT-II) receptors (Majumder, & Wu, 2015). Although its interaction with these receptors is important for the maintenance of normal blood pressure, the effects of excessive ATII levels produced in the pathological state could be detrimental. When in excess, the interaction of AT-II with AT-I receptors leads to vasoconstriction in vascular smooth muscle cells and causes adrenal glands to release aldosterone which stimulates sodium and fluid retention, resulting in vascular volume expansion, more constricted vessels and higher blood pressure (Majumder, & Wu, 2015). Conversely, AT-II receptors found in both vascular smooth muscle and endothelial cells are involved in the release of the vasodilator, nitric oxide (Majumder, & Wu, 2015). The vasodilatory nonapeptide, bradykinin, is an inflammatory mediator and ACE substrate that stimulates the release of nitric oxide and vasodilatory prostaglandins with resultant blood pressure lowering and vasoprotective effects (Majumder, & Wu, 2015). Thus, bradykinin inactivation by ACE contributes to further constriction of blood vessels and elevated blood pressure (Ryan et al., 2011). Although direct inhibition of renin activity is a more effective strategy for controlling hypertension than ACE activity inhibition because the former blocks the synthesis of AT-I, which can be converted to AT-II via an alternative pathway that is independent of ACE but is catalyzed by the enzyme chymase instead (Udenigwe, & Aluko, 2012a), renin inhibition does not prevent the hydrolytic conversion of bradykinin to inactive fragments by ACE. Therefore, pleiotropic agents with the ability to simultaneously inhibit the activities of ACE and renin are needed for the effective prevention and management of hypertension (Udenigwe et al., 2009a).

6. Mechanism of action of ACE-inhibitory peptides

The prospect of using food-derived peptides as blood pressure-lowering agents has gained increasing interest due to their relatively low cost, biogenic origin, and apparent lack of harmful side effects such as those (including skin rashes, dry cough, angioedema and diarrhoea) reported for synthetic ACE and renin inhibitors (Jauhiainen, & Korpela, 2007). ACE-inhibitory peptides work by reacting with the

enzyme and making it unavailable to cleave AT-I, and thus preventing the production of AT-II. In addition to the well-known ACE inhibition pattern in the classic renin-angiotensin system, recent reports have suggested that bioactive peptides could lower blood pressure via an alternative pathway by upregulating ACE-II, an ACE homologue which counteracts the effects of elevated ACE levels by cleaving AT-II to Ang_{1-7} (Wu et al., 2017). Although the mechanisms of ACE inhibition by peptides are yet to be entirely elucidated, partly because different food proteins yield ACE-inhibitory peptides with distinct sequences (Li et al., 2004), the interactions (Guo et al., 2017; Wu et al., 2015) of peptide inhibitors with the enzyme protein (Cushman et al., 1982; Ondetti, & Cushman, 1982). These active site pockets (S1, S1′ and S2′) represent the major sites of ACE interaction with ligands (or inhibitors) and are known to contain mainly hydrophobic amino acids (Ondetti, & Cushman, 1982). It is known that the C-terminal tripeptide of peptide inhibitors, which strongly influences the interaction of peptides with ACE, can interact with the three subsites in the enzyme's active site (Li et al., 2004; Ondetti, & Cushman, 1982). While a variety of peptide ACE inhibitors function as competitive inhibitors (Ryan et al., 2011), indicating their competition with ACE substrate for the enzyme's catalytic site (Li et al., 2004), others such as those purified from hazel nut in a recent study (Liu et al., 2018) function as non-competitive inhibitors. With most competitive ACE-inhibitory peptides containing hydrophobic amino acid residues (Li et al., 2004), the importance of hydrophobic amino acids to ACE inhibition is further underlined by studies suggesting that the crucial last four C- terminal amino acid residues of peptides 4-10 amino acids long (known to be critical for ACE - inhibition), usually contain hydrophobic side chains (Wu et al., 2006). For di- and tripeptides, Cterminal hydrophobic (aromatic or branched) side chains including phenylalanine, proline, tyrosine and tryptophan, and N-terminal aliphatic amino acids such as valine, isoleucine and leucine have been shown to be critical for ACE inhibition, which makes such peptides to be potent inhibitors (Li et al., 2004; Wu et al., 2006). Apart from the more effective interaction between such hydrophobic peptides and the hydrophobic residues present in the ACE active site, other factors that could contribute to their more potent inhibition include hydrogen bond formation and interactions with ACE zinc ions (Erdmann et al., 2008; Lee et al., 2010).

7. Dipeptidyl peptidase-4 inhibitor

Diabetes is a chronic metabolic disorder which leads to high blood sugar levels over a prolonged period. Type 2 diabetes mellitus (T2DM), type 1 diabetes mellitus (T1DM), and gestational diabetes mellitus are the most frequent forms; other specific types exist that are much less common (1). The enzyme, dipeptidyl peptidase (DPP), is a novel target for the treatment of type 2 diabetes. Dipeptidyl peptidase inhibition improves the impaired insulin secretion and decrease postprandial concentrations of glucagon by enhancing the incretin hormone levels lucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide/gastric inhibitory polypeptide (GIP). Recently, DPP inhibitors have attracted more and more attention, several of which have entered pre-clinical and clinical trials, and one has received approval for use as an anti-diabetic agent. Among the DPP inhibitors, two leading agents (sitagliptin and vildagliptin) have been shown to be effective in reducing glycosylated hemoglobin (HbA1c) and fasting plasma glucose (FPG) in patients with type 2 diabetes (Wu et al., 2009). DPP-IV is a homodimeric serine peptidase, which consists of approximately 700 amino acids in each subunit. DPP-IV preferentially cleaves substrate peptides with Pro or Ala at the penultimate position of peptides. DPP-IV is responsible for the degradation of GLP-1 and GIP (Roppongi, et al., 2018). DPP-IV inhibition is a key target in the treatment of T2DM, and DPP-IV inhibitors were one of the first classes of oral antidiabetic drugs to be prospectively designed as anti-hyperglycemic agents (Deacon, & Lebovitz, 2016). To date, more than ten DPP-IV inhibitory drugs, which are classified as gliptins, have been developed and marketed around the world (Sneha, & Doss, 2016). In 2006 Sitagliptin was the first gliptin to be approved by the United States Food and Drug Administration (Thornberry, & Weber, 2007), and new members continue to be approved, such as Vildagliptin, Saxagliptin, Linagliptin, Gemigliptin, Anagliptin, Teneligliptin, Alogliptin, Trelagliptin, Omarigliptin, Evogliptin, and Gosogliptin (Deacon, & Lebovitz, 2016). However, these synthetic DPP-IV inhibitors are reported to have some adverse effects (Filippatos et al., 2014), such as gastrointestinal adverse effects (Nonaka, et al., 2008), allergic reactions (Desai et al., 2010), skin-related side effects (Mas-Vidal et al., 2012), and musculoskeletal disorders (Tarapues et al., 2013). However, DPP-IV inhibitors have been discovered in foods, herbal preparations, natural sources, and traditional Chinese medicines, including

phenolic compounds from blueberry-blackberry wine. It has been reported different peptides showed different DPP-IV inhibitory modes, including competitive, uncompetitive, noncompetitive and mixed-type modes, which means those peptides might exert DPP-IV inhibitory activity by binding either at the active site and/or outside the catalytic site of DPP-IV (Lacroix, & Li-Chan, 2016). It has been suggested that those natural food- or herb-derived constituents should be safer than synthetic forms, and could be used for glycemic management. Among the sources of DPP-IV inhibitors, food protein-derived DPP-IV inhibitory peptides have attracted the attention of more and more researchers, owing to their high efficacy and safety (Lacroix, & Li-Chan, 2014). blends (Fan et al., 2013), alkaloids from seed extract of Castanospermum australe (Bharti et al., 2012), and procyanidins from grape seed (Gonzalez-Abuin et al., 2012), all of which have shown good DPP-IV inhibitory activity.

Table 6 Bioactive peptides of seed

Raw materials	Proteolytic enzymes	Bioactive	Reference
Hemp seed	pepsin and pancreatin	- antioxidant activity	Girgih et al., 2010
Flaxseed	pepsin	- antioxidant activity - inhibit the activity of ACE	Udenigwe et al., 2009
Canola	alcalase, pepsin, papain	- renin-inhibitory activity - inhibit the activity of ACE	Alashi et al., 2014
Soybean	papain	- renin-inhibitory activity - inhibit the activity of ACE	Fitzgerald et al., 2012
Peanut	neutrase / protamex	- renin-inhibitory activity - inhibit the activity of ACE	Gong et al., 2017
Moreton Bay Chestnut	papain	- renin-inhibitory activity - inhibit the activity of ACE	Hsu et al., 2013
Soybean	alcalase	- in-vitro DPP-IV inhibitory	Sato et al., 2018

Ultrafitration membranes for macromolecule processing

Ultrafiltration has been used successfully for years and is an excellent and gentle method to extract, concentrate, purify or fractionate valuable marine molecules from effluents, wastes or by-products from food processing industries. Some works have been carried out to upgrade liquids effluents by recovering proteins (Jaouen, 1992), aromas (Cros et al., 2005) or polysaccharides (Lignot, 2003). The molecular size of the hydrolyzed protein and the specificity of the enzyme are key factors in producing protein hydrolysates with desired functional properties. One method of controlling molecular size is the use of ultrafiltration reactors. It may also produce hydrolysates with functional properties that are superior in some respects to currently available proteins (Deeslie, & Cheryan, 1992). The relation between the structure of peptides and their activities is not known in detail but depends from various characteristics, amino-acid sequence, molecular weight (MW), hydrophobicity, charge and acido-basic character (Pihlanto, 2006). Peptides MW has in particular a great impact so that pressure-driven membrane separations can be used as a second control step to increase their specific activity (i.e. per gram of peptide). Peptides are intermediate products of the decomposition of proteins to amino acids. Peptides are those, in which various combinations of amino acids form a polymer by a dehydration condensation reaction between an amino group and carboxyl group, and generally have a molecular weight (MW) of 10 kDa less (Halim et al. 2016). Protein isolate was hydrolyzed with various proteases to obtain hydrolysates that were separated by membrane ultrafiltration into four molecular size fractions (<1, 1-3, 3-5, and 5-10 kDa). The result show that <1 kDa improved DPPH and superoxide scavenging properties when compared to the unfractionated protein hydrolysates and only the <1 kDa fraction showed ferric reducing antioxidant power and the effect was dose-dependent (He et al., 2013). The Perilla seed meal protein was hydrolyzed with Flavourzyme; the hydrolysate was fractionated by an ultrafiltration and its physiological activity was measured. Peptides with low molecular weights exhibited higher antioxidant activity. The EC50 values of the α-amylase inhibitory activity ranged from 727.89 μg/ml to 757.18 μg/ml, the α-glucosidase inhibitory activity was highest in the < 1 kDa fraction. The < 1 kDa fraction exhibited the strongest angiotensin I-converting enzyme inhibitory activity. As a result, the peptides from PSM protein hydrolysates, particularly peptides

< 1 kDa, exhibited excellent antioxidant, antidiabetic, and antihypertensive activities and thus were highly likely to be developed as a functional food material (Park, & Yoon, 2019). Accord with Arise et al. (2016), the bambara protein isolate was digested with three proteases (Alcalase, Trypsin and Pepsin) through ultrafiltration membranes to obtain peptide fractions of different sizes (<1, 1-3, 3-5 and 5-10 kDa). The membrane fractions showed that peptides with sizes <3 kDa had reduced surface hydrophobicity when compared with peptides >3 kDa. However, for all the hydrolysates, the low molecular weight peptides were more effective DPPH radical scavengers when compared to the bigger peptides. The 5-10 kDa pepsin hydrolysate peptide fractions had greater (88%) hydroxyl scavenging activity than GSH, Alcalase and Trypsin hydrolysates (82%).

Structure and mode of action of gastrointestinal enzymes

The active site of an enzyme (sometimes referred to as the catalytic center) is that portion of the enzyme that interacts with the substrate and converts it into a product. Two distinct models of how an enzyme binds its substrate have been proposed: the lock-and-key (complementary) model of Fischer and the induced fit (conformational change) model of Koshland (DeLuca, & Lyndal York, 2013). In contrast, digestive enzymes, also known as gastrointestinal enzymes are secreted along the gastrointestinal tract to break down food into nutrients that can be easily absorbed into the bloodstream (Kaur, & Sekhon, 2012). The main digestive enzymes that the body uses include protease, amylase, sucrase, lipase, lactase, and maltase (Ho, & Gibaldi, 2013).

The α-amylase (α-1,4-D-glucan-4-glucanohydrolase, EC 3.2.1.1) is a member of glycoside hydrolase family 13 (GH13) of the sequence-based classification of glycoside hydrolases (Janecek et al., 2014). It catalyzes the hydrolysis of the internal α-1,4-glycosidic linkages in starch, glycogen and various malto-oligosaccharides into low molecular weight products such as glucose, maltose, and maltotriose (Kim et al., 2014). The α-amylase is known as a calcium metalloenzyme because it is completely unable to function in the absence of calcium and its active site contains calcium ion which stabilizes the structure of the enzyme, it also contains chloride ion bound underneath the active site that assists in the activity and stability of the enzyme (Tiwari et al., 2015).

The α-glucosidase (EC 3.2.1.20), a member of glycoside hydrolase family GH31 is located in the brush-border surface membrane of intestinal cells and activates the final step of the carbohydrate digestive process (Kim et al., 2014). It is a carbohydrate-hydrolase that releases α-glucose from the non-reducing end by hydrolyzing both α-1,4- and α-1,6-glucosidic linkages (Ho, & Gibaldi, 2013), in other words, it catalyzes the hydrolysis of complex carbohydrates and disaccharides to absorbable monosaccharides such as glucose. it is a calcium-containing enzyme and it performs its catalysis via a double displacement reaction mechanism with retention of the anomeric carbon configuration in the product (Azam et al., 2012).

Trypsin (EC 3.4.21.4), also known as serine proteases are enzymes that cleave peptide bonds in proteins, in which serine serves as the nucleophilic amino acid at the enzyme's active site (Naffin-Olivos et al., 2017). Trypsin is a single chain polypeptide of 223 amino acid residues held together by 2 disulfide bridges formed by 8 homologous cysteine residues in each molecule (Desnuelle et al., 2014). Trypsin is produced in the small intestine when its proenzyme form, trypsinogen produced by the pancreas is activated by the enzyme enteropeptidase (Weiss, 2014). Trypsin cleaves peptide chains mainly at the carboxyl side of the amino acid lysine or arginine (Horn et al., 2014).

Pancreatic lipase (EC 3.1.1.3) belongs to the family of lipolytic enzymes that hydrolyze ester linkages of triglycerides. Pancreatic lipase is secreted from the pancreas (pancreatic acinar cells) and is the primary lipase enzyme that breaks down dietary fat (triglycerides) in the human digestive system to monoglycerides and free fatty acids, which are then absorbed by the body (Lunagariya et al., 2014). It is secreted in its final form however, it becomes effective only in the presence of colipase in the duodenum (Mukherjee, 2003). Structurally, pancreatic lipase is noted to have a tertiary structure suggested by its conserved disulfide bonds and its active site is centered on a serine residue which has been shown to participate with a histidine and an aspartic acid residue, in a charge relay system (Birari, & Bhutani, 2007).

All the above enzymes described in this section are vital for nutrient digestion and absorption in the human body. However, increase in the catalytic activity of these enzymes may result in the inception of certain symptoms of disease conditions such as obesity. Thus, the inhibition of these gastrointestinal enzymes is an important therapeutic strategy in a bid to ameliorate the disease symptoms.

Bioactive peptides with inhibitory activities against gastrointestinal enzymes

A range of various natural compounds and food sources have been investigated and shown to possess inhibitory activities against gastrointestinal enzymes. Several studies have investigated the use of various bioactive compounds (e.g polyphenols, tannins, flavonoids, methanolic extracts from plants) to influence the digestive system as an approach in the management of obesity and diabetesmellitus (Seyedan et al., 2015; Ercan, & El, 2016). This can be achieved by inhibiting the key enzymes involved in food digestion (Lacroix, & Li-Chan, 2013). For example, a chickpea extract was investigated as an inhibitory agent against the digestive enzymes involved in carbohydrate and lipid metabolism (α-amylase, α-glucosidase, and lipase) (Ercan, & El, 2016). The results showed that the chickpea extract inhibited α-amylase (72.6%), lipase (85.4%) and α-glucosidase (10.9%); the inhibitory activity was attributed to the saponin content. A current research study showed that peptides derived from pinto bean were able to inhibit α-amylase (Ngoh, & Gan, 2016). The hydrolysates produced by Protamex were subjected to ultrafiltration to separate peptides based on molecular weight. Six fractions were produced and then tested for the inhibitory activity of α- amylase. Results showed that fractions with molecular weight (MW) less than 3 kDa had the highest inhibitory activity against α-amylase (62.10%). The < 3 kDa fraction was then subjected to mass spectrometry, which identified 7 peptides (PPHMLP, PPMHLP, PLPWGAGF, GDAACCGLPLLP, PPHMGGP, PLPPHDLL, and FNPFPSPHTP). Alcalase hydrolysates were then subjected to ultrafiltration to separate peptides based on molecular weight. Results showed that the <3 kDa fractions had the highest inhibitory activities against α-glucosidase (47.9%). The <3 kDa fraction was then further separated by ion exchange chromatography to yield 2 peaks, with AAIE1 having the highest α- glucosidase inhibitory activity. Evidence from scientific literature has shown that bioactive peptides possess significant inhibitory activities against gastrointestinal enzymes. When the amount of bioactive peptides that can be safely consumed is taken into consideration, these peptides have the potential to be developed into non-drug supplements for the management of obesity and type 2 diabetes mellitus (Uraipong, & Zhao, 2016b).

Protein hydrolysate in beverages

The market segment of plant-based foods and beverages is growing rapidly. Combining new food ingredients with existing technology offers great potential for new product development. The develop a new plant-based beverage with a high protein content, and high nutritional value by a combined process of Sacha Inchi protein hydrolysate and other ingredients. One of the challenges for plant protein ingredients isolated from single crops remains their "incomplete" essential amino acid content. Therefore, combinations of protein sources are often required to deliver a complete diet (Day, 2013). Plant crops that show significant potential as sources of high-quality protein for ingredient and food production in kiwifruit seeds, oilseeds, potato, tree nuts, and wrinkled peas. Many of the requirements for isolating and manufacturing plant proteins are similar to those for dairy proteins, so expertise from the dairy industry could be applied to develop a new plantbased protein food industry. The high demand for plant proteins is still driven by their use in new product development (NPD) in the developing vegetarian/vegan foods sector (Lynch et al., 2016). Protein it is a vital component of a healthy diet, and unlike fats and carbohydrates, our body does not store protein. The amino acids within protein are used to build and repair tissues (bones, muscles, cartilage, skin, and blood) and are necessary for the normal growth and development of infants and children. Functional plant proteins with nutritional attributes continue to meet specific market needs and avoid becoming commoditised. One of the challenges for plant proteins remains the essential amino acid (EAA) content, which is limited from certain plant sources. While complementary proteins may be combined to make complete proteins in finished food products, the individual protein ingredients that have a low EAA content are unlikely to achieve a premium. For cereal crops the essential amino acids lysine and tryptophane are limiting, whereas for legumes it is methionine that is the limiting essential amino acid (Ufaz, & Galili, 2008.). Thus far, functional plant proteins with nutritional attributes continue to meet specific market needs and have avoided becoming commoditised. The prices for functional plant proteins as ingredients (e.g. pea protein and potato protein) remain high because of worldwide shortages. The high demand for plant proteins is driven by their use in NPD in the developing vegetarian/vegan food sector, as well as in non-meat and non-dairy foods for the

general population. Although significant NPD has occurred to date, many of the plant protein foods are still of lower sensory quality than their dairy-based equivalents. There remains a significant opportunity to develop new food technologies and functionalities for plant protein foods that better meet consumer expectations (Sutton et al., 2018).

CHAPTER III

RESEARCH METHODOLOGY

Materials

Sacha Inchi residue (SIR), a by-product of screw press oil extraction process was obtained from the Tai.C.M.S. Standard industrial Co., Ltd. After processing, the samples were ground and sieved to 0.5 mm diameter particle size, using a food mill grinder (Welljun, WJ-1820) and then packed into an aluminum foil bag and stored at -18 °C until the experiment was carried out.

Chemicals

1. Alcalase 2.4L from Bacillus licheniformis (Nutitech, Thailand)
2. Neutrase 0.8L from Bacillus amyloliquefaciens (Nutitech, Thailand)
3. Pepsin from porcine gastric mucosa ≥250 units/mg solid (Sigma-Aldrich, USA)
4. Papain from papaya latex ≥10 units/mg protein (Sigma-Aldrich, USA)
5. Flavourzyme from Aspergillus oryzae ≥500 U/g (Sigma-Aldrich, USA)
6. N-(3-[2-furyl]acryloyl)-phenylalanylglycylglycine (FAPGG) from Sigma-Aldrich (St. Louis, MO, USA)
7. Reduced glutathione (GSH) from Sigma-Aldrich (St. Louis, MO, USA)
8. 2, 2-Diphenyl-1picrylhydrazyl (DPPH) from Sigma-Aldrich (St. Louis, MO, USA)
9. 8-Anilino-1-naphthalenesulfonic acid (ANS) from Sigma-Aldrich (St. Louis, MO, USA)
10. o-Phthaldialdehyde (OPA) from Sigma-Aldrich (St. Louis, MO, USA)
11. Folin-Ciocalteau phenol reagents from Sigma-Aldrich (St. Louis, MO, USA)
12. Angiotensin-1 converting enzyme ACE (from rabbit lung) from Sigma-Aldrich (St. Louis, MO, USA)

13. 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) from Sigma-Aldrich (St. Louis, MO, USA)
14. Alpha-linolenic acid (ALA) from Sigma-Aldrich (St. Louis, MO, USA)
15. glutathione (GSH) from Sigma-Aldrich (St. Louis,MO, USA).
16. 2,4,6-trinitro-benzenesulfonic acid (TNBS)
17. Bovine serum albumin (BSA) from Sigma-Aldrich (St. Louis, MO, USA)
18. Calcium chloride (A.R. grade: Sigma-Aldrich Inc., MO)
19. Sodium carbonate (A.R. grade: RCI Labscan limited, TH)
20. Sodium chloride (A.R. grade: RCI Labscan limited, TH)
21. Hydrogen peroxide (A.R. grade: RCI Labscan limited, TH)
22. Sulfuric acid (A.R. grade: RCI Labscan limited, TH)
23. Methylred (A.R. grade: RCI Labscan limited, TH)
24. Boric acid (A.R. grade: RCI Labscan limited, TH)
25. Hydrochloric (A.R. grade: Sigma-Aldrich Inc., MO)
26. Petroleum ether (A.R. grade: RCI Labscan limited, TH)
27. Sodium dodecyl sulfate- polyacrylamide gel electrophoresis

Apparatuses

1. Twin-screw extruder (chareon tut co., ltd, CET-D25L32, Thailand)
2. Hot air oven (Memmert, UNE 500, Germany)
3. Drum dryer (John-millder, JM-T, Japan)
4. Autoclaves (Kokusan Japan, H-88 Series)
5. Mastersizer 2000 particle size analyzer (Malvern Instruments Ltd., Malvern, U.K.)
6. FP-6300 spectrofluorimeter (JASCO, Tokyo, Japan)
7. Synergy™ H4 Hybrid microplate reader (Biotek™, Vermont, USA)
8. Texture Analyzer (TA-XT. Plus. Stable Micro system Co, Ltd, UK)
9. Kjeldahl Apparatus
10. Digestion Apparatus
11. Distallation
12. Amicon stirred ultrafiltration cell
13. Soxhlet apparatus

14. Analytical balance with 4 decimal points (Sartorius CP 224S, Germany)
15. Muffle furnace
16. Hot plate
17. Centrifuge (Beckman Coulter, Mississauga, ON)
18. pH-stat mode (Radiometer Analytical, Copenhagen, Denmark)
19. Mastersizer 2000 particle size analyzer (Malvern Instruments Ltd., Malvern, U.K.)
20. Microplate reader (BioTek Instruments, Winooski, VT, USA).
21. Freeze dryer (FD8-Cool safe Advance, Scanvac, Denmark)
22. Visible Spectrophotometer (Genesys 20, USA)

Part 1 Stabilization process of Sacha Inchi protein residue

1. Stabilization process

1.1 Extrusion process

Extrusion of the SIR (0.5 mm) was performed in a pilot-scale co-rotating twin-screw extruder (Chareon tut co., ltd, CET-D25L32, Thailand). The extruder was operated at 200 rpm screw speed 10.9 kg/hr feed rate and 4 mm circular die. In-barrel moisture content in the extruder was varied at 39.5, 50.6 and 61.8 %. In-barrel extruder temperature profile was set at B2:45 °C, B3:80 °C, B 4-7: 80-100 °C and die temperature: 80-100 °C (Figure 4). The extrudates were dried in the hot air oven at 50 °C for 8 hr and then were ground to pass a 0.5 mm sieve using a food mill grinder (Welljun, WJ-1820) and packed into an aluminum foil bag and stored at -18 °C until further analysis.

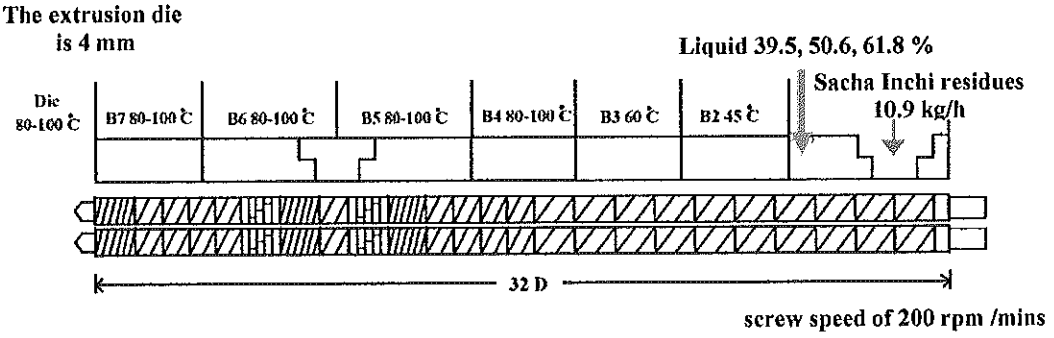


Figure 4 Extrusion parameters; moisture content in barrel and barrel temperature

1.2 Drum-drying processes

The SIR was prepared following the method described by Bencini (1986). The slurries (25% w/w) were dried on double drum drier (John-millder, JM-T, Japan), variable drum speed of 3, 5 and 7 rpm/min. The drum was heated at a steam pressure of 1.4 kg/cm^2 to obtain various drum surface temperature of 100, 120 and 140 °C (Figure 5). The dried flakes were dried in the hot air oven at 50 °C for 8 hr and then were ground to pass a 0.5 mm sieve using a food mill grinder (Welljun, WJ-1820) and packed into an aluminum foil bag and stored at -18 °C until further analysis.

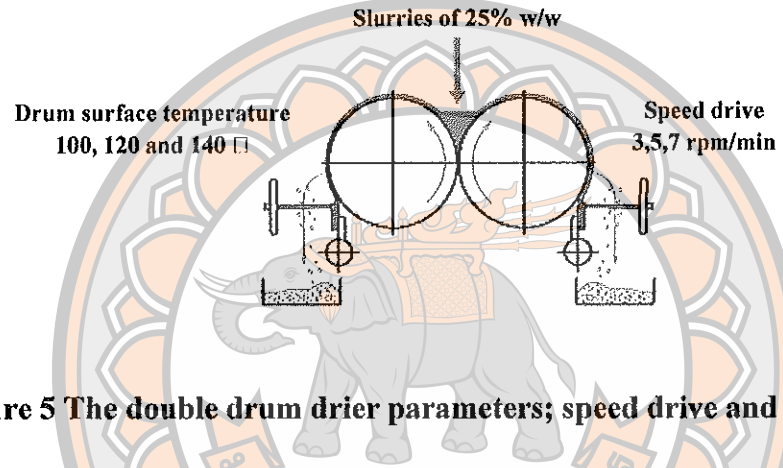


Figure 5 The double drum drier parameters; speed drive and drums heated

1.3 Autoclaving

The SIR was prepared according to the method described by Zhao, & Lin (2009). The Sacha Inchi residue (100 g) was weighed into cheesecloth and sample was then pressure-cooked in an autoclave at three different temperatures (105, 110 and 121 °C) for 10, 15 and 30 min. The autoclaved Sacha Inchi paste was allowed to cool to room temperature and then oven dried at 50 °C for 8 hrs and ground, sifted through 0.5 mm sieve. The samples were packed into an aluminum foil bag and stored at -18 °C until further analysis.

1.4 Heat-moisture treatment

The method of heat-moisture treatment was modified from the method described by Sair (1967). Samples were weighed into glass jars. The SIR (0.5 mm) was weighed into glass jars and the moisture content of the sample was adjusted to 15, 20 and 25% by spraying the proper amounts of water into the jars. The SIR was stirred and mixed constantly during moisture adjustment. The glass jars were sealed, kept at

room temperature for 8 hr and then placed in an air oven at 90, 100 and 110 °C for 16 hr. After they were cooled, the jars were opened, and Sacha Inchi samples had moisture content about 10-8%. The samples were dried in the hot air oven at 50 °C for 8 hr and then packed into an aluminum foil bag and stored at -18 °C until further analysis.

1.5 Combine process

A combine stabilization process of The SIR through autoclaving and extrusion was studied. The SIR (3 kg) was weighed into cheesecloth and sample was then pressure-cooked in an autoclave at temperatures 121°C for three different time (10, 15 and 30 min). The autoclaved Sacha Inchi paste was allowed to room temperature and then oven dried at 50 °C for 8 hr and grounded, sifted through 0.5 mm sieve. The sample through autoclaving was performed in a pilot-scale co-rotating twin-screw extruder (Chareon tut co., ltd, CET-D25L32). The extruder was operated at 200 rpm screw speed 10.9 kg/hr feed rate and 4 mm circular die. In-barrel moisture content in the extruder was 61.8 %. In-barrel extruder temperature profile was set at B2:45 °C, B3:80 °C, B 4-7: 100-150 °C and die temperature: 100-150 °C. The samples were dried in the hot air oven at 50 °C for 8 hr and then packed into an aluminum foil bag and stored at -18 °C until further analysis.

2. Proximate analysis

Determination of proximate composition was determined according to AOAC (2005). Total protein content (N × 6.25) was evaluated from 100 mg of dried meal using the Kjeldahl Method. Moisture content was determined by drying the samples at 105°C to a constant weight. The ash percentage was quantified by incineration at 600 °C for 6 hours. Total dietary fibre content is measured according to the AOAC enzymatic-gravimetric method. The basis of this method is the isolation of dietary fibre by enzymatic digestion of the rest of the constituents of the material. The residue is measured gravimetrically. The total lipid content was determined following an extraction procedure adapted from AOAC (1990). Samples (2 g) was refluxed in Soxhlet apparatus with 150 mL petroleum ether for 6 hr. The excess solvent was removed by evaporation under vacuum and dried in a to a content weight in a forced air oven. Lipid content was expressed as a percentage of dry sample weight. The total carbohydrate content was determined by difference, i.e., 100 - (sum of percentages of moisture, ash, protein and lipids) (Oshodi, 1992).

3. Determination of functional properties

3.1 Water absorption index (WAI) and water solubility index (WSI)

WAI and WSI were determined according to Andersonet et al. (1970). Approximately 2.5g of samples were weighed (W_1) in a 50 mL pre-weighted centrifuge tube. About 30 mL of distilled water was added and placed in a water bath at two different tested temperature levels of 30 while shaking the samples gently to ensure that the samples remained in suspension for 30 min. The dispersions were then centrifuged at 3500 rpm for 10 min. After centrifugation, WAI was recorded as gram of water retained per gram of dried sample. The supernatant was separated from the sediment (W_2), put in a metal dish, weighed, dried at 105 °C for 12 hr, weighed again, and dried again. The weight of the dry-dissolved sample was measured and recorded as W_3. WAI and WSI were calculated as Eq. 1 and 2:

$$WAI = \frac{W2-W1}{W1} \quad \text{..... (Eq.1)}$$

$$\%WSI = \frac{W3}{W1} x100 \quad \text{..... (Eq.2)}$$

3.2 Emulsion formation (EC) and emulsion stability (ES)

The EC and ES were measured according to the method described by Wang, & Kinsella (1976). The sample 3 g, 50 mL of distilled water was added. The slurry was transferred to a blender and blended for 30 s at low speed. The dispersions were blended at high speed (10,000 rpm) while groundnut oil was poured into the blender at a flow rate of 1 mL s^{-1}. After agitation, the samples were centrifuged at 2500 rpm for 5 min. %EC was calculated as Eq. 3:

$$\%EC = \frac{\text{Height of the emulsified layer (cm)}}{\text{Total height (cm)}} x100 \quad \text{..... (Eq.3)}$$

A similar procedure was followed to determine the emulsion stability, but the samples were incubated at 60 °C for 10 min before centrifugation. The emulsion stability was calculated with the same formula.

3.3 Oil binding capacity (OBC)

The OBC to determine these binlding capacities, 1 g of sample was weighed and then stirred into 10 mL of groundnut oil for one minute. These sample suspensions were then centrifuged at 2200 rpm for 30 min. Oil-binding capacity was expressed as g of oil held per g of protein sample (Guerrero et al., 2002).

3.4 Foam formation and measurement

The foam capacity (FC) was determined according to the method described by Sze-Tao, & Sathe (2000). Two hundred and fifty milligrams of sample were mixed with 250 mL of distilled water and homogenization at 20000 rpm for 13 min. The sample solution was then poured into a 100 mL graduated cylinder. FC was then measured as the increased (initial) volume of each sample. FC was calculated as Eq. 4:

$$\%FC = \frac{\text{(after whipping– before whipping) ml}}{\text{(before whipping) ml}} \times 100 \quad \text{.....(Eq. 4)}$$

4. Determination of antioxidant activity

4.1 ABTS radical scavenging assay

Free radical scavenging activity of samples was determined by ABTS radical cation decolorization assay (Re et al., 1999). $ABTS^{\bullet+}$ cation radical was produced by the reaction between 7 mM ABTS in water and 2.45 mM potassium persulfate (1:1), stored in the dark at room temperature for 12-16 hr before use. $ABTS^{\bullet+}$ solution was then diluted with methanol to obtain an absorbance at 730 nm. After the addition of 5 µL of sample extract to 3.995 mL of diluted $ABTS^{\bullet+}$ solution, the absorbance was measured at 30 min after the initial mixing. An appropriate solvent blank was run in each assay. All the measurements were carried out at least three times. Percent $ABTS^{\bullet+}$ scavenging inhibition was calculated as Eq. 5:

$ABTS^{\bullet+}$ scavenging inhibition (%) = ((AB–AA)/AB) ×100 .....(Eq. 5)

Where, AB is absorbance of ABTS radical + methanol; AA is absorbance of ABTS radical + sample extract/standard. Trolox was used as standard substance.

4.2 DPPH radical scavenging assay

Total free radical scavenging capacity of the extracts from different plant samples were estimated according to the previously reported method by Brand-Williams et al. (1995) with slight modification using the stable DPPH radical, which has an absorption maximum at 517 nm. A solution of the radical is prepared by dissolving 2.4 mg DPPH in 100 mL methanol. A test solution (5 µL) was added to 3.995 ml of methanolic DPPH. The mixture was shaken vigorously and kept at room temperature for 30 min in the dark. Absorbance of the reaction mixture was measured at 517 nm spectrophotometrically. Absorbance of the DPPH radical without antioxidant, i.e. blank was also measured. All the determinations were performed in triplicate. The capability to scavenge the DPPH radical was calculated as Eq. 6:

DPPH Scavenged (%) = ((AB–AA)/AB) ×100..... (Eq. 6)

Where, AB is absorbance of blank at t= 0 min; AA is absorbance of the antioxidant at t= 30 min. A calibration curve was plotted with % DPPH scavenged versus concentration of standard antioxidant (Trolox).

4.3 Ferric reducing antioxidant power (FRAP)

The antioxidant capacity of the medicinal sample was estimated spectrophotometrically following the procedure described by Benzie, & Strain (1996). The method is based on the reduction of Fe^{3+} TPTZ complex (colorless complex) to Fe^{2+}-tripyridyltriazine (blue colored complex) formed by the action of electron donating antioxidants at low pH. This reaction was monitored by measuring the change in absorbance at 593 nm. The Ferric reducing antioxidant power (FRAP) reagent was prepared by mixing 300 mM acetate buffer, 10 mL TPTZ in 40 mM HCl and 20 mM $FeCl_3.6H_2O$ in the proportion of 10:1:1. Freshly prepared working FRAP reagent was pipetted using 1-5 mL variable micropipette (3.995 ml) and mixed with 5 µL of the appropriately diluted plant sample and mixed thoroughly. An intense blue color complex was formed when ferric tripyridyl triazine (Fe^{3+} TPTZ) complex was reduced to ferrous (Fe^{2+}) form and the absorbance at 593 nm was recorded against a reagent blank (3.995 ml FRAP reagent+5 µL distilled water) after 30 min incubation at 37 °C. All the determinations were performed in triplicates. The calibration curve was prepared by plotting the absorbance at 593 nm versus different concentrations of $FeSO_4$. The concentrations of $FeSO_4$ were in turn plotted against concentration of standard antioxidant trolox. The FRAP values were obtained by comparing the absorbance change in the test mixture with those obtained from increasing concentrations of Fe^{3+} and expressed as mg $FeSO_4$ equiv. /g sample.

4.4 Determination of total phenolic content (TPC)

The TPC was determined using the method described by Pinsirodom, & Changnoi (2001) with some modifications. To create a standard curve, gallic acid solution was used and the absorbance was measured at 734 nm. Each result was reported as gallic acid equivalent (mg) in 1 g dry sample (mg GAE/1g DM).

5. Determination of microbial analysis

Microbial analysis was carried out aseptically by mixing 25 g of samples, with 225 mL 0.1 peptone. In order to evaluate total bacteria count, samples were plated

on agar medium and incubated at 30 °C for 3 days. Yeast and molds enumeration were made by spreading samples on the surface of a rose bengal chloramphenicol agar and incubation at 25 °C for 5 days (Siva et al., 2001).

6. Determination of in vitro digestibility

In vitro digestion was carried out according to Garrett et al. (1999) with slight modifications. Briefly, the control and SSIR (5 g dry protein) were homogenized in distilled water (250 mL) using an IKA homogenizer (IKA Works Asia, Digital 3725001 T25 digital) for 1 min and adjusted to pH 2.0 with 5 M HCl. Pepsin was added (2.86% [w/w], based on dry substrate), and the samples were incubated in a shaking incubator at 37 °C for 1 hr. The pH was adjusted to 5.3 with a 0.9 M $NaHCO_3$ solution and then further to pH 7.5 with 5 M NaOH. Pancreatin was then added (4.00% [w/w], based on dry substrate), and the samples were further incubated under the same conditions for 2 hr. Subsequently, all digested samples were submerged in a 95 °C water bath for 10 min, cooled on ice to room temperature, and centrifuged at 10000 x g for 10 min. The peptide content of the supernatant was determined using the Lowry method with tyrosine as a standard. The degree of digestibility was determined based on TNBS using leucine as a standard. Digestion was calculated as Eq. 7:

$$\text{Digestibility (\%)} = \frac{(LA3 - LA0)}{LAt} \times 100\% \quad \text{..... (Eq.7)}$$

Where LA_3 is the α-amino content of the digesta, LA_0 is the α-amino content of the sample at time 0, and LA_t is the total α-amino content of raw or products samples obtained from hydrolyzing the samples with 6 M HCl at 110 °C for 24 h.

7. Antinutrients and aflatoxin

7.1 Tannin Contents

Tannin contents of the control and SSIR were chemically determined according to Pizzaro et al. (1994) based on the AOAC (1995) as follows: tannins were extracted using triplicate samples of 25 g of the dried samples. The chemical determination was performed using the Folin-Denis reagent and 5 mL Na_2CO_3 which give a dark blue color with tannins. The absorbance of the resulting color was measured by spectrophotometer at 760 nm wavelength using a quartz cuvette. Then the hydrolyzable tannin concentration was calculated using a tannin-standard curve based on the reaction of Folin-Denis reagent with known concentration of tannic acid.

7.2 Phytic acid and total phosphorus

Phytic acid and total phosphorus were determined according to according to the manufacturer's instruction (K-PHYT; Megazyme International, Wicklow, Ireland). One g of sample material was transferred into beaker, then 20 mL of hydrochloric acid (0.66 M) was added and the beaker was covered with foil and vigorously stirred for a minimum of 3 hr at room temperature. The sample was transfer 1 mL of extract to a 1.5 mL microfuge tube and centrifuge at 13,000 rpm for 10 min and neutralise by addition of 0.5 mL of sodium hydroxide solution (0.75 M).

phytic acid and total Phosphorus; pipette sample (phytic acid or total Phosphorus) or phosphorus standard 1 mL of each supernatant was added to 0.5 mL of colour reagent (Ascorbic acid (10% w/v)/Sulphuric acid (1 M) + Ammonium molybdate (5% w/v)) in a 1.5 mL microfuge tube. Mix by vortex and incubate in a water bath set at 40°C for 1 hr. After 1 hr, mix by vortex and then transfer 1 mL to a cuvette and read the absorbance at 655 nm within 3 hr Phosphorus solution (50 µg/mL) was used as the standard. The concentration of phosphorus and phytic acid were calculated as Eq. 8-9:

phosphorus; $c = [(\text{mean } M \times 20 \times F)/ (10\,000 \times 1.0 \times v)] \times \Delta A_{phosphorus}$ ……..…. (Eq.8)

phytic acid; c = phosphorus [g/100 g] / 0.282 ……..…. (Eq.9)

where: mean M = mean value of phosphorus standards [µg/ΔAphosphorus]

20 = original sample extract volume [mL]

F = dilution factor

ΔA = absorbance change of sample

10000 = conversion from µg/g to g/100 g

1.0 = weight of original sample material [g]

v = sample volume (used in the colourimetric determination step)

7.3 Trypsin inhibition (TI)

The TI was determined according to Hamerstrand et al. (1981). A sample (1.00 g) was extracted with 50 mL of 0.01N NaOH (the pH adjusted, when required, to 8.4-10.0) for 3 hr. This suspension was then diluted so that 2 mL of the sample extract inhibited 40-60% of the trypsin used as a standard in the analysis. To each of

four test tubes 2 mL aliquots of the diluted sample extract were added with a wide-tip pipette. A fifth tube was prepared for the trypsin standard by adding 2 mL of distilled water. To three of the four tubes containing the sample extract and the tube containing distilled water. Two mL of the trypsin solution was added. and the tubes were placed in a constant temperature bath (37°C) for 10 min. Five milliliters of BAPA solution (prewarmed to 37°C) was rapidly blown into each tube. The contents were stirred immediately on a vortex mixer. and the tubes were replaced in the constant temperature bath. The reaction was terminated exactly 10 min later by blowing in 1 mL of 30% acetic acid with immediate mixing with a vortex mixer. A sample blank (the fourth tube containing sample extract) was prepared by the same procedure except that the trypsin solution was added after the reaction was terminated by the addition of acetic acid. The absorbance of each solution was determined at 410 nm against the sample blank. Values obtained from each of the three sample extracts were subtracted from the trypsin standard. The TI content was calculated as Eq. 10:

$TI_{\text{mg/g of sample}}$ = (differential absorbance/ 0.019x1,000) x dilution factor……. (Eq.10)

7.4 Aflatoxin assay

For seed samples, AOAC method 991.31 and 994.08 (Association of Official Analytical Chemists, 2016) was used. Its efficacy was tested by analyzing blank samples and samples spiked with 5.25 ng g^{-1} aflatoxin standard. Calibration curves were prepared from standard solutions having concentrations of 0.75, 2.25, 3.75, 5.25, 6.75 and 8.25 ng g^{-1}. Peak area was plotted against concentration to give regression equations for aflatoxin B1, B2, G1 and G2, which were used to determine the concentration of each respective aflatoxin.

8. Oxidation reaction

8.1 Peroxide value (PV)

Five grams of the sample was weighed into a 250 mL conical flask with a glass stopper. Thirty mL of 3:2 v/v glacial acetic acid-chloroform solvent was added and swirled to dissolve the sample. Point five mL of saturated KI solution was added. The solution was left in the dark with occasional shaking for exactly 1min, and 30mL of distilled water added immediately. The mixture was titrated with 0.1N sodium

thiosulphate using 1.0 mL of starch indicator solution. A blank was also performed at the same time. The test was done in triplicate. The PV was calculated as Eq. 11:

$$PV = [1000(V_1-V_2)/N] \times W \quad \text{..... (Eq.11)}$$

When: N = normality of $Na_2S_2O_3$ solution,

v_1 = volume (ml) of $Na_2S_2O_3$ solution used in test,

v_2 = volume (ml) of $Na_2S_2O_3$ solution used in blank,

W =weight of oil sample.

8.2 Free fatty acids (FFA)

Five grams of the sample was dissolved in a well-mixed neutral solvent consisting of 25mL of diethyl ether and 25ml 95% ethanol. It was then titrated with aqueous 0.1N NaOH using 1ml of phenolphthalein indicator solution and shaking constantly until a pink colour that persisted for at least 15s was obtained. The FFA was calculated as Eq.12:

$$\text{Acid value mg KOH / g of sample} = (56.1 \times N \times V) / W \quad \text{..... (Eq.12)}$$

When: N = normality of NaOH solution,

v = volume (ml) of NaOH solution,

W = weight of oil sample.

8.3 Thio barbituric acid reactive substance (TBARS)

TBARS were measured following Buege and Aust (1978), using a thiobarbituric acid (TBA) reagent, which consists of 0.335% (w/v) TBA solution containing 15% (w/v) of trichloroacetic acid (Enoki et al., 1999). Concentrated extracts (100 mL) were treated with 3.0 mL of TBA reagent for 15 min in a boiling water bath. After cooling, the flocculent precipitates were removed by centrifugation at 1000 g for 10 min. The amount of TBARS was determined in the soluble fraction by measuring their absorbance at 532 nm considering an absorption coefficient of 1.56×10^5 M^{-1} cm^{-1} (Buege, & Aust, 1978). The results were expressed as mmol of MDA/L.

9. Shelf life evaluation

A shelf-life study is an objective means to determine how long a product can reasonably be expected to keep, without an appreciable change in quality (microbial, moisture or oxidation), safety and character. Determination of shelf life by the indirect methods of a product without running a full-length storage trial and are useful for products with long shelf life such as accelerated shelf-life studies. This is usually done

by increasing the storage temperature and predictive models; using information from a database to predict change e.g. oxidation reaction, moisture contents or microbial analysis and then can be used to calculate the shelf life of a food.

The SIR obtained from the optimal stabilization process were selected to analyze for oxidation reaction, moisture contents and microbial analysis for by accelerated shelf-life test (ASLT) at temperature of 30, 40 and 50 ℃.

ASLT

The SSIR samples were packed in an aluminum foil bag (50g/ bags) and stored at 30, 40 and 50 ℃ (Fig 6). The samples were sampling every 2 weeks until 12 weeks to analyze the oxidation reaction, moisture content, microbial analysis and evaluate the shelf life based on Q_{10} values (Robertson, 2009).

Figure 6 Stabilized Sacha Inchi residue packed into an aluminum foil bag (50g/ bags) and storage at 30, 40 and 50 ℃.

The Q_{10} value is a temperature quotient that reflects the change in reaction rate for every 10 ℃ rise in temperature. A shelf-life was calculated as Eq. 13, 14 and 15:

$$Q_{10} = \square_{st} / \square_{st+10} \quad \text{..... (Eq.13)}$$

$$Q_1 = (Q_{10})^{0.1} \quad \text{..... (Eq.14)}$$

$$(Q_1)^{\Delta t} = \square_{st} / \square_{st+\Delta t} \quad \text{..... (Eq.15)}$$

When: $\square_{st}$ = the shelf-life at temperature T (℃)

$\square_{st+\Delta t}$ = the shelf life at temperature T+10 (℃)

10. Pasta development from Sacha Inchi residue

10.1 Pasta formulations

Treatment details for formula pasta are given in Table 1. All the formulations contained various contents of SSIR (0-50%, w/w basis), semolina flour, and salt.

Table 7 Formulations for protein fortified stabilized Sacha Inchi residue pasta

Treatment	Combinations (g)
C (0%)	100 SF+10 S
T1 (10%)	90 SF+10 SSIR + 10S
T2 (20%)	80 SF+20 SSIR + 10S
T3 (30%)	70 SF+30 SSIR + 10S
T4 (40%)	60 SF+40 SSIR + 10S
T5 (50%)	50 SF+50 SSIR + 10S

Note: * semolina flour (SF) and salt (S)

10.2 Pasta production

The pasta was produced using a pilot-scale co-rotating twin-screw extruder (moisture content in the extruder barrel was fixed as constant at 61.8 % and extrusion barrel temperature employed was B2:45 °C, B 3:80 °C, B 4-7: 100°C and die: 100 °C) using the round die (2 mm.). Extrudates were cut to short pieces of 8.0 cm length. The screw speed of 200 rpm and feed rate of 10.9 kg/hr were fixed. The samples were dried in the hot air oven at 50 °C for 6 hr, and packed in the polypropylene bag and kept at room temperature prior to analysis.

10.3 Determination of proximate composition

Determination of proximate composition was determined according to AOAC (2005). Total protein content (N × 6.25) was evaluated from 100 mg of dried meal using the Kjeldahl Method. Moisture content was determined by drying the samples at 105°C to a constant weight. The ash percentage was quantified by incineration at 600 °C for 6 hours. Total dietary fibre content is measured according to

to the AOAC enzymatic-gravimetric method. The basis of this method is the isolation of dietary fibre by enzymatic digestion of the rest of the constituents of the material. The residue is measured gravimetrically. The total lipid content was determined following an extraction procedure adapted from AOAC (1990). Samples (2 g) was refluxed in Soxhlet apparatus with 150 mL petroleum ether for 6 hr. The excess solvent was removed by evaporation under vacuum and dried in a to a content weight in a forced air oven. Lipid content was expressed as a percentage of dry sample weight. The total carbohydrate content was determined by difference, i.e., 100 - (sum of percentages of moisture, ash, protein and lipids) (Oshodi, 1992).

10.4 In vitro digestion

In vitro digestion was carried out according to Garrett et al. (1999) with slight modifications. Briefly, the pastas (5 g dry protein) were homogenized in distilled water (250 mL) using an IKA homogenizer (IKA Works Asia, Digital 3725001 T25 digital) for 1 min and adjusted to pH 2.0 with 5 M HCl. Pepsin was added (2.86% [w/w], based on dry substrate), and the samples were incubated in a shaking incubator at 37 °C for 1 hr. The pH was adjusted to 5.3 with a 0.9 M $NaHCO_3$ solution and then further to pH 7.5 with 5 M NaOH. Pancreatin was then added (4.00% [w/w], based on dry substrate), and the samples were further incubated under the same conditions for 2 hr. Subsequently, all digested samples were submerged in a 95 °C water bath for 10 min, cooled on ice to room temperature, and centrifuged at 10000 x g for 10 min. The peptide content of the supernatant was determined using the Lowry method with tyrosine as a standard. The degree of digestibility was determined based on TNBS using leucine as a standard and calculated as previously shown in Eq.7:

10.5 Pasta quality

10.5.1 Cooked weight

The protein fortified pastas were according to Fari (2011). The protein fortified pastas were cooked in boiling water for their respective cooking time as per the determined cooking time. Then cooked pasta were rinsed with 20 ml distilled water and drained for 2 min. Cooked weight was calculated as Eq.16:

Cooked weight (%) = (Weight of wet protein fortified pastas/Weight of dry protein fortified pastas) x 100 ..... (Eq.16)

10.5.2 Cooking loss

Cooking loss were determined by the method of (Hormdok, 2007). Protein fortified pastas were cooked their respective cooking time. The cooked protein fortified pastas were then washed with water and drained for 2 min. It was dried in an oven at 105°C till constant weight was obtained. The residues were weighed after cooling in desiccators to determine the cooking loss. The cooking loss was calculated by measuring the amount of solid residues remained in the cooking and rinse water after drying. Cooking loss was calculated as Eq.17:

Cooking loss (%) = (Weight of dried residues /Weight of uncooked protein fortified pastas) x 100..... (Eq.17)

10.5.3 Swelling Index (SI)

SI of cooked pasta (g water absorbed per g of dry pasta) was determined as (weight of cooked pasta-weight of dry pasta)/weight of dry pasta (Mestres et al., 1988)

10.5.4 Texture analysis

Texture analysis of pasta products was analyzed using a TA.XT plus texture analyzer (Stable Micro System Co. Ltd., Godalming, UK) equipped with a 5-kg load cell and the Pasta Quality Ring made of plexiglass (A/LKB-F, Stable Micro System Co. Ltd., Godalming, UK). Five pieces of cooked pasta were placed centrally under the knife blade onto the measurement plate of the texture analyzer (n = 3). The measurement was performed at the test speed: 0.17 mm/s and the posttest speed: 10 mm/s. TA-XT Plus measures hardness, cohesiveness, springiness, gumminess, chewiness and adhesiveness of the pasta (Kowalczewski et al., 2015).

11. Statistical Analysis

Data were collected in triplicates and subjected to one way analysis of variance using statistical analysis of variance (ANOVA) was done to determine the significant differences among means followed by Duncan's new multiple range tests were carried out to determine statistical significance ($p < 0.05$) using SPSS 11.0 software (SPSS Inc., Chicago, Ill., U.S.A.).

Part 2 Enzymatic proteolysis process of Sacha Inchi protein

1. Sacha Inchi protein isolate (SIPI)

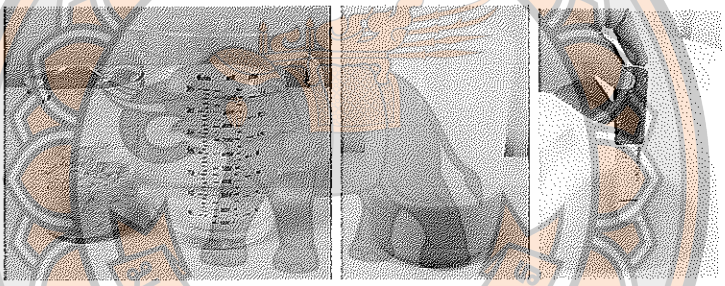
The SIPI was produced according to the method described by Adebowale et al. (2009) with slight modifications. SIR was dispersed in deionized water (1:10, w/v) and adjusted to pH 11.5 with 2M NaOH to solubilize the proteins. The resultant dispersion was stirred at 60 °C for 1 hr followed by centrifugation (7000 x g, 30 min at 4 °C). The supernatant and adjusted to pH 7.0 with 2M HCl to precipitate most of the proteins. Thereafter, the mixture was centrifuged (7000 x g, 30 min at 4 °C), the resultant precipitate was freeze-dried to produce SIPI (Fig 7). The protein content of SIPI was determined using Kjeldahl Method (AOAC, 1995).

2. Sacha Inchi protein hydrolysates

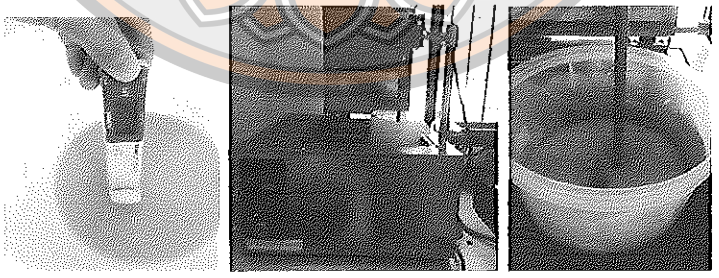
Enzymatic hydrolysis of the SIPI was carried out to produce protein hydrolysates using five different proteases (Alcalase, Pepsin, Papain, Neutrase and Flavourzyme). These enzymes were chosen because they are food grade status and are compliant with regulatory laws. Briefly, in a 1 L beaker, the SIPI was prepared in distilled water (1:10, w/v). Under constant stirring, the beaker containing the protein mixture was placed on a magnetic stirring hot plate equipped with an external temperature probe for accurate temperature control. The mixture was heated to the optimal temperature and adjusted to the optimal pH of each protease using either 1 M NaOH or 1 M HCl. The optimal temperature and pH of each protease are shown in Table 2. Hydrolysis was initiated by the addition of each protease at a ratio of 1% (on basis of protein content w/w). Once an enzyme has been added, the temperature and pH of the reaction was monitored and maintained constant for 1.5 hr in Alcalase and Neutrase hydrolysis and 4 hr in Pepsin, Papain and Flavourzyme, after which hydrolysis was terminated by adjusting to pH 7.0 with either 2 M NaOH or 2 M HCl followed by heating at 100°C for 15 min to ensure complete denaturation of the enzymes. The reaction mixture was then cooled to room temperature and centrifuged at 10,000g for 30 min at 4°C using an Allegra TM 6R centrifuge (Beckman Coulter, Mississauga, ON) to separate the soluble hydrolyzed materials (peptides) from the unhydrolyzed residue (mainly undigested proteins). The supernatants were freeze-dried and preserved at -20 °C until further tests (Figure 8).

Table 8 Optimal temperature and pH of proteases used for enzymatic hydrolysis

Enzyme	Temperature (°C)	pH	Time (hour)
1% Alcalase (SPHA)	55	7.5	1.5
1% Pepsin (SPHPe)	37	2.0	4.0
1% Papain (SPHPa)	55	7.0	4.0
1% Neutrase (SPHN)	55	7.5	1.5
1% Flavourzyme (SPHFl)	50	6.5	4.0
0.5% Alcalase combined 0.5% Neutrase (SPHAN)	55	7.5	1.5

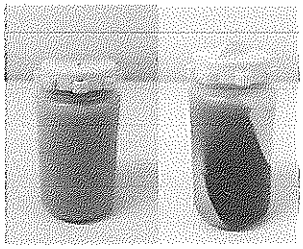


SIR was dispersed in deionized water (1:10, w/v) and check initiate pH (pH about 5.5)

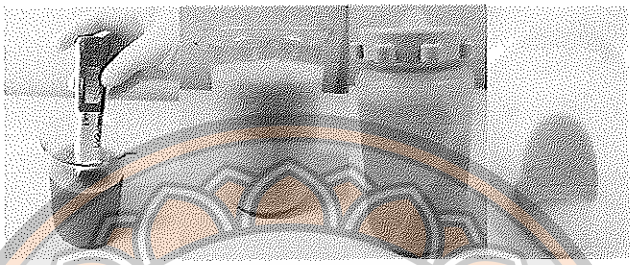


pH Adjust to 11.5 with 2M NaOH and stirred for 1 hr at 60 °C

Figure 7 Flow chart for preparation of the SIPI

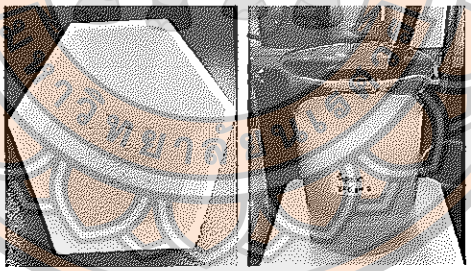


centrifugation for 30 min at 10000 rpm at 4 °C



pH Adjust to 7.0 with either 2 M HCl followed by centrifuged and collected as the SIPI

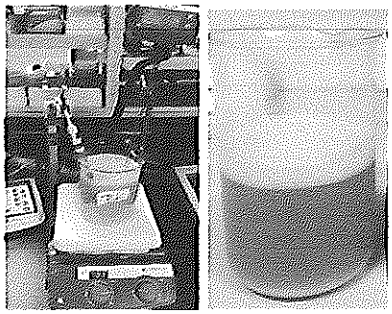
Figure 7 (cont.)



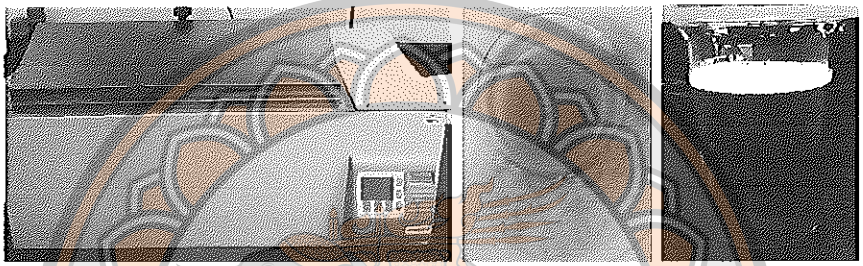
SIPI was prepared in distilled water (1:10, w/v)

The temperature and pH adjust to the optimal of each enzyme with 1 M NaOH or 1 M HCl (Table 2). Hydrolysis was carried out with 1% (on basis of protein content w/w).

Figure 8 Flow chart for preparation of Sacha Inchi protein hydrolysates



Enzymatic treatment of 90 mins by Alcalase and Neutrase and
4 hr in Pepsin, Papain and Flavourzyme. pH Adjust to 7.0 with 1 M NaOH or 1 M HCl



Enzymes were inactivated by heating the reaction mixture for 15 min at 100°C and then cooling samples at room temperature and centrifugation

The supernatants were freeze-dried and preserved
at -20 °C until further tests.

Figure 8 (cont.)

3. Peptide fractionation by ultrafiltration technique

The collected supernatant (hydrolysate) was then passed through an amicon stirred ultrafiltration cell using 1 kDa, 3 kDa, 5 kDa and 10 kDa molecular weight (MW) cut-off membranes respectively, to produce peptides of different sizes. Briefly, the supernatant was first passed through the ultrafiltration cell using 1 kDa membrane, the permeate (<1 kDa) was collected and freeze-dried, while the retentate was kept in the fridge. Distilled water was then added to the retentate and passed through a 3 kDa membrane; the permeate (1-3 kDa) was collected and freeze-dried, while the retentate was kept in the fridge. This process was repeated by passing the 3 kDa retentate through a 5 kDa membrane to collect a permeate (3-5 kDa). The 5 kDa retentate was

then passed through a 10 kDa membrane and the permeate (5-10 kDa) collected. The resulting permeates and 10 kDa retentate were lyophilized and stored at -20°C for further analysis. The freeze-dried hydrolysates and membrane fractions were weighed and their PC was determined using the modified Lowry method as described above. The yield of the hydrolysates and peptide fractions were calculated by dividing the final weight of the protein hydrolysates and peptide fractions after ultrafiltration by the initial weight of the protein used for enzymatic hydrolysis. The PY (%) was calculated as Eq.18:

PY (%) = (final total units / initial total units) x 100 ..... (Eq.18)

4. PC

The PC of SIPI and hydrolysate were determined using the modified Lowry method with bovine serum albumin (BSA) as the standard (Markwell et al., 1978). Briefly, 100 parts of Reagent A (which consisted of 2% Na_2CO_3, 0.4% NaOH, 0.16% C_4O_6HKNa and 1% SDS) was mixed homogeneously with 1 part of Reagent B (4% $CuSO_4.5H_20$) to form Reagent C. Reagent D was a mixture of 1 part of Folin-Ciocalteu phenol reagent and 1 part of double distilled water. A stock solution of BSA (in distilled water) and protein sample (dissolved in 0.1 M NaOH) at 10 mg/ml was used to prepare serial dilutions of 10-100 µg/ml. A 3 ml aliquot of reagent C was then added to each tube, mixed and incubated at room temperature for 1 hr. After incubation, 0.3 ml of reagent D was added to each tube, vortexed thoroughly and left to stand in the dark for 45 min. Finally, absorbance was read at 660 nm using UV-visible spectrophotometer (Biochrom Ultrospec 4300 Pro, Hollister, MA, USA). The analysis was done in triplicates. The PC was calculated as Eq.19 and 20:

$(Absorbance_{sample}) / (Absorbance_{standard}) \times Concentration_{standard}$ = Corresponding sample concentration.....(Eq.19)

$Concentration_{sample} / Concentration_{standard} \times 100$ = PC (%)..... (Eq.20)

5. Amino acid analysis

The amino acid composition of samples was analyzed using an HPLC system with a Pico-Tag column after hydrolyzing the samples with 6 M HCl for 24 h (Bidlingmeyer, Cohen, & Tarvin, 1984). A separate digestion with performic acid was carried out in order to enable the determinations of methionine and cysteine (Gehrke,

Wall, & Absheer, 1985), while the tryptophan content was determined following hydrolysis with NaOH (Landry, & Delhaye, 1992).

6. Surface hydrophobicity (S_o)

The S_o of the Sacha Inchi protein hydrolysates was determined using a hydrophobic fluorescence probe, 1-anilino-8naphthalene sulfonate (ANS) method as described by Hayakawa, & Nakai (1985) with some modifications. Samples were serially diluted to 0.5-0.25% (w/v) in 0.01 M phosphate buffer (pH 3.0, 5.0, 7.0, or 9.0) for 30 min followed by centrifugation (16000×g, 25 °C, 30 min) and the supernatant saved. Ten µl of ANS (8.0 mM in 0.1 M phosphate buffer, pH 7.0) was added to 200 ml of the sample solution. Fluorescence intensity of ANS-peptide conjugates was measured with an FP-6300 spectrofluorometer (JASCO, Tokyo, Japan) at the excitation and emission wavelengths of 390 and 470 nm, respectively.

7. Emulsion formation and determination of oil droplet size

A slight modification of the protocol earlier described by Aluko et al. (2009). was used to prepare oil-in-water emulsions of the Sacha Inchi protein isolate and its 5 enzymatic hydrolysates. Samples were prepared by mixing 10, 20 or 30 mg (protein weight basis) of each sample with 5 mL of 0.1 M sodium phosphate buffer pH 3.0, 5.0, 7.0, or 9.0 and 1 mL of pure canola oil. Homogenization (20000 rpm for 1 min) of each oil/water mixture was carried out with the aid of a Polytron PT 3100 homogenizer to which a 20 mm non-foaming shaft had been attached. A mastersizer 2000 particle size analyzer (Malvern Instruments Ltd., Malvern, U.K.) was used to measure mean oil droplet size ($d_{3,2}$) of the emulsions while ultrapure water was used as a dispersant. Samples were carefully transferred (drop-wise) with the aid of a transfer pipet (under steady shearing) into about 100 mL of ultrapure water contained in the small volume wet sample dispersion component (Hydro 2000S) of the particle size analyzer until a sufficient level of obscuration was achieved. After taking triplicate readings, the emulsions were stored at room temperature without agitation for 30 min; the mean particle diameter was recorded again in order to determine the ES was calculated as Eq. 21:

ES = (initial mean oil droplet size at time zero/ mean oil droplet size at 30 min) x100..... (Eq.21)

8. Foam formation and stability

Foams were formed as previously described Aluko et al. (2009) with the following modifications. Samples were prepared by mixing 10, 20 or 30 mg (protein weight basis) of each sample with 5 mL of 0.1 M sodium phosphate buffer pH 3.0, 5.0, 7.0 followed by homogenization at 20,000 rpm for 1 min using a 20 mm foaming shaft on the Polytron PT 3100 homogenizer. The foam was formed in a 50 mL graduated centrifuge tube, which enabled determination of foam volume (mL). Each sample was analyzed in triplicate, and the mean value is reported. The volume of foam remaining after 30 min at room temperature was expressed as a percent value of original foam volume to obtain foam stability.

9. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Polypeptides present in the freeze-dried the Sacha Inchi meal and the Sacha inchi protein isolate were separated using a previously described SDS-PAGE method (Aluko, & McIntosh, 2004). Each protein sample was dispersed to a final concentration of 5 and 10 mg/mL in Tris-HCl buffer pH 8.0 that contained SDS (10%, w/v) for non-reducing condition and SDS plus mercaptoethanol for reducing condition, respectively. The dispersed samples were heated at 95 °C for 10 min, cooled, centrifuged (16000 x g, 24 °C, 10 min) and the supernatant saved. A 4 µL aliquot of the supernatant of each sample was then separately loaded onto PhastGel® 8-25 % gradient gels (GE Healthcare, Montreal, Canada) before initiating the electrophoretic separation of the proteins using a PhastSystem Separation and Development unit (GE Healthcare, Montreal, Canada). The gels were stained with Coomassie Brilliant Blue while a blend of protein standards (Promega) ranging in size from 10 to 200 kDa was used as the molecular weight marker.

10. Determination of degree of hydrolysis (DH)

The OPA reagent was prepared according to Church et al. (1983). A volume of 12.5 mL of sodium tetraborate solution (100 mmol L-1) was mixed with 1.25 mL of 20% (w/v) sodium dodecyl sulphate solution, 20 mg of OPA (dissolved in 0.5 ml of methanol) and 50 µL of 2-mercaptoethanol. The reagent was always prepared immediately before use and placed in a vial covered with aluminium foil to protect it from light. The method described by Spellman et al. (2003) was used with some modifications for the derivatization. A volume of 10 µL of the sample was mixed with

200 µL of the OPA reagent and this mixture was incubated at 37°C for 100 sec. Subsequently, the absorbance was read at 340 nm. The DH was calculated as Eq 22:

$$DH\ (\%) = h/h_{tot} * 100\% \quad \text{..... (Eq.22)}$$

where, h_{tot} is the total number of peptide bonds per protein equivalent, and h is the number of hydrolyzed bonds.

11. Determination of ACE

The effect of hydrolysates on inhibition of in vitro ACE activity was measured according to the method reported by Udenigwe et al. (2009) with some modifications. A 0.5mMN-[3-(2-furyl) acryloyl]-L-phenylalanylglycylglycine (FAPGG) was prepared in 50 mMTris-HCl buffer to contain 0.3 M NaCl and adjusted to pH 7.5. The FAPGG was used as substrate and samples were dissolved in the same buffer as the FAPGG. When conducting assays, 170 µL of 0.5mM FAPGG was mixed with 10µL ACE (0.5U/mL, the final activity of 25mU) and 20 µL sample. The rate of decrease in absorbance at 345nm was recorded for 30 min at 37°C using Synergy H4 microplate reader (BioTek Instruments, Winooski, VT, USA). The buffer was used instead of sample solutions for the control (uninhibited reaction). The ACE activity was expressed as the rate of reaction (ΔA/min) and inhibitory activity calculated as Eq.23:

$$\text{ACE inhibition } (\%) = [(\Delta A/min)_{(blank)} - (\Delta A/min)_{(sample)} / (\Delta A/min)_{(blank)}] \text{ X } 100 \text{ . (Eq.23)}$$

The IC_{50} values (concentrations of samples required to cause a 50% inhibition of the enzyme activity) were determined from the cubic regression equations generated by fitting the data from the plot of percent ACE inhibition against hydrolysate concentrations ranging from 0.2 to 1.0 mg/mL (final assay concentration). The N-[3-(2-Furyl)acryloyl]-Phe-Gly-Gly (FAPGG) was used as a reference inhibitor.

12. Determination of DPP-IV

The effect of hydrolysates on the activity of DPP-IV was determined using the chromogenic substrate Gly-Pro-p-NA as described by Lacroix, & Li-Chan (2012) with some modifications. The samples were dissolved to the desired concentrations in 100 mM Tris-HCl buffer, pH 8.0, to concentrations ranging from 0.5 to 1.5 mg/mL (final assay concentrations). In a 96-well microplate, 20 µL of the test sample, 100 µL of substrate Gly-Pro-pnitroanilide (0.75 mM), 30 µL of DPP-IV (0.0375 mM) were added and the mixture incubated at 37 °C for 30 min. Each test

sample was analyzed in triplicate, and the absorbance values were corrected for sample blanks in which DPP-IV was replaced with Tris-HCl buffer (100 mM, pH 8.0). The positive control (test sample was replaced with Tris-HCl buffer (100 mM, pH 8.0) and negative control (no DPP-IV activity and test sample) were prepared by using Tris-HCl buffer (100 mM, pH 8.0). The percent DPP-IV inhibition was calculated as Eq. 24:

% DPP-IV inhibition = 100 x {1-[(A_{405} (test sample) - A_{405} (test sample blank)]/ [(A_{405} (positive control) - A_{405} (negative control)]}.....(Eq.24)

The IC_{50} values (concentrations of samples required to cause a 50% inhibition of the enzyme activity) were determined from the cubic regression equations generated by fitting the data from the plot of percent DPP-IV inhibition against hydrolysate concentrations ranging from 0.2 to 1.0 mg/mL (final assay concentration). The tripeptide diprotin A (Ile-Pro-Ile) was used as a reference inhibitor.

13. Determination of the antioxidant activity

13.1 DPPH assay

Reduction of DPPH by an antioxidant usually results in a loss of absorbance at 517 nm. The extent of discoloration of the solution indicates the scavenging efficiency of the added compound. Determination of the antioxidant activity of hydrolysates was adapted from the method described by Pownall et al. (2010). using a 96-well microplate. The hydrolysates (final assay concentration of 1.5 to 0.0625 mg protein/ml) were dissolved in 0.1 M sodium phosphate buffer, pH 7.0 containing 1% (w/v) Triton X-100. A solution of DPPH was dissolved in methanol to a final concentration of 100 µM. Samples (100 µl) were added to 100 µl of DPPH in a 96-well microplate. A blank well contained only DPPH and the sodium phosphate buffer. The plate was then covered and incubated in the dark at room temperature for 30 min. Thereafter, the absorbance of the sample (As) and blank (Ab) at 517 nm was measured in a spectrophotometer. The scavenging activities of SIH and the peptide fractions were compared to that of GSH. The percent scavenging activity of GSH and the samples was calculated as Eq.25:

DPPH radical scavenging activity (%) = (Ab-As/Ab) × 100 ..... (Eq.25)

13.2 Hydroxyl radical scavenging assay (HRSAs)

The HRSAs was modified based on a method described by Girgih et al. (2011). Protein hydrolysate and GSH were each separately dissolved in 0.1 M sodium phosphate buffer (pH 7.4) while and 1, 10-phenanthroline (3 mM), $FeSO_4$ (3 mM) and 0.01% hydrogen peroxide were each separately dissolved in distilled water. An aliquot (50 µL) of 1, 10-phenanthroline and 50 µL of $FeSO_4$ were added consecutively to 50 µL of protein hydrolysate, GSH, or buffer (control) in a clear, flat bottom 96-well microplate. Final assay concentration of samples was 1.5 to 0.25 mg protein/ml. To initiate a reaction in the wells, 50 µL of hydrogen peroxide (H_2O_2) solution was added to the mixture, which was then covered and incubated at 37 °C for 1 hr with shaking. Thereafter, the absorbance of the mixtures was measured at 536 nm every 10 min for a period of 1 hr. The absorbance was also determined for a blank (without peptides and H_2O_2) and a blank (without peptides). The $^{\cdot}OH$ scavenging activity was calculated as described by Ajibola et al. (2011).

13.3 Determination of Fe^{2+} chelating activity

The iron-chelating activity of protein hydrolysates were measured using a slightly modified version of a previous method Ajibola et al. (2011). Protein hydrolysates or GSH solutions (final concentration of 1.5 to 0.0625 mg protein/mL) was mixed with 0.025 mL of 2 mM $FeCl_2$ and 0.925 mL distilled water in a reaction tube. Thereafter, 0.05 mL of 5 mM Ferrozine [3-(2-pyridyl)-5, 6diphenyl-1, 2, 4-triazine-4', 4"-disulfonic acid, sodium salt] solution was added and mixed thoroughly. The mixture was allowed to stand at room temperature for 10 min followed by removal of 200 µL aliquot of the reaction mixture and added to a clear bottom 96- well microplate well. The control experiment contained all the reaction mixtures except that distilled water was used to replace the sample. The absorbance of sample (A_s) and blank (A_b) was measured using a spectrophotometer at 562 nm and the metal chelating activity of the sample was compared to that of GSH. The percentage chelating effect (%) was calculated as Eq. 26:

$$Fe^{2+} \text{ chelating activity } (\%) = (A_b\text{-}A_s/A_b) \times 100 \quad \text{(Eq.26)}$$

The EC_{50} values (concentrations of samples required to cause a 50% inhibition of the enzyme activity) were determined from the cubic regression equations generated by fitting the data from the plot of percent inhibition against

hydrolysate concentrations ranging from 1.5 to 0.0625 mg/mL (final assay concentration). The glutathione (GSH) was used as a reference inhibitor.

13.4 FRAP assay

The ability of the hydrolysate to reduce iron (III) was determined according to the method of Benzie and Strain (1996) with some modifications. Briefly, the FRAP reagent was freshly prepared by mixing 300 mM acetate buffer (sodium acetate buffer, pH 3.6), 10 mM 4,6-tripryridyls-triazine (TPTZ) in 40 mM HCl and 20 mM ferric chloride in a ratio 5:1:1 (v/v) before evaluation. Two hundred mL of FRAP reagent (preheated to 37 °C) was added to 40 mL of SIH, peptide fractions or GSH in a 96-well microplate. Absorbance at 593 nm was measured relative to a reagent blank. Ferrous sulfate (0.025-0.25 mM) was used to prepare a standard curve and the results of the samples were expressed in mM $FeSO_4$. Increased absorbance of the reaction mixture indicated increased reducing power.

13.5 Inhibition of linoleic acid oxidation

Linoleic acid oxidation was measured according to a method described by Li et al (2008). Protein hydrolysates or GSH were dissolved in 2.5 mL of 0.1 M phosphate buffer, pH 7.0 at a final concentration of 1 mg protein/mL. Each mixture was added to 1.5 mL of 50 mM linolenic acid dissolved in 99.5% ethanol and stored in a glass test tube kept at 60 °C in the dark for 7 days. Daily, 100 mL of the reaction mixture was removed and mixed with 4.7 mL of 75% aqueous ethanol, 100 µL of ammonium thiocyanate (30%, w/v) and 100 µL of 0.02 M acidified ferrous chloride (dissolved in 1 M HCl). An aliquot (200 µL) of the resulting solution was added to a clear bottom 96-well microplate and the degree of color development was measured using the spectrophotometer at 500 nm after 3 min incubation at room temperature. An increase in absorbance indicates an increase in linoleic acid oxidation.

14. In vitro enzyme inhibition assays

The hydrolysates and membrane fractions were tested for in vitro inhibition of the following digestive enzymes: α-amylase, α-glucosidase, trypsin and pancreatic lipase. The appropriate assay methods to determine the activity of each enzyme was obtained from the literature and adapted for the samples. All assays were conducted in triplicates.

14.1 α-amylase inhibition assays

The α-amylase inhibitory activity was assayed following the method described by Siow et al. (2017) with slight modifications. Briefly, lyophilized protein hydrolysates and membrane fractions were re-dissolved in 1 mL of 0.02 M sodium phosphate containing 0.006 M NaCl, pH 6.9. A 100 µL aliquot of each sample (final peptide concentration = 1-6 mg/mL) and 100 µL of α-amylase solution (final concentration = 3 µg/mL) were added to test tubes and allowed to incubate for 10 min at 25 °C. After incubation, 100 µL of 1% starch solution (dissolved in the above buffer) was added and incubated at 25 °C for 10 min. The reaction was terminated by adding 200 µL of dinitrosalicylic acid (DNSA) color reagent (96 mM DNSA, 2 M sodium potassium tartrate tetrahydrate and 2 M NaOH) followed by incubation in a boiling water bath at 100 °C for 5 min. The reaction mixture was allowed to cool to room temperature, after which 3 mL of double-distilled water was added. A 200 µL aliquot of the reaction mixture was then transferred to a 96-well microplate and the absorbance read at 540 nm using a Synergy™ H4 Hybrid microplate reader (Biotek™, Vermont, USA) set at 25 °C. A blank reading (addition of buffer to replace enzyme) was subtracted from each well. The enzyme activity was quantified by measuring the maltose equivalents released from starch at 540 nm. The pharmacological α-amylase inhibitor acarbose was assayed the same way and used as a positive control. The 50% inhibition concentration (IC_{50}) values of the protein isolate and hydrolysates were obtained reduced by half. The α-amylase inhibitory activity (%) was calculated as Eq. 27:

$$\alpha\text{-amylase inhibition (\%)} = [((A_c - (A_s - A_{sb})) / A_c] * 100 \quad \text{(Eq.27)}$$

A_c = Absorbance of the control; A_s = Absorbance of the sample; A_{sb} = Absorbance of the sample blank

14.2 α-glucosidase inhibition assays

The α-glucosidase inhibitory activity was assayed according to previously described methods (Awosika, 2018) with slight modifications. Briefly, 300 mg of rat intestinal acetone powder was homogenized in 9 ml of 0.9% NaCl solution and centrifuged at 12,000 x g for 30 min; the clear supernatant was used as a source of α-glucosidase enzyme. Lyophilized protein hydrolysates and membrane fractions were redissolved in 0.1 M sodium phosphate buffer, pH 6.9. Fifty µl of samples (final peptide

concentration = 1-6 mg/mL) were pre-mixed with 50 µl of α-glucosidase enzyme (final concentration = 8.33 mg/mL) in a 96-well microplate and incubated at 37 °C for 10 min. Following incubation, 100 µl of 5 mM 4-nitrophenyl α-D-glucopyranoside (PNP-glycoside) solution (in 0.1 M sodium phosphate buffer, pH 6.9) was added to each well and absorbance read continuously at 405 nm for 30 min (at every 30 seconds interval) using a microplate reader set at 37 °C. A blank reading (no enzyme added) was subtracted from each well. α-glucosidase activity was quantified by measuring the p-nitrophenol released from the PNP-glycoside at 405 nm. The pharmacological α- glucosidase inhibitor acarbose was assayed using the same protocol and served as a positive control. The 50% inhibition concentration (IC_{50}) values of the protein isolate and hydrolysates were obtained reduced by half. The α-glucosidase inhibitory activity (%) was calculated as Eq. 28:

$$\alpha\text{-glucosidase inhibition (\%)} = [((A_c - A_{cb}) - (A_s - A_{sb})) / [A_c - A_{cb}] * 100 \quad \text{.....(Eq.28)}$$

A_c = Absorbance of the control; A_{cb} = Absorbance of the control blank;

A_s = Absorbance of the sample; A_{sb} = Absorbance of the sample blank

14.3 Trypsin inhibition assays

Trypsin inhibitory activity was determined following the method described by Awosika (2018) Briefly, 200 µl of trypsin (dissolved in 20 mM Tris-HCl buffer, pH = 7.5; final concentration = 60 µg/ml) was premixed with 200 µL of samples (re-dissolved in the above buffer; final peptide concentration = 1-6 mg/ml) and incubated for 5 min at 37 °C. The reaction was started by the addition of 500 µL of 1 mM BApNA (N-Benzoyl-D-L arginine paranitroanilide) prepared in Tris-HCl buffer (pH 7.5) that contained 1% (v/v) dimethyl sulfoxide. Following incubation for 10 min at 37 °C, the reaction was terminated by the addition of 100 µL of 30 % (v/v) acetic acid. A 200 µL aliquot of the reaction mixture was transferred to a 96-well microplate and the absorbance measured using a microplate reader at 410 nm. Trypsin inhibitory activity was determined by measuring the release of p-nitroaniline from the substrate BApNA. 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) was used as a positive control. The 50% inhibition concentration (IC_{50}) values of the protein isolate

and hydrolysates were obtained reduced by half. The trypsin inhibitory activity (%) was calculated as Eq. 29:

Trypsin inhibition (%) = [Ac − As] /Ac] *100 .....(Eq.29)

A_c = Absorbance of the control; A_s = Absorbance of the sample

14.4 Pancreatic lipase inhibition

Pancreatic lipase inhibitory activity was assayed following the method described by Awosika, (2018) with slight modifications. Pancreatic lipase inhibitory activity was determined by measuring the release of 4-methylumbelliferone (4-MU) from the substrate 4-methylumbelliferyl oleate (4-MU oleate). A 25 µL aliquot of samples (final peptide concentration = 1-5 mg/mL) dissolved in Tris buffer (13 mM Tris-HCl, 150 mM NaCl and 1.3 mM $CaCl_2$, pH = 8) and 225 µL of a 0.5 mM 4-MU oleate solution were mixed in a 96-well microplate and incubated for 15 min at 37 °C. After incubation, 25 µL of pancreatic lipase solution (final concentration = 3.125 U/mL) was added to start the enzyme reaction and then incubated at 37 °C for 1 hr. After incubation, the amount of 4-methylumbelliferone released by the lipase was measured with a microplate reader at a wavelength of 400 nm. The pharmacological pancreatic lipase inhibitor (orlistat) was used as a positive control. The 50% inhibition concentration (IC_{50}) values of the protein isolate and hydrolysates were obtained reduced by half. The pancreatic lipase inhibitory activity (%) was calculated as Eq. 30:

Pancreatic lipase inhibition (%) = [Ac - As] /Ac] * 100..... (Eq.30)

A_c = Absorbance of the control

A_s = Absorbance of the sample

15. Development of beverage from Sacha Inchi protein hydrolysate

As plant-based eating becomes more and more popular, alternative protein sources have been flooding the food market. Additionally, they have no major allergens. A beverage was formulated with hydrolyzed SPHPe, organic brown rice protein isolate, organic pea protein isolate, stevia, cocoa, coffee and vanilla powder as shown in Table 3 and analyzed for their proximate compositions, functional properties and protein digestion.

Table 9 Beverage from Sacha Inchi protein hydrolysate

Ingredients/ Chocolate formula	**g.**
Sacha Inchi protein hydrolysate (SPHPe)	10
Low-fat-cocoa power	6
Stevia	9
Organic brown rice protein isolate	2.5
organic pea protein isolate [from yellow peas (pisum sativum)]	2.5
Ingredients / Coffee formula	**g.**
Sacha Inchi protein hydrolysate (SPHPe)	10
Coffee power	2
Soy creamer	4
Stevia	9
Organic brown rice protein isolate	2.5
organic pea protein isolate [from yellow peas (pisum sativum)]	2.5
Ingredients / Vanilla formula	**g.**
Sacha Inchi protein hydrolysate (SPHPe)	10
Vanilla power	3
Soy creamer	5
Stevia	7
Organic brown rice protein isolate	2.5
organic pea protein isolate [from yellow peas (pisum sativum)]	2.5

Note: Net Wt. 30/150 mL

Directions: mix one sachet (30 g) with 150 mL of plain water or your favorite beverage and blend well.

Storage: Store in cool and dry place (Below 30 °C). Keep away from direct sunlight.

15.1 Determination of proximate composition

Determination of proximate composition was determined according to AOAC (2005). Total protein content (N × 6.25) was evaluated from 100 mg of dried meal using the Kjeldahl Method. Moisture content was determined by drying the

samples at 105°C to a constant weight. The ash percentage was quantified by incineration at 600 °C for 6 hours. Total dietary fibre content is measured according to the AOAC enzymatic-gravimetric method. The basis of this method is the isolation of dietary fibre by enzymatic digestion of the rest of the constituents of the material. The residue is measured gravimetrically. The total lipid content was determined following an extraction procedure adapted from AOAC (1990). Samples (2 g) was refluxed in Soxhlet apparatus with 150 mL petroleum ether for 6 hr. The excess solvent was removed by evaporation under vacuum and dried in a to a content weight in a forced air oven. Lipid content was expressed as a percentage of dry sample weight. The total carbohydrate content was determined by difference, i.e., 100 - (sum of percentages of moisture, ash, protein and lipids) (Oshodi, 1992).

15.2 In vitro digestion

In vitro digestion was carried out according to Garrett et al. (1999) with slight modifications. Briefly, the beverage (5 g dry protein) were homogenized in distilled water (250 mL) using an IKA homogenizer (IKA Works Asia, Digital 3725001 T25 digital) for 1 min and adjusted to pH 2.0 with 5 M HCl. Pepsin was added (2.86% [w/w], based on dry substrate), and the samples were incubated in a shaking incubator at 37 °C for 1 hr. The pH was adjusted to 5.3 with a 0.9 M $NaHCO_3$ solution and then further to pH 7.5 with 5 M NaOH. Pancreatin was then added (4.00% [w/w], based on dry substrate), and the samples were further incubated under the same conditions for 2 hr. Subsequently, all digested samples were submerged in a 95 °C water bath for 10 min, cooled on ice to room temperature, and centrifuged at 10000 x g for 10 min. The peptide content of the supernatant was determined using the Lowry method with tyrosine as a standard. The degree of digestibility was determined based on TNBS using leucine as a standard and calculated as previously shown in Eq.7:

15.3 Total soluble solid (TSS)

the samples were subjected to TSS measurement using a digital refractometer, which provided the °Brix value with an accuracy of +/- 0.1%. The TSS content of date samples was measured using the procedure described by Dadzie, & Orchard (1997) and some modification. Protein shake was prepared by protein chake 30 g mix in 1 Cups (250 mL) of ice-cold water or blend. Then one drop was placed on the prism of the refractometer and °Brix value was recorded.

15.4 Dispersibility

Dispersibility was determined as described by Takashi, & Seibi (1988). Dispersibility of the sample was determined by dissolving approximately 10 g of each sample in 100 mL of distilled water at 27°C. The mixture was manually stirred continuously for 1 min and allowed to rest for 30 min for the suspended particles to settle down before the supernatant was carefully decanted. The density of the supernatant was then determined by filling an aliquot of the supernatant into a 50 mL density bottle. The mass of the filled bottle was noted. The weight of the dispersed solid was calculated as twice the difference in the mass of the supernatant and an equal volume (50 mL) of distilled water. All the weight determinations were done in duplicate using a digital balance (0.001 g accuracy).

15.5 Solubility index

Solubility was determined as described by Takashi, & Seibi (1988). About 5 g of each sample were suspended in 50 mL of water at 30 C in a centrifuge tube. The suspension was stirred intermittently for 30 min before it was finally centrifuged at 9500 rpm for 10 min. The supernatant was completely drained into an evaporating dish and dried to constant weight at 105 °C. The weight of the solids recovered after drying was used to calculate the water solubility (%). Analyses were done in triplicate.

15.6 Wettability (or wetting time)

Wettability (or wetting time) was determined as described by Schubert (1980). The wetting time was regarded as the time (in seconds) required for all the powder to become wetted and penetrate the surface of the distilled water at 27 °C. Analyses were done in triplicate.

16. Statistical Analysis

Data were collected in triplicates and subjected to one way analysis of variance using statistical analysis of variance (ANOVA) was done to determine the significant differences among means followed by Duncan's new multiple range tests were carried out to determine statistical significance ($p < 0.05$) using SPSS 11.0 software (SPSS Inc., Chicago, Ill., U.S.A.).

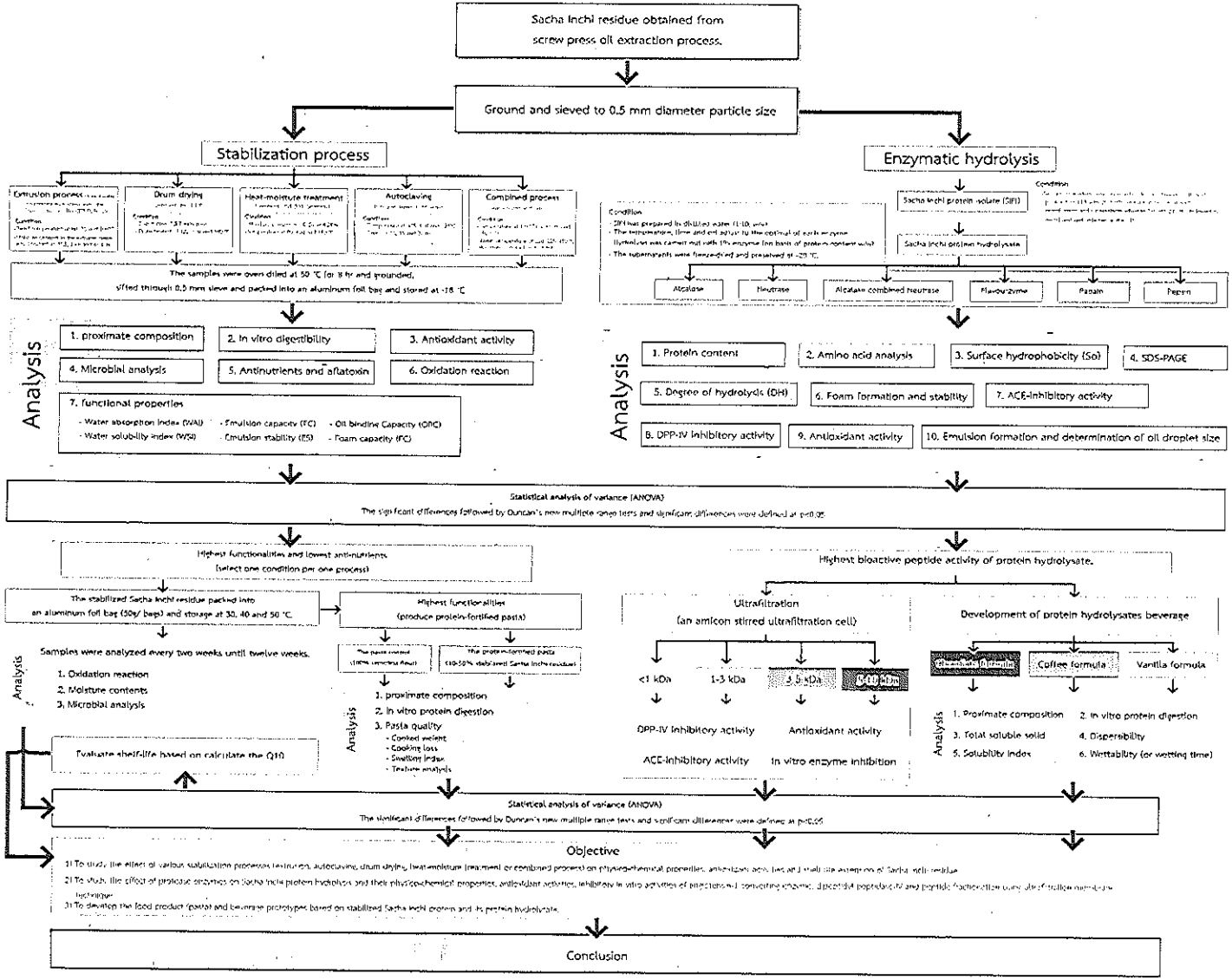


Figure 9 Research flow chart

CHAPTER IV

RESULT AND DISCUSSION

This chapter provides the results and discussions of the study which were divided into 2 sections as following.

Part 1 Stabilization process of Sacha Inchi protein residue

Part 2 Enzymatic proteolysis process of Sacha Inchi protein

Part 1 Stabilization process of Sacha Inchi Protein residue

1. Proximate composition

The proximate composition of the control (non-stabilized) and SSIR are presented in Table 4-8. All SSIR had a significant ($p<0.05$) higher protein content than the control due to the loss of moisture and fat in the samples during thermal stabilization processes. Oduro et al. (2011) observed that heat processing contributed in reducing of moisture content. Similarly, Chukwu (2009) reported that fat loss is intensive in heat processing, due to fat exude with the moisture evaporation. The protein content of SSIR through extrusion process at barrel temperature 100 °C and in-barrel moisture level 61.8% was also found to be a significantly highest ($p <0.05$), as presented in Table 9.

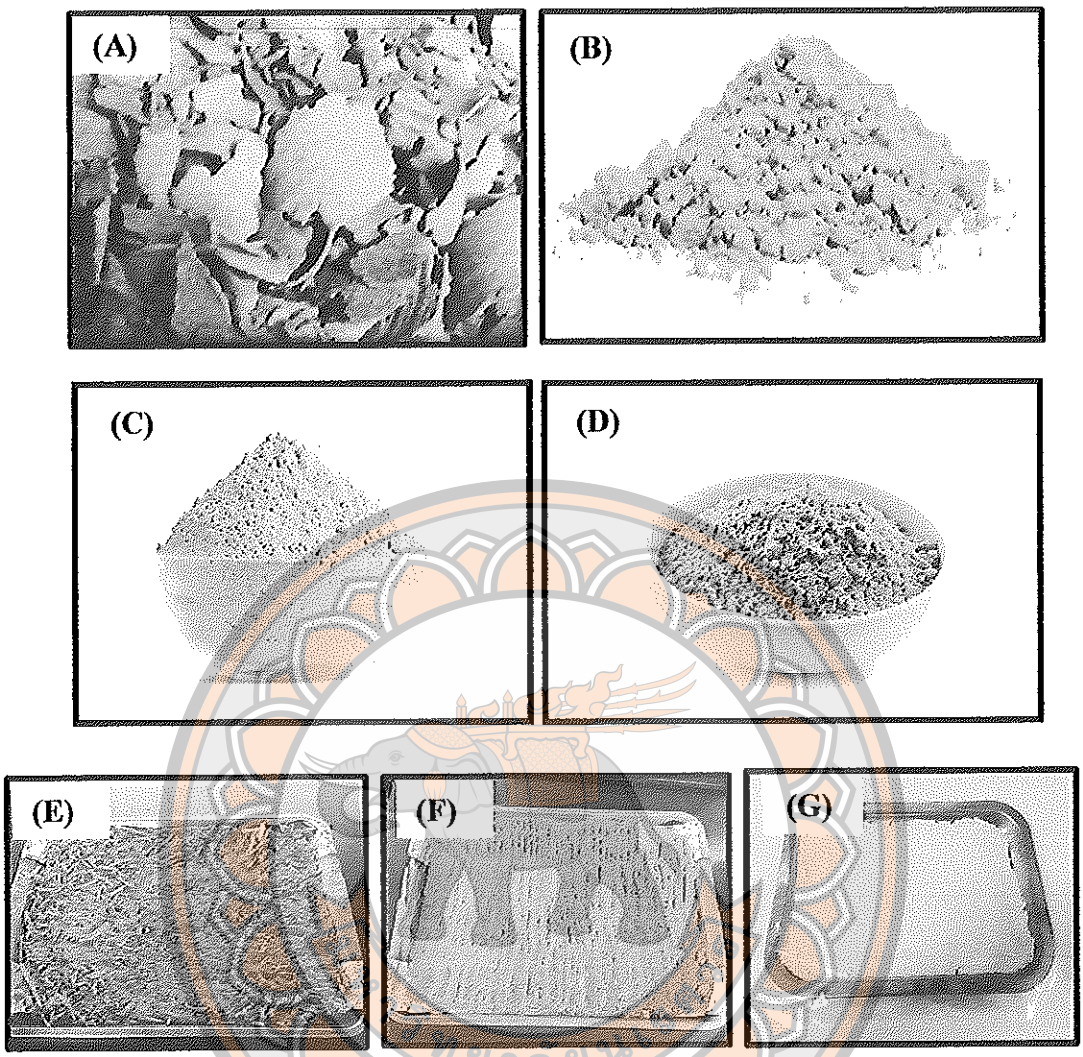


Figure 10 The SSIR through thermal process. SIR, a by-product (A), SIR was ground and sieved (B), SSIR by extrusion (C), combined process (autoclaving combined with extrusion) (D), drum-drying processes (E), autoclaving (F) and heat-moist treatment (G)

Table 10 Proximate composition (dry basis) of SSIR by the extrusion process

Barrel temperature (°C) / In-barrel moisture (%)	Protein (%)	Fat (%)	Ash (%)	Moisture (%)	Fiber (%)	Carbohydrate (%)
80 / 39.5	55.54±0.10^{h}	3.49±0.02^{b}	5.82±0.33^{ab}	3.33±0.16^{b}	5.31±0.09^{d}	31.82±0.15^{a}
80 / 50.6	55.74±0.11^{h}	3.45±0.06^{b}	5.66±0.23^{b}	3.74±0.06^{b}	5.34±0.07^{d}	31.41±0.11^{a}
80 / 61.8	56.85±0.11^{g}	3.45±0.02^{b}	6.07±0.21^{ab}	3.72±0.16^{b}	5.71±0.06^{b}	29.91±0.14^{c}
90 / 39.5	57.02±0.03^{f}	3.17±0.08^{d}	5.69±0.24^{b}	3.14±0.03^{b}	5.36±0.03^{d}	30.98±0.08^{b}
90 / 50.6	58.11±0.06^{e}	3.15±0.09^{c}	5.85±0.27^{ab}	3.10±0.05^{b}	5.41±0.03^{c}	29.79±0.15^{c}
90 / 61.8	58.85±0.01^{d}	3.17±0.01^{c}	6.12±0.07^{a}	3.97±0.01^{b}	5.32±0.03^{d}	27.89±0.11^{d}
100 / 39.5	60.03±0.12^{c}	3.04±0.02^{d}	6.05±0.19^{ab}	3.04±0.52^{b}	5.99±0.02^{a}	27.84±0.12^{d}
100 / 50.6	60.11±0.03^{b}	3.11±0.07^{c}	6.04±0.14^{ab}	3.21±0.57^{b}	5.30±0.04^{d}	27.53±0.05^{d}
100 / 61.8	61.33±0.02^{a}	3.11±0.07^{c}	5.69±0.15^{b}	3.21±0.17^{b}	5.35±0.04^{d}	26.66±0.06^{e}
The control	54.30±0.37^{i}	10.54±0.03^{a}	6.16±0.48^{a}	6.04±0.04^{a}	5.23±0.01^{e}	22.96±0.02^{f}

Note: Data are expressed as the mean ± SD (n=3)

$^{a-f}$ Values in each column for each analytical property with differences are significantly different ($p<0.05$)

Table 11 Proximate composition (dry basis) of SSIR by drum drying process

Drums heated (°C) / Rotation speeds (rpm)	Protein (%)	Fat (%)	Ash (%)	Moisture (%)	Fiber (%)	Carbohydrate (%)
100 / 3	55.02 ± 0.02^{h}	8.84 ± 0.03^{c}	5.33 ± 0.13^{a}	4.14 ± 0.04^{b}	5.14 ± 011^{c}	26.67 ± 0.13^{a}
100 / 5	55.11 ± 0.12^{g}	8.82 ± 0.01^{c}	5.35 ± 0.08^{a}	4.15 ± 0.07^{b}	5.11 ± 0.13^{c}	26.57 ± 0.13^{a}
100 / 7	55.07 ± 0.08^{h}	8.85 ± 0.12^{c}	5.33 ± 0.03^{a}	4.21 ± 0.15^{b}	5.23 ± 0.09^{b}	26.54 ± 0.08^{a}
120 / 3	56.34 ± 0.05^{f}	8.60 ± 0.06^{c}	5.14 ± 0.14^{c}	3.84 ± 0.03^{c}	5.08 ± 0.07^{d}	26.08 ± 0.15^{b}
120 / 5	56.55 ± 0.05^{e}	8.62 ± 0.09^{c}	5.21 ± 0.03^{b}	3.99 ± 0.06^{c}	5.16 ± 0.11^{c}	25.84 ± 0.13^{b}
120 / 7	56.86 ± 0.04^{d}	7.97 ± 0.11^{d}	5.18 ± 0.13^{c}	4.06 ± 0.04^{b}	5.11 ± 0.13^{c}	25.93 ± 0.06^{b}
140 / 3	57.99 ± 0.03^{a}	7.09 ± 0.01^{b}	5.17 ± 0.04^{ab}	3.74 ± 0.06^{c}	5.14 ± 0.08^{c}	26.01 ± 0.07^{c}
140 / 5	57.67 ± 0.07^{b}	7.11 ± 0.13^{b}	5.22 ± 0.07^{b}	3.82 ± 0.11^{c}	5.12 ± 0.03^{c}	26.18 ± 0.05^{c}
140 / 7	57.35 ± 0.07^{c}	7.14 ± 0.08^{b}	5.19 ± 0.11^{c}	3.85 ± 0.02^{c}	5.34 ± 0.17^{a}	26.47 ± 0.09^{d}
The control	54.30 ± 0.37^{i}	10.54 ± 0.03^{a}	6.16 ± 0.48^{a}	6.04 ± 0.04^{a}	5.23 ± 0.01^{b}	22.96 ± 0.02^{c}

Note: Data are expressed as the mean ± SD (n=3)

$^{a-i}$ Values in each column for each analytical property with different are significantly different ($p<0.05$)

Table 12 Proximate composition (dry basis) of SSIR by autoclaving

Temperature (°C) / Time (min)	Protein (%)	Fat (%)	Ash (%)	Moisture (%)	Fiber (%)	Carbohydrate (%)
105 / 10	55.23±0.03^{e}	9.60±0.03^{c}	5.16±0.06^{b}	4.11±0.03^{b}	5.26±0.05^{a}	25.90±0.05^{a}
105 / 15	55.28±0.04^{d}	9.54±0.03^{c}	5.18±0.10^{b}	4.08±0.06^{b}	5.21±0.05^{b}	25.92±0.04^{a}
105 / 30	55.26±0.06^{d}	9.59±0.07^{c}	5.06±0.11^{b}	4.27±0.09^{b}	5.27±0.08^{a}	25.82±0.05^{a}
110 / 10	56.31±0.05^{c}	9.14±0.10^{d}	5.11±0.06^{b}	4.21±0.02^{b}	5.19±0.08^{c}	25.23±0.06^{a}
110 / 15	56.38±0.02^{c}	9.17±0.08^{d}	5.16±0.03^{b}	4.27±0.02^{b}	5.20±0.08^{b}	25.02±0.03^{a}
110 / 30	56.33±0.04^{c}	9.18±0.05^{d}	5.17±0.11^{b}	4.17±0.04^{b}	5.22±0.01^{b}	25.15±0.06^{a}
121 / 10	56.76±0.06^{b}	9.61±0.04^{c}	5.19±0.17^{b}	3.26±0.05^{c}	5.28±0.04^{a}	25.18±0.13^{a}
121 / 15	56.87±0.05^{a}	9.95±0.05^{b}	5.16±0.13^{b}	3.28±0.08^{c}	5.24±0.09^{b}	24.74±0.04^{b}
121 / 30	56.92±0.06^{a}	9.93±0.03^{b}	5.13±0.08^{b}	3.74±0.03^{c}	5.21±0.12^{b}	24.28±0.05^{b}
The control	54.30±0.37^{f}	10.54±0.03^{a}	6.16±0.48^{a}	6.04±0.04^{a}	5.23±0.01^{b}	22.96±0.02^{c}

Note: Data are expressed as the mean ± SD (n=3)

$^{a\text{-}f}$ Values in each column for each analytical property with different are significantly different ($p<0.05$)

Table 13 Proximate composition (dry basis) of SSIR by heat-moisture treatment

Temperatures (°C) / Moisture content (%)	Protein (%)	Fat (%)	Ash (%)	Moisture (%)	Fiber (%)	Carbohydrate (%)
90 / 15	54.55±0.04^{e}	10.49±0.12^{a}	5.22±0.05^{b}	4.88±0.13^{b}	5.11±0.02^{c}	24.86±0.05^{d}
90 / 20	54.58±0.05^{e}	10.44±0.13^{a}	5.24±0.03^{b}	4.98±0.08^{b}	5.13±0.05^{c}	24.76±0.04^{d}
90 / 25	54.61±0.11^{d}	10.47±0.07^{a}	5.22±0.03^{b}	5.00±0.05^{a}	5.16±0.04^{b}	24.70±0.12^{d}
100 / 15	55.06±0.12^{c}	9.29±0.09^{b}	5.28±0.02^{b}	4.75±0.04^{b}	5.15±0.05^{b}	25.62±0.09^{c}
100 / 20	55.13±0.09^{b}	9.23±0.06^{b}	5.25±0.04^{b}	4.78±0.11^{b}	5.14±0.04^{c}	25.61±0.07^{c}
100 / 25	55.13±0.07^{b}	9.31±0.16^{b}	5.29±0.03^{b}	4.79±0.06^{b}	5.11±0.05^{c}	25.48±0.05^{c}
110 / 15	55.34±0.07^{a}	9.16±0.08^{c}	5.27±0.04^{b}	3.71±0.08^{c}	5.09±0.04^{d}	26.52±0.08^{b}
110 / 20	55.36±0.08^{a}	9.11±0.08^{c}	5.28±0.07^{b}	3.78±0.05^{c}	5.11±0.03^{c}	26.47±0.03^{b}
110 / 25	55.39±0.04^{a}	8.33±0.18^{d}	5.29±0.05^{b}	3.46±0.05^{d}	5.16±0.03^{b}	27.53±0.07^{a}
The control	54.30±0.37^{i}	10.54±0.03^{a}	6.16±0.48^{a}	6.04±0.04^{a}	5.23±0.01^{a}	22.96±0.02^{e}

Note: Data are expressed as the mean ± SD (n=3)

$^{a-i}$ Values in each column for each analytical property with different are significantly different ($p<0.05$)

Table 14 Proximate composition (dry basis) of SSIR by combined process

Time (min) / Temperatures (°C)	Protein (%)	Fat (%)	Ash (%)	Moisture (%)	Fiber (%)	Carbohydrate (%)
30/100	55.11±0.07^{b}	7.24±0.12^{b}	5.09±0.08^{b}	4.36±0.05^{c}	5.14±0.12^{b}	28.20±0.07^{a}
45/100	55.03±0.13^{c}	7.13±0.13^{b}	5.16±0.09^{b}	4.42±0.09^{b}	5.11±0.05^{b}	28.26±0.11^{a}
60/100	55.08±0.11^{c}	7.22±0.07^{b}	5.09±0.11^{b}	4.46±0.11^{b}	5.14±0.11^{b}	28.15±0.03^{a}
30/125	55.11±0.12^{b}	7.06±0.09^{b}	5.06±0.12^{b}	4.48±0.06^{b}	5.06±0.15^{c}	28.29±0.07^{a}
45/125	55.13±0.11^{b}	7.21±0.07^{b}	5.11±0.09^{b}	4.45±0.03^{b}	5.08±0.04^{c}	28.10±0.10^{a}
60/125	55.13±0.03^{b}	7.31±0.11^{b}	5.18 ±0.05^{b}	4.42±0.09^{b}	5.11±0.03^{b}	27.96±0.02^{b}
30/150	56.14±0.04^{a}	6.16±0.08^{c}	5.06±0.09^{b}	4.06±0.01^{d}	5.12±0.08^{b}	28.58±0.13^{a}
45/150	56.14±0.02^{a}	6.11±0.07^{c}	5.14±0.12^{b}	4.03±0.09^{d}	5.13±0.09^{b}	28.58±0.06^{a}
60/150	56.89±0.04^{a}	6.13±0.03^{c}	5.05±0.11^{b}	4.09±0.03^{d}	5.14±0.11^{b}	27.84±0.07^{b}
The control	54.30±0.37^{d}	10.54±0.03^{a}	6.16±0.48^{a}	6.04±0.04^{a}	5.23±0.01^{a}	22.96±0.02^{c}

Note: Data are expressed as the mean ± SD (n=3)

$^{a-d}$ Values in each column for each analytical property with different are significantly different ($p<0.05$)

autoclaving at 121 °C for 30, 45, 60 min combined extrusion at barrel temperature employed of 100, 125, 150 °C (in-barrel moisture level was 61.8)

Table 15 Proximate composition (dry basis) of the control and SSIR

Stabilization process	Protein (%)	Fat (%)	Ash (%)	Moisture (%)	Fiber (%)	Carbohydrate (%)
Extrusion (100 °C / 61.8%)	61.33±0.02^{a}	3.11±0.07^{f}	5.69±0.15^{b}	3.21±0.17^{e}	5.35±0.04^{a}	26.66±0.06^{b}
Drum drying (140 °C / 3 rpm)	57.99±0.03^{b}	7.09±0.01^{d}	5.17±0.04^{d}	3.74±0.06^{c}	5.14±0.08^{d}	26.01±0.07^{b}
Autoclaving (121 °C/ 30 min)	56.92±0.06^{c}	9.93±0.03^{b}	5.13±0.08^{d}	3.74±0.03^{c}	5.21±0.12^{c}	24.28±0.05^{c}
Heat-moisture treatment (110 °C/ 25%)	55.39±0.04^{d}	8.33±0.18^{c}	5.29±0.05^{c}	3.46±0.05^{d}	5.16±0.03^{d}	27.53±0.07^{a}
Combined process (60 min /150 °C)	56.89±0.04^{c}	6.13±0.03^{e}	5.05±0.11^{e}	4.09±0.03^{b}	5.14±0.11^{d}	27.84±0.07^{a}
The control	54.30±0.37^{e}	10.54±0.03^{a}	6.16±0.48^{a}	6.04±0.04^{a}	5.23±0.01^{b}	22.96±0.02^{d}

Note: Data are expressed as the mean ± SD (n=3)

$^{a-f}$ Values in each column for each analytical property with different are significantly different ($p<0.05$)

2. Functional properties

The functional properties of protein are affected by both intrinsic and extrinsic factors. The intrinsic factors include shape, size, amino acid composition and sequence, the distribution of net charges, the ration between hydrophobicity/ hydrophilicity, secondary, tertiary and quaternary structures of the protein as well as the protein's capacity to interact with other components in the food system (Damodaran, 1997). The extrinsic factors that affect the functionality of proteins are pH, temperature, moisture, chemical additives, mechanical processing, enzymes and ionic strength (Kinsella, 1982). The functional properties that are required from a protein vary with different food applications and food systems. In general, the three most important functional properties of food proteins are solubility, emulsification, and foaming (Kinsella, 1982). One reason why protein possess such different functional properties is the fact that all proteins are built up with different amino acids (Nakai, 1983). The amino acid composition affects the functional properties of a protein according to how they are disposed of in the polypeptide chain, as well as what type and how many of those amino acids are present (Kinsella, 1981).

2.1 WAI, WSI and SP

With an increase in temperature process, the WSI and SP of SSIR increased, while the WAI decreased, as presented in Table 10-15. WSI of SSIR through extrusion process at barrel temperature 100 °C and in-barrel moisture level 61.8% was highest ($p < 0.05$). The change in the protein system caused by high heat, high pressure, shear forces could increase intermolecular interactions owing to the partial unfolding of protein molecules then the breaking and forming of protein molecules by disulfide bonds, non-covalent bonds and peptide bonds especially hydrophobicity and hydrophilicity group. WAI decreased with an increase in temperature process, which may be attributed to the reduction of elasticity of protein and starch gelatinization and swelling of the crude fiber, which occurred during the process, could all be responsible for the decreased WAI (Christ, 2005). It can be seen from Table 10 (stabilized Sacha Inchi residues through extrusion process at barrel temperature 100 °C and in-barrel moisture level 61.8%) and Table 14 (autoclaving at temperature 121 °C for 60 min and combined extrusion process at barrel temperature 150 °C and in-barrel moisture level 61.8%) that WAI decrease when increased

temperature and may be attributed to high mechanical shear and higher expansion due to gelatinization.

The SP of SSIR shown in Table 10-15 indicate that autoclaving combined with extrusion caused significantly ($p < 0.05$) higher soluble protein than that of other stabilization processes, due to high heat and high pressure would lead to the protein degradation and the release of low-molecular-weight compounds. At high heat, high pressure and shear forces especially strong process such as extrusion, autoclaving and combined process can change the molecular structure of proteins or breakdown of S-S bonds with the release of H_2S, the release of NH_3 from amide groups, dissociation of subunits and /or breakdown of these subunits into compounds of small molecular weights (Shimada, & Cheftel, 1988), which may be increase protein solubility. In accordance with Ng, A. (1999), it was reported that increasing the die temperature during the extrusion of onion waste decreased WAI and increased WSI, SP. The change in the protein system caused by heat and shear forces, increasing intermolecular interactions owing to the partial unfolding of protein molecules then the aggregation of protein molecules by sulfhydryl-disulphide interchange and sulfhydryl oxidation within the preformed aggregates and perhaps between the aggregates to form the gel network and finally is the occurrence of multiple hydrogen bonding (Christ, 2005). Solubility can be described as when equilibrium exists between hydrophilic and hydrophobic interactions. Heating, drying, and shearing are also factor that have an influence on protein solubility in food systems (Vaclavik, & Christian, 2003).

2.3 EC, ES, FC and OBC

An increase in temperature enhanced the hydrophobicity of proteins as observed from the improvement of FC, EC, ES and OBC values of the samples. The emulsifying, foaming and oil binding of SSIR through stabilization processes were also found to be significantly ($p < 0.05$) higher than those of the control. The SSIR by extrusion process (barrel temperature 100 °C and in-barrel moisture level 61.8%) was highest (Table 10-15). The stabilization process especially at high heat, high pressure and shear forces increasing intermolecular interactions owing to release the partial unfolding of protein molecules such as hydrophobicity group. Hydrophobicity regions directly influence surfactant properties, e.g. emulsifying,

foaming and oil binding. The view was that the hydrophobic interactions increase as the temperature is raised from a low value, that the extended polypeptide chain to produce an active protein so may be due to breaking or forming and releasing hydrophobic or and hydrophilicity group. Proteins contain hydrophilic and hydrophobic groups in their primary structure, these groups are SIR and all over the primary structure of proteins. Thus, when a protein adsorbs to air-water interface or the oil-water interface, only a fraction of the hydrophobic residues is positioned in the interface facing the oil phase and most the protein molecule is suspended into the bulk aqueous phase. That is, hydrophobic group is of great importance to add an emulsifier. The more hydrophobic the proteins, the greater the decrease in interfacial tensions and the increase in emulsifying activity. In other words, the hydrophobic proteins readily interact with oil and the effective hydrophobicity of proteins plays a major role in stabilizing the protein-lipid interaction (Kato, & Nakai, 1980). According to Damodaran (1997), foam capacity is enhanced by greater thermal process because it increases the viscosity and facilitates the formation of a multilayer, cohesive protein film at the interface. Nakai (1983) observed an increase in the hydrophobicity upon heating of proteins, indicating the unfolding of the molecules.

Table 16 Functional properties of SSIR by the extrusion process

Barrel temperature (°C) / In-barrel moisture (%)	SP (%)	WSI (%)	WAI (%)	EC (%)	ES (%)	OBC (%)	FC (%)
80 / 39.5	13.44±0.11^{h}	4.24±0.02^{h}	3.83±0.04^{a}	44.44±0.09^{h}	45.71±0.01^{g}	15.62±0.03^{e}	5.56±0.03^{i}
80 / 50.6	13.48±0.08^{h}	4.47±0.08^{f}	3.55±0.73^{b}	44.49±0.02^{h}	45.87±0.21^{g}	15.64±0.03^{e}	5.70±0.05^{h}
80 / 61.8	13.56±0.04^{g}	4.53±0.68^{e}	3.27±0.13^{c}	46.51±0.23^{g}	47.11±0.32^{f}	15.71±0.07^{d}	5.99±0.02^{g}
90 / 39.5	13.72±0.05^{f}	4.32±0.06^{g}	2.99±0.24^{d}	48.60±0.13^{f}	50.95±0.31^{e}	15.72±0.07^{d}	8.11±0.10^{e}
90 / 50.6	13.82±0.09^{e}	4.51±0.08^{e}	2.93±0.18^{d}	50.95±0.14^{e}	51.43±0.53^{d}	15.74±0.12^{d}	7.41±0.03^{f}
90 / 61.8	13.95±0.21^{d}	4.71±0.04^{d}	2.75±0.33^{e}	53.33±0.11^{d}	51.46±0.08^{d}	15.84±0.01^{c}	9.26±0.04^{d}
100 / 39.5	14.26±0.09^{c}	5.12±0.08^{c}	1.97±0.01^{f}	56.61±0.09^{b}	57.94±0.06^{c}	16.01±0.31^{b}	11.11±0.05^{c}
100 / 50.6	14.98±0.03^{b}	5.48±0.49^{b}	1.68±0.03^{g}	58.19±0.09^{b}	58.89±0.06^{b}	16.18±0.34^{a}	12.96±0.03^{b}
100 / 61.8	16.53±0.07^{a}	5.63±0.04^{a}	1.47±0.24^{h}	60.00±0.14^{a}	60.74±0.07^{a}	16.32±0.07^{a}	13.60±0.03^{a}
The control	5.48±0.03^{i}	3.22±0.04^{i}	0.39±0.06^{i}	46.50 ±0.08^{g}	37.00±0.11^{h}	2.18±0.06^{f}	4.90±0.02^{j}

Note: Data are expressed as the mean ± SD (n=3)

$^{a-j}$ Values in each column for each analytical property with different are significantly different ($p<0.05$)

Table 17 Functional properties of SSIR by drum drying

Drums heated (°C) / Rotation speeds (rpm)	SP (%)	WSI (%)	WAI (%)	EC (%)	ES (%)	OBC (%)	FC (%)
100 / 3	12.28±0.02^{g}	3.14±0.34^{g}	2.29±0.06^{a}	48.00±0.01^{g}	48.89±0.01^{g}	14.50±0.07^{e}	4.24±0.04^{h}
100 / 5	12.40±0.23^{f}	3.55±0.26^{f}	1.93±0.03^{b}	50.67±0.09^{f}	50.67±0.21^{f}	14.81±0.08^{c}	4.41±0.07^{g}
100 / 7	12.53±0.02^{e}	3.56±0.16^{f}	1.76±0.04^{c}	52.01±0.13^{e}	52.44±0.19^{d}	14.97±0.03^{c}	4.85±0.11^{f}
120 / 3	12.69±0.07^{e}	3.62±0.03^{e}	1.70±0.03^{e}	50.67±0.13^{f}	51.56±0.02^{e}	14.61±0.07^{d}	5.26±0.08^{e}
120 / 5	12.91±0.08^{d}	3.67±0.05^{d}	1.66±0.03^{d}	52.00±0.23^{e}	52.01±0.11^{d}	14.71±0.03^{c}	5.81±0.04^{e}
120 / 7	13.20±0.06^{c}	3.71±0.14^{d}	1.63±0.03^{d}	54.67±0.08^{d}	53.33±0.21^{c}	14.82±0.07^{c}	6.41±0.06^{d}
140 / 3	15.46±0.05^{a}	4.43±0.32^{a}	1.51±0.03^{f}	57.33±0.11^{a}	57.33±0.09^{a}	15.92±0.01^{a}	10.2±0.04^{a}
140 / 5	14.69±0.02^{b}	4.28±0.14^{b}	1.54±0.01^{f}	56.12±0.06^{b}	56.01±0.21^{b}	15.59±0.01^{b}	8.97±0.16^{b}
140 / 7	13.83±0.05^{c}	4.12±0.11^{c}	1.61±0.02^{a}	53.33±0.08^{c}	53.33±0.12^{c}	15.58±0.05^{b}	7.42±0.06^{c}
The control	5.48±0.03^{i}	3.22±0.04^{h}	0.39±0.06^{g}	46.50 ±0.08^{h}	37.00±0.11^{h}	2.18±0.06^{f}	4.90±0.02^{j}

Note: Data are expressed as the mean ± SD (n=3)

$^{a-j}$ Values in each column for each analytical property with different are significantly different ($p<0.05$)

Table 18 Functional properties of SSIR by autoclaving

Temperature (°C) / Time (min)	SP (%)	WSI (%)	WAI (%)	EC (%)	ES (%)	OBC (%)	FC (%)
105 / 10	12.66±0.04^{f}	3.26±0.16^{f}	2.91±0.02^{a}	47.67±0.81^{h}	45.73±0.03^{e}	13.81±0.08^{g}	4.12±0.03^{h}
105 / 15	12.64±0.05^{f}	3.25±0.14^{f}	2.52±0.03^{b}	48.33±0.09^{g}	49.83±0.04^{c}	13.85±0.09^{g}	4.23±0.05^{h}
105 / 30	13.20±0.22^{e}	3.33±0.11^{e}	2.52±0.02^{b}	49.56±0.01^{f}	50.97±0.03^{b}	13.92±0.01^{f}	4.86±0.06^{g}
110 / 10	13.39±0.13^{e}	3.37±0.09^{e}	2.52±0.13^{b}	50.02±0.11^{c}	47.67±0.03^{d}	14.19±0.01^{e}	5.11±0.06^{f}
110 / 15	13.50±0.04^{d}	3.42±0.12^{d}	2.51±0.11^{b}	51.22±0.12^{d}	50.87±0.23^{b}	14.32±0.02^{d}	5.75±0.11^{e}
110 / 30	13.56±0.04^{d}	3.48±0.13^{c}	2.47±0.17^{c}	52.67±0.11^{c}	52.33±0.13^{a}	14.55±0.14^{d}	5.98±0.08^{d}
121 / 10	14.32±0.08^{c}	3.72±0.15^{b}	2.42±0.09^{d}	52.67±0.01^{c}	49.73±0.21^{c}	14.72±0.21^{c}	6.14±0.05^{c}
121 / 15	14.37±0.13^{b}	3.78±0.03^{b}	2.39±0.12^{e}	53.11±0.17^{b}	50.77±0.06^{b}	14.97±0.26^{b}	7.85±0.03^{b}
121 / 30	14.93±0.11^{a}	3.83±0.01^{a}	2.35±0.11^{f}	53.58±0.12^{a}	52.42±0.27^{a}	15.21±0.08^{a}	8.22±0.05^{a}
The control	5.48±0.03^{i}	3.22±0.04^{g}	0.39±0.06^{g}	46.50 ±0.08^{i}	37.00±0.11^{f}	2.18±0.06^{h}	4.90±0.02^{i}

Note: Data are expressed as the mean ± SD (n=3)

$^{a-i}$ Values in each column for each analytical property with different are significantly different ($p<0.05$)

Table 19 Functional properties of SSIR by heat-moisture treatment

Temperatures (°C) / Moisture content (%)	SP (%)	WSI (%)	WAI (%)	EC (%)	ES (%)	OBC (%)	FC (%)
90 / 15	12.36±0.14^{f}	3.36±0.03^{e}	2.92±0.02^{a}	46.67±0.03^{f}	45.33±0.09^{e}	13.71±0.08^{d}	3.51±0.07^{f}
90 / 20	12.44±0.14^{f}	3.35±0.21^{e}	2.63±0.03^{b}	49.33±0.31^{e}	49.33±0.07^{c}	13.75±0.07^{d}	3.57±0.18^{f}
90 / 25	13.22±0.09^{e}	3.41±0.13^{d}	2.52±0.09^{c}	51.56±0.21^{b}	50.17±0.07^{b}	13.82±0.03^{c}	3.83±0.10^{e}
100 / 15	13.49±0.13^{d}	3.47±0.07^{d}	2.42±0.09^{d}	48.02±0.08^{d}	46.67±0.05^{d}	13.79±0.03^{d}	4.81±0.11^{d}
100 / 20	13.62±0.11^{c}	3.53±0.06^{c}	2.41±0.11^{d}	50.22±0.01^{c}	50.27±0.02^{b}	13.82±0.04^{c}	5.22±0.07^{c}
100 / 25	13.66±0.08^{c}	3.55±0.03^{c}	2.47±0.01^{c}	50.67±0.03^{c}	52.01±0.05^{a}	13.85±0.11^{c}	5.43±0.07^{c}
110 / 15	14.42±0.04^{b}	3.82±0.01^{b}	2.42±0.11^{d}	50.67±0.03^{c}	49.36±0.11^{c}	13.92±0.21^{b}	6.54±0.05^{b}
110 / 20	14.47±0.11^{b}	3.88±0.31^{b}	2.29±0.13^{e}	51.11±0.21^{b}	50.67±0.19^{b}	13.97±0.06^{b}	6.68±0.06^{a}
110 / 25	14.53±0.12^{a}	3.93±0.13^{a}	2.25±0.11^{f}	52.09±0.12^{a}	52.21±0.04^{a}	14.03±0.06^{a}	6.71±0.05^{a}
The control	5.48±0.03^{i}	3.22±0.04^{f}	0.39±0.06^{g}	46.50 ±0.08^{f}	37.00±0.11^{f}	2.18±0.06^{e}	4.90±0.02^{g}

Note: Data are expressed as the mean ± SD (n=3)

$^{a\text{-}i}$ Values in each column for each analytical property with different are significantly different ($p<0.05$)

Table 20 Functional properties of SSIR by combined process

Temperatures (°C) / Time (min)	SP (%)	WSI (%)	WAI (%)	EC (%)	ES (%)	OBC (%)	FC (%)
30/100	13.34±0.01^{c}	4.34±0.04^{b}	3.33±0.04^{a}	46.54±0.09^{c}	45.81±0.01^{c}	15.61±0.05^{b}	5.55±0.05^{c}
45/100	13.38±0.06^{c}	4.36±0.08^{b}	3.37±0.03^{a}	46.55±0.07^{c}	45.87±0.07^{c}	15.66±0.03^{b}	5.58±0.03^{c}
60/100	13.36±0.04^{c}	4.37±0.03^{b}	3.35±0.03^{a}	46.53±0.03^{c}	45.81±0.02^{c}	15.63±0.06^{b}	5.59±0.07^{c}
30/125	13.42±0.05^{c}	4.35±0.06^{b}	2.57±0.04^{b}	53.65±0.03^{b}	51.55±0.07^{b}	15.81±0.09^{b}	8.67±0.07^{b}
45/125	13.42±0.07^{c}	4.39±0.08^{b}	2.55±0.05^{b}	53.95±0.04^{b}	51.53±0.06^{b}	15.85±0.07^{b}	8.65±0.03^{b}
60/125	13.45±0.01^{c}	4.37±0.07^{b}	2.51±0.03^{b}	53.83±0.06^{b}	51.56±0.03^{b}	15.88±0.09^{b}	8.66±0.04^{b}
30/150	14.56±0.09^{b}	5.35±0.09^{a}	1.36±0.09^{c}	56.66±0.09^{a}	57.64±0.06^{a}	16.25±0.08^{a}	12.16±0.05^{a}
45/150	14.58±0.03^{b}	5.37±0.09^{a}	1.36±0.03^{c}	56.63±0.09^{a}	57.69±0.06^{a}	16.26±0.04^{a}	12.19±0.07^{a}
60/150	16.54±0.07^{a}	5.37±0.04^{a}	1.38±0.04^{c}	56.78±0.04^{a}	57.64±0.06^{a}	16.29±0.07^{a}	12.19±0.05^{a}
The control	5.48±0.03^{d}	3.22±0.04 ^{c}	0.39±0.06^{d}	46.50 ±0.08^{e}	37.00±0.11^{d}	2.18±0.06^{c}	4.90±0.02^{d}

Note: Data are expressed as the mean ± SD (n=3)

$^{a-i}$ Values in each column for each analytical property with different are significantly different ($p<0.05$)

Table 21 Functional properties of SIR through stabilization process

Stabilization process	SP (%)	WSI (%)	WAI (%)	EC (%)	ES (%)	OBC (%)	FC (%)
Extrusion (100 °C / 61.8%)	16.53±0.07[a]	5.63±0.04[a]	1.47±0.24[b]	60.00±0.14[a]	60.74±0.07[a]	16.32±0.07[a]	13.60±0.03[a]
Drum drying (140 °C / 3 rpm)	15.46±0.05[b]	4.43±0.32[b]	1.51±0.03[b]	57.33±0.11[b]	57.33±0.09[b]	15.92±0.01[b]	10.2±0.04[c]
Autoclaving (121 °C/ 30 min)	14.93±0.11[c]	3.83±0.01[c]	2.35±0.11[a]	53.58±0.12[d]	52.42±0.27[c]	15.21±0.08[b]	8.22±0.05[d]
Heat-moisture treatment (110 °C/ 25%)	14.53±0.12[c]	3.93±0.13[c]	2.25±0.11[a]	52.09±0.12[e]	52.21±0.04[c]	14.03±0.06[c]	6.71±0.05[e]
Combined process (60 min /150 °C)	16.54±0.07[a]	5.37±0.04[a]	1.38±0.04[c]	56.78±0.04[c]	57.64±0.06[b]	16.29±0.07[a]	12.19±0.05[b]
The control	5.48±0.03[d]	3.22±0.04[d]	0.39±0.06[d]	46.50 ±0.08[f]	37.00±0.11[d]	2.18±0.06[d]	4.90±0.02[f]

Note: Data are expressed as the mean ± SD (n=3)

[a-f] Values in each column for each analytical property with different are significantly different ($p<0.05$)

3. Antioxidant activity

The results showed that changes in temperature levels have an effect on the content total phenolic compounds and total antioxidant activities in the samples. The antioxidant activities of the SIR were significantly enhanced by the thermal process ($p < 0.05$). The SSIR obtained by extrusion process at barrel temperature of 80 °C and in-barrel moisture level of 39.5% had the highest $ABTS^{\bullet+}$ (2.4 mg Trolox /g powder), FRAP (0.7 mg $FeSO_4$ /g powder), DPPH (1.5 mg Trolox /g powder) and TPC (2.4 mg gallic acid /g powder) compared to The control ($ABTS^{\bullet+}$=0.2 mg Trolox /g powder, FRAP=0.2 mg $FeSO_4$ /g powder, DPPH=0.3 mg Trolox /g powder and TPC=0.6 mg gallic acid /g. powder) as presented in Table 16. The SSIR through extrusion showed significantly ($p < 0.05$) higher TPC and total antioxidant activities when compared to drum drying, autoclaving, autoclaving combined extrusion, and heat-moisture treatment (Table 16-21). Further, a higher antioxidant activity was observed which could be possibly due to evaporation of most of the water during the processing which led to concentration of phenolic compounds on the SSIR. An increase in temperature caused the softness of the cell walls and finally enhanced the extraction effect to antioxidant activity increase. However, a lowest in antioxidant activity, as observed in heat-moisture treatment, can be explained as a result of non-violent process. The antioxidant activity of all studied compounds decreased with increasing working temperature. Temperature is one of the most important factors affecting antioxidant activity. However, variations in temperature may change the mechanism of action of some antioxidants. Dewanto et al. (2002) reported that the total antioxidant activity of sweet corn was elevated by 44% after thermal processing. Nayak et al. (2011) reported that the TPC of extruded purple potato flour mix increased compared with the non-extruded product. Lima et al. (2009) observed a dramatic loss of phenolic content on convectional as a result of thermal treatment.

Table 22 Antioxidant activity of SSIR through extrusion process

Barrel temperature (°C) / In-barrel moisture (%)	$ABTS^{\bullet+}$	DPPH	FRAP	TPC
80 / 39.5	2.37±0.01[a]	1.55±0.03[a]	0.68±0.03[a]	2.43±0.01[a]
80 / 50.6	2.22±0.01[e]	1.32±0.07[f]	0.22±0.04[e]	2.41±0.03[b]
80 / 61.8	2.34±0.04[b]	1.50±0.07[b]	0.59±0.01[b]	2.16±0.04[e]
90 / 39.5	1.30±0.06[h]	1.48±0.13[c]	0.48±0.04[c]	1.85±0.05[d]
90 / 50.6	1.95±0.04[d]	1.40±0.06[d]	0.32±0.01[d]	1.82±0.01[e]
90 / 61.8	1.82±0.11[e]	1.37±0.08[e]	0.31±0.02[d]	1.75±0.07[f]
100 / 39.5	1.69±0.07[f]	1.30±0.07[f]	0.23±0.04[e]	1.53±0.03[g]
100 / 50.6	1.42±0.06[g]	1.12±0.14[g]	0.12±0.05[f]	1.25±0.02[h]
100 / 61.8	1.10±0.04[i]	0.92±0.05[h]	0.08±0.05[g]	1.11±0.05[i]
The control	0.22±0.01[j]	0.21±0.04[i]	0.32±0.07[d]	0.60±0.09[j]

Note: -$ABTS^{\bullet+}$, DPPH = mgTrolox equiv. /g sample, db, FRAP=mgFeSO4 equiv. /g sample, db and TPC = mggallic acid equiv./g sample, db. Data are expressed as the mean ± SD (n=3)

[a-j] Values in each column for each analytical property with different are significantly different ($p<0.05$)

Table 23 Antioxidant activity of SSIR through drum drying

Drums heated (°C) / Rotation speeds (rpm)	$ABTS^{\bullet+}$	DPPH	FRAP	TPC
100 / 3	2.24±0.03[a]	0.52±0.02[a]	1.42±0.03 [a]	2.37±0.01[a]
100 / 5	2.15±0.01[b]	0.52±0.07[b]	1.35±0.01[d]	2.29±0.04[b]
100 / 7	2.12±0.01[c]	0.51±0.12[b]	1.32±0.01[e]	2.14±0.06[e]
120 / 3	1.62±0.02[d]	0.32±0.02[c]	1.22±0.02[d]	1.75±0.03[d]
120 / 5	1.56±0.02[e]	0.31±0.02[c]	1.26±0.02[d]	1.47±0.05[e]
120 / 7	1.36±0.01[f]	0.22±0.07[d]	1.16±0.01[e]	1.36±0.04[f]
140 / 3	1.33±0.01[f]	0.20±0.12[e]	1.13±0.01[e]	0.87±0.07[g]
140 / 5	1.26±0.01[g]	0.10±0.03[f]	1.06±0.01[f]	0.79±0.02[h]

Table 23 (cont.)

Drums heated (°C) / Rotation speeds (rpm)	$ABTS^{\bullet+}$	DPPH	FRAP	TPC
140 / 7	1.11 ± 0.01^{h}	0.08 ± 0.03^{g}	1.01 ± 0.01^{f}	0.69 ± 0.03^{i}
non-stabilized sample	0.22 ± 0.01^{i}	0.21 ± 0.04^{h}	0.32 ± 0.07^{g}	0.60 ± 0.09^{i}

Note: -$ABTS^{\bullet+}$, DPPH = mgTrolox equiv. /g sample, db, FRAP=mg$FeSO_4$ equiv. /g sample, db and TPC = mggallic acid equiv./g sample, db. Data are expressed as the mean ± SD (n=3)

$^{a-i}$ Values in each column for each analytical property with different are significantly different ($p<0.05$)

Table 24 Antioxidant activity of SSIR through autoclaving

Temperature (°C) / Time (min)	$ABTS^{\bullet+}$	DPPH	FRAP	TPC
105 / 10	1.98 ± 0.08^{a}	0.48 ± 0.09^{a}	1.24 ± 0.06^{a}	1.98 ± 0.08^{a}
105 / 15	1.97 ± 0.05^{b}	0.46 ± 0.07^{b}	1.21 ± 0.11^{b}	1.92 ± 0.08^{b}
105 / 30	1.95 ± 0.08^{c}	0.45 ± 0.09^{c}	1.07 ± 0.15^{c}	1.86 ± 0.06^{c}
110 / 10	1.77 ± 0.03^{d}	0.43 ± 0.12^{d}	0.97 ± 0.13^{d}	1.84 ± 0.04^{d}
110 / 15	1.76 ± 0.07^{e}	0.35 ± 0.04^{e}	0.96 ± 0.19^{e}	1.65 ± 0.04^{e}
110 / 30	1.68 ± 0.09^{f}	0.25 ± 0.11^{f}	0.85 ± 0.09^{e}	1.54 ± 0.09^{f}
121 / 10	1.46 ± 0.07^{g}	0.18 ± 0.08^{g}	0.83 ± 0.07^{f}	1.51 ± 0.09^{g}
121 / 15	1.35 ± 0.06^{h}	0.16 ± 0.09^{h}	0.75 ± 0.06^{g}	1.39 ± 0.07^{h}
121 / 30	1.26 ± 0.04^{h}	0.12 ± 0.09^{i}	0.73 ± 0.05^{g}	1.36 ± 0.02^{i}
The control	0.22 ± 0.01^{i}	0.22 ± 0.04^{f}	0.32 ± 0.07^{h}	0.60 ± 0.09^{j}

Note: -$ABTS^{\bullet+}$, DPPH = mgTrolox equiv. /g sample, db, FRAP=mg$FeSO_4$ equiv. /g sample, db and TPC = mggallic acid equiv./g sample, db. Data are expressed as the mean ± SD (n=3)

$^{a-j}$ Values in each column for each analytical property with different are significantly different ($p<0.05$)

Table 25 Antioxidant activity of SSIR through heat-moisture treatment

Temperatures (°C) / Moisture content (%)	$ABTS^{\bullet+}$	DPPH	FRAP	TPC
90 / 15	0.94 ± 0.01^{a}	0.21 ± 0.01^{a}	0.34 ± 0.07^{a}	0.63 ± 0.08^{a}
90 / 20	0.93 ± 0.02^{b}	0.20 ± 0.04^{b}	0.32 ± 0.04^{b}	0.62 ± 0.05^{b}
90 / 25	0.91 ± 0.02^{c}	0.17 ± 0.09^{c}	0.31 ± 0.11^{c}	0.60 ± 0.06^{c}
100 / 15	0.78 ± 0.01^{d}	0.15 ± 0.01^{d}	0.28 ± 0.12^{d}	0.53 ± 0.06^{d}
100 / 20	0.65 ± 0.01^{e}	0.13 ± 0.07^{e}	0.26 ± 0.05^{e}	0.51 ± 0.05^{e}
100 / 25	0.60 ± 0.02^{f}	0.13 ± 0.06^{e}	0.26 ± 0.05^{e}	0.48 ± 0.09^{f}
110 / 15	0.57 ± 0.01^{g}	0.11 ± 0.08^{f}	0.21 ± 0.08^{f}	0.36 ± 0.12^{g}
110 / 20	0.53 ± 0.02^{h}	0.10 ± 0.05^{g}	0.20 ± 0.07^{g}	0.34 ± 0.07^{h}
110 / 25	0.52 ± 0.02^{h}	0.10 ± 0.09^{g}	0.20 ± 0.04^{g}	0.63 ± 0.12^{i}
The control	0.22 ± 0.01^{i}	0.21 ± 0.04^{a}	0.32 ± 0.07^{b}	0.60 ± 0.09^{c}

Note: -$ABTS^{\bullet+}$, DPPH = mgTrolox equiv. /g sample, db, FRAP=mg$FeSO_4$ equiv. /g sample, db and TPC = mggallic acid equiv./g sample, db. Data are expressed as the mean ± SD (n=3)

$^{a-i}$ Values in each column for each analytical property with different are significantly different ($p<0.05$)

Table 26 Antioxidant activity of SSIR through combined process

Temperatures (°C) / Time (min)	$ABTS^{\bullet+}$	DPPH	FRAP	TPC
30/100	1.21 ± 0.05^{a}	0.36 ± 0.05^{a}	1.13 ± 0.04^{a}	1.61 ± 0.08^{a}
45/100	1.11 ± 0.03^{b}	0.31 ± 0.04^{c}	1.12 ± 0.06^{b}	1.45 ± 0.08^{b}
60/100	1.05 ± 0.11^{c}	0.33 ± 0.09^{b}	1.03 ± 0.05^{c}	1.40 ± 0.06^{c}
30/125	1.07 ± 0.13^{d}	0.29 ± 0.05^{d}	0.87 ± 0.03^{d}	1.34 ± 0.04^{d}
45/125	0.86 ± 0.14^{e}	0.25 ± 0.08^{e}	0.81 ± 0.04^{e}	1.22 ± 0.04^{e}
60/125	0.77 ± 0.06^{f}	0.25 ± 0.11^{e}	0.75 ± 0.08^{f}	1.18 ± 0.09^{f}
30/150	0.57 ± 0.07^{g}	0.14 ± 0.03^{f}	0.70 ± 0.04^{g}	1.03 ± 0.09^{g}
45/150	0.35 ± 0.05^{h}	0.12 ± 0.04^{g}	0.55 ± 0.04^{g}	0.79 ± 0.07^{h}

Table 26 (cont.)

Temperatures (°C) / Time (min)	ABTS$^{•+}$	DPPH	FRAP	TPC
60/150	0.29±0.08i	0.12±0.11g	0.53±0.07h	0.76±0.02i
The control	0.22±0.01j	0.21±0.04h	0.32±0.07i	0.60±0.09j

Note: -ABTS$^{•+}$, DPPH = mgTrolox equiv. /g sample, db, FRAP=mg$FeSO_4$ equiv. /g sample, db and TPC = mggallic acid equiv./g sample, db. Data are expressed as the mean ± SD (n=3)

$^{a-j}$ Values in each column for each analytical property with different are significantly different ($p<0.05$)

Table 27 Antioxidant activity of SIR through stabilization process

Stabilization process	ABTS$^{•+}$	DPPH	FRAP	TPC
Extrusion (80 °C / 39.5%)	2.37±0.01^{a}	1.55±0.03^{a}	0.68±0.03^{d}	2.43±0.01^{a}
Drum drying (100 °C / 3 rpm)	2.24±0.03^{b}	0.52±0.02^{b}	1.42±0.03^{a}	2.37±0.01^{b}
Autoclaving (105 °C/ 10 min)	1.98±0.08^{c}	0.48±0.09^{c}	1.24±0.06^{b}	1.98±0.08^{c}
Heat-moisture treatment (90 °C/ 15%)	0.94±0.01^{e}	0.21±0.01^{e}	0.34±0.07^{e}	0.63±0.08^{e}
Combined process (30 min /100 °C)	1.21±0.05^{d}	0.36±0.05^{d}	1.13±0.04^{c}	1.61±0.08^{d}
The control	0.22±0.01^{f}	0.21±0.04^{f}	0.32±0.07^{f}	0.60±0.09^{f}

Note: -ABTS$^{•+}$, DPPH = mgTrolox equiv. /g sample, db, FRAP=mg$FeSO_4$ equiv. /g sample, db and TPC = mggallic acid equiv./g sample, db. Data are expressed as the mean ± SD (n=3)

$^{a-j}$ Values in each column for each analytical property with different are significantly different ($p<0.05$)

4. Microbial analysis

Extrusion processing at barrel temperature of 100 °C and in-barrel moisture level of 61.9 % (w/w), drum drying at rotation speeds 3 rpm/min and drum surface temperature of 140 °C, heat-moisture treatment at moisture content of 25% and temperatures of 110 °C could destroy total microbial populations, yeast, and mold by approximately 1.4×10^3 UFC/g and 1.2×10^3 UFC/g, 1.8 $x10^3$ UFC/g and 1.5 $x10^3$ UFC/g, and 1.4 $\times10^3$ UFC/g and 1.9 $x10^3$ UFC/g compared with the control (Total microbial, yeast and molds as 7×10^3 UFC/g). Temperatures above 121 °C for 15 min of autoclaving or autoclaving combined extrusion can kill these microorganisms. The pressure and high temperature can inhibit protein synthesis in microorganisms or lead to denaturation of proteins and damage to the cell membrane and the internal structure of the cell. Increasing the pressure or temperature causes irreversible denaturation of the enzymes and proteins, resulting in rupturing of cell membranes and withdrawal of intracellular contents, which will lead to bacteria death. Thus, sterilization process can be accomplished through temperature or pressure increase, which can be reduced/ destroy microorganisms. There has been little research on the destruction of bacterial spores during sterilization process. In 1990, a study by Likimani, & Sofos (1990) was performed. The results of their study indicated that extrusion processing at low barrel temperatures (i.e., 80/100 °C) resulted in injury to spores of B. globigii. Extrusion at the higher barrel temperatures in zone 2 i.e. 120 and 140 °C resulted in extensive spore destruction. Two related studies examined destruction of liquid or freeze-dried spore suspensions of Bacillus stearothermophilus during extrusion processing of a starch protein-sucrose biscuit mix with a twin-screw extruder (Bouveresse, 1982). Their results indicated major reduction (105-108) in spore numbers during extrusion at high temperatures (150-180 ° C).

5. In vitro digestibility, antinutrients and aflatoxin.

There were significant ($p < 0.05$) differences between the values obtained for in vitro protein digestibility, antinutrients (tannin and phytic acid) of the control and SSIR (Table 22-27). In vitro protein digestibility of the control was relatively low (15%), which could be partly attributed to the presence of tannin and phytic acid. The combined process showed a significant ($p<0.05$) increase in in vitro protein digestion (63.1%) compared to extrusion at barrel temperature of 100 °C and in-barrel moisture

level of 61.8% (49.7%), drum drying at rotation speeds 3 rpm/min and drum surface temperature of 140 °C (36.3%), heat-moisture treatment at moisture content 25% and temperatures of 110 °C (32.1%) and autoclaving at 121 °C for 30 minutes (55.8%). The rise of protein digestibility could be related to the degradation of the protein complexes within the extruded samples and the denaturation of protein due to the heat and shear. The alterations in protein structure thus make the extruded products more susceptible to degradation and hence the release of the products of digestion are increased-the bioavailability of the protein may be elevated. Studies by Nwokolo (1987) using African breadfruit seed show that the raw seed had a low protein digestibility and protein efficiency ratio. These observations were attributed to antinutritional components such as phytic acid, polyphenols, and oxalate, among others, in the seed. Improvements in vitro protein digestibility as a result of thermal processing have been reported for winged beans (Kadam et al., 1987) and groundnuts (Singh et al., 1991). For instance, Linsberger-Martin et al. (2013) reported that applying pressure and cooking to legume seeds at a high temperature increases the protein digestibility of such legume seeds, possibly by increasing the solubility of the protein and fragmenting the long polymer chains of intact proteins.

The extrusion process, autoclaving and combined process were more effective in reducing phytic acid and tannin than the drum drying process and heat-moisture treatment. The level of phytic acid and tannin in the control (1.63 g/g, 8.56 mg/g) was higher than SSIR (extrusion at barrel temperature 100 °C and in-barrel moisture level of 61.8% = 0.87 g/g, 1.42 mg/g), drum drying at rotation speeds 3 rpm/mins and drum surface temperature of 140 °C (1.39 g/g, 5.34 mg/g), heat-moisture treatment at moisture content 25% and oven at higher temperatures 110 °C (1.55 g/g, 7.10 mg/g) and autoclaving at 121 °C for 30 minutes (0.28 g/g, 0.08 mg/g). Results show that combined process is very effective in reducing the phytic acid and tannin from SIR (Table 22-27). The reduction of tannin and phytic acid after processing may be partly attributed to its heat-labile nature. Additionally, the formation of insoluble complexes by phytate and calcium or magnesium during heat treatment may also lead to tannin and phytic acid reduction. Alonso et al. (2000) reported the effects of extrusion processing methods, reduced condensed tannin and polyphenol levels in legumes. Extrusion was the best method to abolish phytic acid and tannin.

The oil seeds such as Sacha Inchi, are fairly rich in protein but presence of anti-nutritional factors limits their use. The TI are generally considered to be the main a number of antinutritional factors (ANFs) in oil seeds. The reduction of TI in the samples was found to be highest (100%) with autoclaving at 60 min combined with extrusion at barrel temperature 150 °C. The level of the TI in the control (8.52 mg/g) was higher than the SSIR (extrusion at barrel temperature 100 °C and in-barrel moisture level of 61.8% (7.74 mg/g)), autoclaving at 121 °C for 15 min (6.66 mg/g), combined process (autoclaving at 60 min combined extrusion at barrel temperature 100 °C and 125 °C value as 2.52 and 1.92 mg/mg). Results show that combined process between autoclaving and extrusion process is very effective in reducing the trypsin in the residue. High temperature and shear forces also cause physical deformation of proteins and therefore possibly have an additional denaturation effect on TI. In conclusion, high temperature, high pressure and shear forces are the key variables for the destruction of TI. In another study that was conducted in 2000 by Singh et al, extrusion (300-rpm screw speed, 27 kg/hr) feed rate, 5/32 inches die size and 93–97 °C outlet temperature) causes complete destruction of TI activity in extruded blends of broken rice and wheat bran containing up to 20% wheat bran.

The level of aflatoxins in terms of aflatoxin B1, B2, and G1 of SIR were 9.92 µg/kg, 1.55 µg/kg, and 1.22 µg/kg, respectively. It was found that all aflatoxins can be destroyed by extrusion at barrel temperature 80 °C and in-barrel moisture level of 61.8%. Thermal still provides a feasible mechanism for reducing the aflatoxins concentration. Extrusion cooking generally decreases the mycotoxins levels at rates depending on different factors such as the initial mycotoxin concentration, the barrel temperature, the screw speed and the moisture content of the raw material. Similar results were achieved for peanut meal, when extrusion at barrel temperature 60 °C reduced aflatoxins by 23-66 % (Cheftel, 1989). Hameed (1993) also showed that extrusion reduced the aflatoxin content by 50-80%.

Table 28 In vitro digestibility and antinutrients of SSIR by extrusion process

Barrel temperature (°C) / In-barrel moisture (%)	Total digestibility (%)	Tannin (mg gallic acid /g)	Phytic acid (g/100g)
80 / 39.5	37.13±0.06^{h}	2.39±0.06^{b}	1.12±0.09^{b}
80 / 50.6	40.04±0.06^{f}	2.37±0.09^{b}	1.17±0.08^{b}
80 / 61.8	41.83±0.03^{e}	2.39±0.07^{b}	1.16±0.11^{b}
90 / 39.5	38.86±0.04^{g}	1.74±0.09^{c}	0.95±0.09^{c}
90 / 50.6	42.56±0.07^{d}	1.73±0.08^{c}	0.95±0.08^{c}
90 / 61.8	44.43±0.06^{b}	1.71±0.09^{c}	0.96±0.07^{c}
100 / 39.5	43.52±0.06^{c}	1.42±0.06^{d}	0.86±0.09^{d}
100 / 50.6	42.38±0.05^{d}	1.42±0.08^{d}	0.87±0.09^{d}
100 / 61.8	49.71±0.04^{a}	1.42±0.37^{d}	0.87±0.07^{d}
The control	15.40±0.06^{i}	8.56±0.53^{a}	1.63±0.07^{a}

Note: Data are expressed as the mean ± SD (n=3)

$^{a-i}$ Values in each column for each analytical property with different are significantly different ($p<0.05$)

Table 29 In vitro digestibility and antinutrients of SSIR by drum drying

Drums heated (°C) / Rotation speeds (rpm)	Total digestibility (%)	Tannin (mg gallic acid /g)	Phytic acid (g/100g)
100 / 3	23.74±0.07^{h}	6.79±0.06^{b}	1.37±0.04^{b}
100 / 5	29.47±0.05^{g}	6.78±0.07^{b}	1.39±0.06^{b}
100 / 7	31.38±0.03^{f}	6.78±0.08^{b}	1.39±0.03^{b}
120 / 3	34.16±0.05^{c}	6.45±0.09^{c}	1.03±0.09^{d}
120 / 5	32.13±0.07^{e}	6.44±0.10^{c}	1.11±0.07^{c}
120 / 7	33.55±0.05^{d}	6.46±0.11^{c}	1.15±0.06^{c}
140 / 3	36.36±0.04^{a}	5.36±0.12^{d}	0.84±0.10^{e}

Table 29 (cont.)

Drums heated (°C) / Rotation speeds (rpm)	Total digestibility (%)	Tannin (mg gallic acid /g)	Phytic acid (g/100g)
140 / 5	35.94±0.05b	5.35±0.13d	0.89±0.11e
140 / 7	31.33±0.04^{f}	5.34±0.14^{d}	0.89±0.06^{e}
The control	15.40±0.06^{i}	8.56±0.53^{a}	1.63±0.07^{a}

Note: Data are expressed as the mean ± SD (n=3)

$^{a-i}$ Values in each column for each analytical property with different are significantly different ($p<0.05$)

Table 30 In vitro digestibility and antinutrients of SSIR by autoclaving

Temperature (°C) / Time (min)	Total digestibility (%)	Tannin (mg gallic acid /g)	Phytic acid (g/100g)
105 / 10	33.05±0.05^{g}	1.02±0.06^{b}	0.87±0.06^{b}
105 / 15	37.02±0.06^{f}	1.01±0.07^{b}	0.81±0.05^{b}
105 / 30	38.87±0.07^{e}	0.96±0.08^{c}	0.77±0.06^{c}
110 / 10	38.00±0.07^{e}	0.98±0.09^{c}	0.74±0.08^{c}
110 / 15	41.33±0.06^{d}	0.93±0.10^{d}	0.53±0.04^{d}
110 / 30	42.87±0.05^{c}	0.89±0.14^{e}	0.47±0.09^{e}
121 / 10	43.57±0.04^{b}	0.75±0.09^{f}	0.41±0.11^{e}
121 / 15	43.86±0.05^{b}	0.11±0.07^{f}	0.34±0.06^{f}
121 / 30	55.84±0.07^{a}	0.08±0.08^{f}	0.28±0.07^{g}
The control	15.40±0.06^{h}	8.56±0.53^{a}	1.63±0.07^{a}

Note: Data are expressed as the mean ± SD (n=3)

$^{a-i}$ Values in each column for each analytical property with different are significantly different ($p<0.05$)

Table 31 In vitro digestibility and antinutrients of SSIR by heat-moisture treatment

Temperatures (°C) / Moisture content (%)	Total digestibility (%)	Tannin (mg gallic acid/g)	Phytic acid (g/100g)
90 / 15	21.13±0.04^{g}	7.80±0.05^{b}	1.59±0.04^{b}
90 / 20	22.89±0.05^{f}	7.81±0.05^{b}	1.58±0.01^{b}
90 / 25	24.96±0.06^{d}	7.81±0.16^{b}	1.59±0.08^{b}
100 / 15	23.72±0.07^{e}	7.52±0.09^{c}	1.21±0.09^{c}
100 / 20	22.40±0.06^{f}	7.53±0.13^{c}	1.19±0.07^{c}
100 / 25	25.95±0.07^{c}	7.52±0.09^{c}	1.19±0.08^{c}
110 / 15	30.97±0.06^{b}	7.10±0.09^{d}	0.93±0.09^{d}
110 / 20	30.81±0.04^{b}	7.11±0.04^{d}	0.95±0.07^{d}
110 / 25	32.10±0.06^{a}	7.10±0.09^{d}	0.97±0.09^{d}
The control	15.40±0.06^{h}	8.56±0.53^{a}	1.63±0.07^{a}

Note: Data are expressed as the mean ± SD (n=3)

$^{a-i}$ Values in each column for each analytical property with different are significantly different ($p<0.05$)

Table 32 In vitro digestibility and antinutrients of SSIR by combined process

Temperatures (°C) / Time (min)	Total digestibility (%)	Tannin (mg gallic acid /g)	Phytic acid (g/100g)
30/100	47.66±0.07^{g}	0.09±0.06^{b}	0.07±0.06^{b}
45/100	47.96±0.06^{g}	0.04±0.02^{c}	0.06±0.05^{c}
60/100	49.51±0.04^{e}	0.00±0.00^{d}	0.00±0.00^{d}
30/125	47.02±0.06^{h}	0.00±0.00^{d}	0.00±0.00^{d}
45/125	48.90±0.07^{f}	0.00±0.00^{d}	0.00±0.00^{d}
60/125	59.82±0.06^{b}	0.00±0.00^{d}	0.00±0.00^{d}
30/150	55.34±0.09^{d}	0.00±0.00^{d}	0.00±0.00^{d}

Table 32 (cont.)

Temperatures (°C) / Time (min)	Total digestibility (%)	Tannin (mg gallic acid /g)	Phytic acid (g/100g)
45/150	57.24±0.06^{c}	0.00±0.00^{d}	0.00±0.00^{d}
60/150	63.13±0.10^{a}	0.00±0.00^{d}	0.00±0.00^{d}
The control	15.40±0.07^{i}	8.56±0.53^{a}	1.63±0.07^{a}

Note: Data are expressed as the mean ± SD (n=3)

$^{a\text{-}i}$ Values in each column for each analytical property with different are significantly different ($p<0.05$)

Table 33 In vitro digestibility and antinutrients of SIR through stabilization process

Stabilization process	Total digestibility (%)	Tannin (mg gallic acid /g)	Phytic acid (g/100g)
Extrusion (100 °C / 61.8%)	49.71±0.04^{c}	1.42±0.37^{d}	0.87±0.07^{c}
Drum drying (140 °C / 3 rpm)	36.36±0.04^{d}	5.36±0.12^{c}	0.84±0.10^{d}
Autoclaving (121 °C/ 30 min)	55.84±0.07^{b}	0.08±0.08^{e}	0.28±0.07^{e}
Heat-moisture treatment (110 °C/ 25%)	32.10±0.06^{e}	7.10±0.09^{b}	0.97±0.09^{b}
Combined process (60 min /150 °C)	63.13±0.10^{a}	0.00±0.00^{f}	0.00±0.00^{f}
The control	15.40±0.07^{f}	8.56±0.53^{a}	1.63±0.07^{a}

Note: Data are expressed as the mean ± SD (n=3)

$^{a\text{-}f}$ Values in each column for each analytical property with different are significantly different ($p<0.05$)

6. Oxidation reaction

Stabilization can significantly reduce the oxidation of the samples. The results show that SSIR enriched with TPC and the antioxidant activity (DPPH, FRAP, and $ABTS^{\bullet+}$) as indicated in Table 16-21. Therefore, it can suppress oxidation reactions by participating in or interfering with the lipid autoxidation reaction cascade through various mechanisms. Results also suggested that the SSIR especially SSIR by extrusion (at barrel temperature of 80 °C and moisture level of 39.5 %) can also be used as potential natural antioxidant source. The results show that stabilization influenced the oxidation reaction as indicated by PV, FFA and TBA values. The oxidation reaction of the SSIR was significantly lower by the thermal process ($p < 0.05$). With temperature increased, an increase in PV of all the samples was observed (Table 28-33). However, highest PV was observed for the control sample followed by combined process (60 mins/ 150 °C), heat-moisture treatment (110 °C/ 25%), autoclaving (121°C / 30 min), drum drying (140°C/3 rpm) and extrusion process (100°C / 61.8%). The statistically analyzed data (Table 28-33) indicate that FFA values increased with the temperature of process. Highest FFA contents (0.88%) were observed in control ($p < 0.05$) while extrusion process (at barrel temperature of 80 °C and moisture level of 39.5 %) yielded lowest FFA contents (0.03%). TBARS measures the secondary oxidation product of the oil and important to check the rancidity. The results indicated thermal process resulted in a significant inhibition of TBARS. The control exhibited highest TBA value (0.27 µg MAD/kg) whereas least TBA values (0.09 µg MAD/kg) were observed with reference extrusion process (at barrel temperature of 80 °C and moisture level of 39.5 %). Radical reactions of proteins promoted by lipid hydroperoxides fall into three general categories: (a) protein-protein or lipid-protein crosslinking, (b) protein scission, and (c) protein oxidation. In addition, Jacks et al. (1982) observed that rancid oil (P.V. = 144) had no effect on the storage protein of peanuts.

Table 34 Oxidation reaction of SSIR by extrusion process

Barrel temperature (°C) / In-barrel moisture (%)	FFA (%)	PV (meq /kg)	TBA value (μg MAD/kg)
80 / 39.5	0.03±0.02^{g}	0.06±0.02^{e}	0.09±0.03^{g}
80 / 50.6	0.08±0.03^{f}	0.07±0.04^{d}	0.11±0.05^{f}
80 / 61.8	0.07±0.07^{f}	0.08±0.01^{d}	0.12±0.05^{e}
90 / 39.5	0.15±0.09^{e}	0.16±0.08^{c}	0.12±0.05^{e}
90 / 50.6	0.15±0.06^{e}	0.16±0.11^{c}	0.11±0.03^{f}
90 / 61.8	0.22±0.09^{d}	0.12±0.09^{c}	0.12±0.07^{e}
100 / 39.5	0.29±0.11^{c}	0.33±0.03^{b}	0.18±0.03^{c}
100 / 50.6	0.35±0.03^{b}	0.31±0.07^{b}	0.17±0.04^{d}
100 / 61.8	0.39±0.05^{b}	0.33±0.04^{b}	0.19±0.02^{b}
The control	0.88±0.01^{a}	0.62±0.03^{a}	0.27±0.02^{a}

Note: Data are expressed as the mean ± SD (n=3)

$^{a\text{-}f}$ Values in each column for each analytical property with different are significantly different ($p<0.05$)

Table 35 Oxidation reaction of SSIR by drum drying

Drums heated (°C) / Rotation speeds (rpm)	FFA (%)	PV (meq /kg)	TBA value (μg MAD/kg)
100 / 3	0.15±0.09^{d}	0.11±0.06^{d}	0.10±0.08^{f}
100 / 5	0.18±0.05^{d}	0.13±0.07^{d}	0.14±0.04^{e}
100 / 7	0.18±0.07^{d}	0.12±0.06^{d}	0.17±0.03^{d}
120 / 3	0.35±0.05^{c}	0.21±0.05^{c}	0.16±0.07^{d}
120 / 5	0.35±0.06^{c}	0.23±0.09^{c}	0.16±0.09^{d}
120 / 7	0.38±0.04^{c}	0.21±0.07^{c}	0.16±0.04^{d}
140 / 3	0.44±0.06^{b}	0.31±0.10^{b}	0.22±0.04^{b}

Table 35 (cont.)

Drums heated (°C) / Rotation speeds (rpm)	FFA (%)	PV (meq /kg)	TBA value (μg MAD/kg)
140 / 5	0.45±0.08[b]	0.39±0.11[b]	0.21±0.07[b]
140 / 7	0.47±0.07[b]	0.35±0.09[b]	0.19±0.03[c]
The control	0.88±0.01[a]	0.62±0.03[a]	0.27±0.02[a]

Note: Data are expressed as the mean ± SD (n=3)

[a-f] Values in each column for each analytical property with different are significantly different ($p<0.05$)

Table 36 Oxidation reaction of SSIR by autoclaving

Temperature (°C) / Time (min)	FFA (%)	PV (meq /kg)	TBA value (μg MAD/kg)
105 / 10	0.17±0.06[g]	0.12±0.07[h]	0.13±0.06[g]
105 / 15	0.19±0.05[f]	0.15±0.04[f]	0.15±0.05[f]
105 / 30	0.20±0.07[e]	0.13±0.09[g]	0.16±0.01[e]
110 / 10	0.21±0.04[d]	0.21±0.09[e]	0.19±0.04[c]
110 / 15	0.21±0.08[d]	0.22±0.13[d]	0.19±0.05[c]
110 / 30	0.29±0.07[c]	0.21±0.08[e]	0.18±0.07[d]
121 / 10	0.29±0.08[c]	0.45±0.06[b]	0.21±0.04[b]
121 / 15	0.31±0.02[h]	0.39±0.00[c]	0.19±0.04[c]
121 / 30	0.31±0.04[b]	0.41±0.04[b]	0.18±0.02[d]
The control	0.88±0.01[a]	0.62±0.03[a]	0.27±0.02[a]

Note: Data are expressed as the mean ± SD (n=3)

[a-g] Values in each column for each analytical property with different are significantly different ($p<0.05$)

Table 37 Oxidation reaction of SSIR by heat-moisture treatment

Temperatures (°C) / Moisture content (%)	FFA (%)	PV (meq /kg)	TBA value (μg MAD/kg)
90 / 15	0.26±0.11^{f}	0.49±0.07^{f}	0.17±0.06^{e}
90 / 20	0.28±0.09^{e}	0.50±0.04^{e}	0.19±0.05^{c}
90 / 25	0.29±0.05^{d}	0.50±0.09^{e}	0.18±0.07^{d}
100 / 15	0.51±0.04^{c}	0.55±0.09^{c}	0.19±0.04^{c}
100 / 20	0.51±0.06^{c}	0.53±0.13^{d}	0.18±0.06^{d}
100 / 25	0.51±0.04^{c}	0.54±0.08^{c}	0.18±0.07^{d}
110 / 15	0.62±0.07^{b}	0.57±0.11^{b}	0.21±0.06^{b}
110 / 20	0.63±0.06^{b}	0.53±0.06^{d}	0.19±0.03^{c}
110 / 25	0.62±0.08^{b}	0.53±0.03^{d}	0.19±0.07^{c}
The control	0.88±0.01^{a}	0.62±0.03^{a}	0.27±0.02^{a}

Note: Data are expressed as the mean ± SD (n=3)

$^{a\text{-}f}$ Values in each column for each analytical property with different are significantly different ($p<0.05$)

Table 38 Oxidation reaction of SSIR by combined process

Temperatures (°C) / Time (min)	FFA (%)	PV (meq /kg)	TBA value (μg MAD/kg)
30/100	0.39±0.01^{g}	0.53±0.07^{g}	0.19±0.08^{h}
45/100	0.41±0.09^{f}	0.55±0.04^{e}	0.20±0.07^{g}
60/100	0.41±0.05^{f}	0.54±0.04^{f}	0.21±0.04^{f}
30/125	0.53±0.07^{c}	0.60±0.11^{b}	0.24±0.09^{e}
45/125	0.49±0.08^{d}	0.58±0.13^{c}	0.25±0.08^{d}
60/125	0.51±0.08^{c}	0.56±0.07^{d}	0.24±0.04^{e}
30/150	0.51±0.03^{c}	0.58±0.06^{c}	0.26±0.01^{b}

Table 38 (cont.)

Temperatures (°C) / Time (min)	FFA (%)	PV (meq /kg)	TBA value (µg MAD/kg)
45/150	0.46±0.07^{e}	0.55±0.07^{e}	0.25±0.03^{d}
60/150	0.56±0.05^{b}	0.56±0.06^{d}	0.26±0.07^{c}
The control	0.88±0.01^{a}	0.62±0.03^{a}	0.27±0.02^{a}

Note: Data are expressed as the mean ± SD (n=3)

$^{a-h}$ Values in each column for each analytical property with different are significantly different ($p<0.05$)

Table 39 Oxidation reaction of SIR through stabilization process

Stabilization process	FFA (%)	PV (meq /kg)	TBA value (µg MAD/kg)
Extrusion (80 °C / 39.5%)	0.03±0.02^{f}	0.06±0.02^{f}	0.09±0.03^{f}
Drum drying (100 °C / 3 rpm)	0.15±0.09^{e}	0.11±0.06^{e}	0.10±0.08^{e}
Autoclaving (105 °C/ 10 min)	0.17±0.06^{d}	0.12±0.07^{d}	0.13±0.06^{d}
Heat-moisture treatment (90 °C/ 15%)	0.26±0.11^{c}	0.49±0.07^{c}	0.17±0.06^{c}
Combined process (30 min /100 °C)	0.39±0.01^{b}	0.53±0.07^{b}	0.19±0.08^{b}
The control	0.88±0.01^{a}	0.62±0.03^{a}	0.27±0.02^{a}

Note: Data are expressed as the mean ± SD (n=3)

$^{a-f}$ Values in each column for each analytical property with different are significantly different ($p<0.05$)

7. Quality changes during storage and shelf-life evaluation

Stabilization generally improved functional properties of SIR. For shelf life evaluation, the samples treated with optimal stabilization condition were selected regarding the least antinutrients and most effective in improving the functional properties. It was found that the best conditions are extrusion process (barrel temperature at 100 °C and in-barrel moisture level of 61.8%), drum drying (rotation speeds 3 rpm/mins and drums heated 140 °C), autoclaving (temperature at 121 °C for 30 minutes), heat-moisture treatment (moisture content of sample at 25% and stabilization by hot air oven at higher temperatures 110 °C) and combined process (autoclaving at 121 °C for 60 minutes and extrusion process at barrel temperature 100 °C and in-barrel moisture level of 61.8%).

7.1 Oxidation reaction

The PV measures the content of hydroperoxides and is often used as an indicator of the primary products of lipid oxidation. There is an increase ($p < 0.05$) in PV over incubation time at all storage temperatures. At 30 °C storage, the control show higher oxidation, reaching 17.81 meq/kg of peroxide compound to drum drying (12.05 meq/kg), heat-moisture treatment (13.37 meq/kg) and extrusion process (10.07 meq/kg) at 12 weeks. Interestingly, the PV of the autoclaving and combined process value was equal to 0 meq/kg when stored at 30, 40 and 50 ° C for 12 weeks (Tables 34-36). However, the PV was beyond the acceptable limits (10 meq/kg) when stored at 30°C for 10, 8 and 6 weeks in extrusion process, drum drying, heat-moisture treatment and the control. The PV of SSIR increased continuously when stored at 40 °C and 50 °C. The increased PV may be due to advanced disruption in the SSIR structures at the later stage of storage which caused oil separation from samples and increased the accessibility to the solvent. Therefore, it is significantly important to the physicochemical properties changes such as rancid of SSIR during storage. This phenomenon may be due to the fact that the initial steps of lipid oxidation involve chain reactions that form hydroperoxides which are classified as primary lipid oxidation products and could generate secondary lipid oxidation products (Andersen, & Skibsted, 2002). According to Codex Alimentarius (2009), seed oils have a maximum of 10 meq/kg of peroxide in order to be considered as good-quality. This is in accordance with the fact that the PV level correlated well with the rancid, as demonstrated by Lee et al. (2002). In addition, Poskocilova et al.

(1988) reported that extruded oats can be stored for 1.5 months without loss of quality. In the same way Negedu et al. (2013) reported the effect of autoclaving at 121°C for 30 minutes on castor seeds. The level of PV was lower in sample through autoclaved compared to the un-autoclaved seeds after 180 days of storage period.

The SSIR by five processes and the control were non-significant ($p>0.05$) differences in FFA and TBA value for 12 weeks of storage at 30-50 °C. The control show the value of FFA as 0.88- 0.91% and TBA value as 0.24-0.29 µg MAD/kg. While, SSIR by drum drying show the value of FFA as 0.47-0.52% and TBA value as 0.18-0.22 µg MAD/kg, heat-moisture treatment of FFA as 0.63-0.68% and TBA value as 0.18-0.22 µg MAD/kg and extrusion process of FFA as 0.02-0.09% and TBA value as 0.07-0.09 µg MAD/kg were found to stable for up to 12 weeks of storage (acceptable limits 15%). However, SSIR by autoclaving and combined process provided undetectable during storage. The FFA did not change during storage at 30-50 °C. This is probably because the FFA content at more saturated state and conversion of double bonds configuration from cis to trans after disruption of seed cell walls at higher temperature that led towards the destruction of essential fatty acids activity. Manthey et al. (2002) reported an apparent decline in FFA contents for flaxseed during extrusion processing. The decrease in FFA may be attributed to holding time effect on feed material, low rotation of screws and increased barrel temperature (Aldapa et al., 1996). A similar trend was observed in Shin et al. (1993) found that the cooking temperature had no significant effects on the TBA values of products. TBARS very little effect on flavor, texture, and other important sensory properties (Yosuf et al., 2007).

7.2 Moisture content

The SSIR had the low moisture content (low than 5%) for 12 weeks of storage at 30-50 °C, except for the control had high moisture content equity 6.16% and 5.33% during the storage period at 30 °C and 40 °C, respectively. It was visually observed that all stabilized samples had low moisture content with loose and soft characteristics, while the control had higher moisture content with more agglutinated characteristics. In general, thermal processes make samples drier, and a variation in their moisture content during storage until equilibrium. This variation in moisture content and equilibrium rate might be also a consequence of sample composition after treatment. The higher the temperature or sample heat contact during treatment, the drier

the sample, and the faster and more expressive the moisture recovery during storage. These results show the importance of moisture control at the storage environment and the use of appropriate packages to keep the desirable traits of the samples.

7.3 Microbiological

The relationship between moisture content and bacteria, yeast and molds growth are in agreement with Vieira (1999), who stated that moisture and temperature are critical factors for fungi growth and mycotoxins production. According to the National Agency for Sanitary Surveillance (ANVISA), which establishes the microbial contamination limits of 3×10^3 UFC/g for flour (Brasil, 2001). The results found show SSIR did not change growth of microorganism in any of the storage at 30-50 °C. However, the highest numbers for bacteria, yeast, and molds were found in the control averaging 9×10^3 UFC/ g and 8.3×10^3 UFC /g at stored of 30 °C, 5.8×10^3 UFC/ g and 4.6×10^3 UFC/ g at stored of 40 °C and 4.4×10^3 UFC/ g and 3.1×10^3 UFC/ g at stored at 50 °C from initial time was 7×10^3 UFC/ g.

7.4 Shelf-life evaluation

The calculated Q_{10} values were found to be in the range of 1.5 to 2.0, and which was reported to be the range for lipid oxidation in various food products (Labuza, 1982a). The Q_{10} values of all samples concerning with PV values were calculate and presented in Table 37. It indicates that SSIR can be stored at 4 °C to 50 °C. The results from accelerated shelf-life testing conducted at higher temperatures (30-50 °C) were extrapolated to predict the shelf-life at normal storage conditions. The results indicate that all samples had shelf-life for more than 90 days or 3 months when stored at 4°C. Nevertheless, the extrusion process (barrel temperature at 100 °C and in-barrel moisture level of 61.8%) had shelf-life for 28-509 days, drum drying (rotation speeds 3 rpm/mins and drums heated 140 °C) had shelf-life for 14-112 days, heat-moisture treatment (moisture content of sample at 25% and stabilization by hot air oven at higher temperatures 110 °C) had shelf-life for 14-100 days and The control (control) had shelf-life for 14-51 days stored at 25-90 °C. Moreover, SSIR by autoclaving and combined process had shelf-life more than 3 months stored at 4-50 °C. The quality of most foods decreased over time. Thus, there will be a time that the product becomes unacceptable (Robertson, 2009).

Table 40 Effect of storage period at 30 °C on the PV (meq./kg) of SSIR

Stabilization	30 °C						
	0	2	4	6	8	10	12
Extrusion	0.00±0.00^{c}	0.88±0.05^{d}	1.57±0.11^{d}	4.14±0.06^{d}	7.05±0.09^{c}	7.57±0.05^{d}	10.07±0.07^{d}
Drum drying	0.31±0.10^{b}	2.48±0.04^{c}	4.13±0.13^{c}	7.89±0.07^{c}	10.89±0.07^{b}	11.61±0.12^{c}	12.05±0.02^{c}
Heat-moisture treatment	0.33±0.03^{b}	2.66±0.08^{b}	5.16±0.03^{b}	8.96±0.05^{b}	10.86±0.04^{b}	12.34±0.09^{b}	13.37±0.08^{b}
Autoclaving	0.00±0.00^{c}	0.00±0.00^{e}	0.00±0.00^{e}	0.00±0.00^{e}	0.00±0.00^{d}	0.00±0.00^{e}	0.00±0.00^{e}
Combined process	0.00±0.00^{c}	0.00±0.00^{e}	0.00±0.00^{e}	0.00±0.00^{e}	0.00±0.00^{d}	0.00±0.00^{e}	0.00±0.00^{e}
The control	2.62±0.03^{a}	4.97±0.08^{a}	8.32±0.03^{a}	10.27±0.04^{a}	13.12±0.07^{a}	15.62±0.08^{a}	17.81±0.03^{a}

Note: Data are expressed as the mean ± SD (n=3)

$^{a-c}$ Values in each column for each analytical property with different are significantly different ($p<0.05$)

Table 41 Effect of storage period at 40 °C on the PV (meq./kg) of SSIR

Stabilization	40 °C						
	0	2	4	6	8	10	12
Extrusion	0.00±0.00^{c}	5.27±0.03^{c}	9.89±0.07^{c}	10.22±0.04^{d}	12.43±0.07^{c}	13.13±0.08^{c}	13.97±0.01^{c}
Drum drying	0.31±0.10^{b}	6.19±0.09^{b}	10.22±0.05^{b}	11.07±0.07^{c}	12.85±0.06^{b}	15.67±0.03^{b}	16.87±0.03^{b}
Heat-moisture treatment	0.33±0.03^{b}	6.24±0.08^{b}	10.28±0.08^{b}	11.53±0.05^{b}	13.81±0.09^{b}	15.63±0.07^{b}	16.83±0.09^{b}
Autoclaving	0.00±0.00^{c}	0.00±0.00^{e}	0.00±0.00^{c}	0.00±0.00^{e}	0.00±0.00^{d}	0.00±0.00^{e}	0.00±0.00^{e}
Combined process	0.00±0.00^{c}	0.00±0.00^{e}	0.00±0.00^{e}	0.00±0.00^{e}	0.00±0.00^{d}	0.00±0.00^{e}	0.00±0.00^{e}
The control	2.62±0.03^{a}	7.59±0.11^{a}	10.87±0.11^{a}	12.34±0.07^{a}	14.06±0.09^{a}	16.26±0.08^{a}	18.11±0.08^{a}

Note: Data are expressed as the mean ± SD (n=3)

$^{a-c}$ Values in each column for each analytical property with different are significantly different ($p<0.05$)

Table 42 Effect of storage period at 50 °C on the PV (meq./kg) of SSIR

Stabilization	50 °C						
	0	2	4	6	8	10	12
Extrusion	0.00±0.00^{c}	7.22±0.07^{d}	10.57±0.06^{c}	12.94±0.06^{c}	13.33±0.04^{c}	15.96±0.07^{d}	17.28±0.06^{d}
Drum drying	0.31±0.10^{b}	10.49±0.03^{c}	12.11±0.09^{b}	13.87±0.07^{b}	15.57±0.03^{b}	19.93±0.06^{c}	21.39±0.11^{c}
Heat-moisture treatment	0.33±0.03^{b}	10.71±0.05^{b}	12.13±0.07^{b}	13.95±0.06^{b}	15.64±0.07^{b}	20.11±0.07^{b}	23.78±0.04^{b}
Autoclaving	0.00±0.00^{c}	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{e}	0.00±0.00^{e}
Combined process	0.00±0.00^{c}	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{e}	0.00±0.00^{e}
The control	2.62±0.03^{a}	10.86±0.09^{a}	14.48±0.08^{a}	16.57±0.05^{a}	18.67±0.06^{a}	22.49±0.07^{a}	25.06±0.05^{a}

Note: Data are expressed as the mean ± SD (n=3)

$^{a-c}$ Values in each column for each analytical property with different are significantly different ($p<0.05$

Table 43 Shelf life (days) of SSIR were calculated from Q_{10}

Conditions	temperature (°C) /shelf-life (day)			Q_{10} =	Q_1 =	$(Q_1)^{\Delta t} = \square_{st} / \square_{st+\Delta t}$		
	30	40	50	$\square_{st} / \square_{st+1}0$	$(Q_{10})^{0.1}$	shelf-life at 4°C (day)	shelf-life at 25°C (day)	shelf-life at 35°C (day)
Extrusion	84	42	28	2.0	1.07	509.3	118.8	59.4
Drum drying	44	28	14	1.6	1.05	112.0	55.2	35.1
Heat-moisture treatment	43	28	14	1.5	1.04	100.7	53.3	34.7
Autoclaving	84	84	84	-	-	-	-	-
Combined process	84	84	84	-	-	-	-	-
The control	42	28	14	1.5	1.04	90.4	51.4	34.3

8. Pasta development from SSIR

From past development studies, it was found that SSIR by extrusion processing at high temperature 100 °C and moisture level 61.8% yielded pasta with highest PC, nutritional and functionality. This research was to study the impact of fortified with SSIR (by extrusion processing at high temperature 100 °C and moisture level 61.8%) at levels 0-50% and pasta control (100% semolina flour) on pasta quality, namely in terms of texture and cooking quality. The fortified with SSIR at levels 0-50% and the pasta control appearnces were demonstrated in Fig 11. Fortification pasta with various protein sources such as plants protein has been attempted by several workers, with a view to enhancing the nutritional value of pasta. Over the last few years, many studies have been made on the production of protein-fortified pasta. It was observed that fortification affects pasta quality, namely in terms of texture and cooking quality.

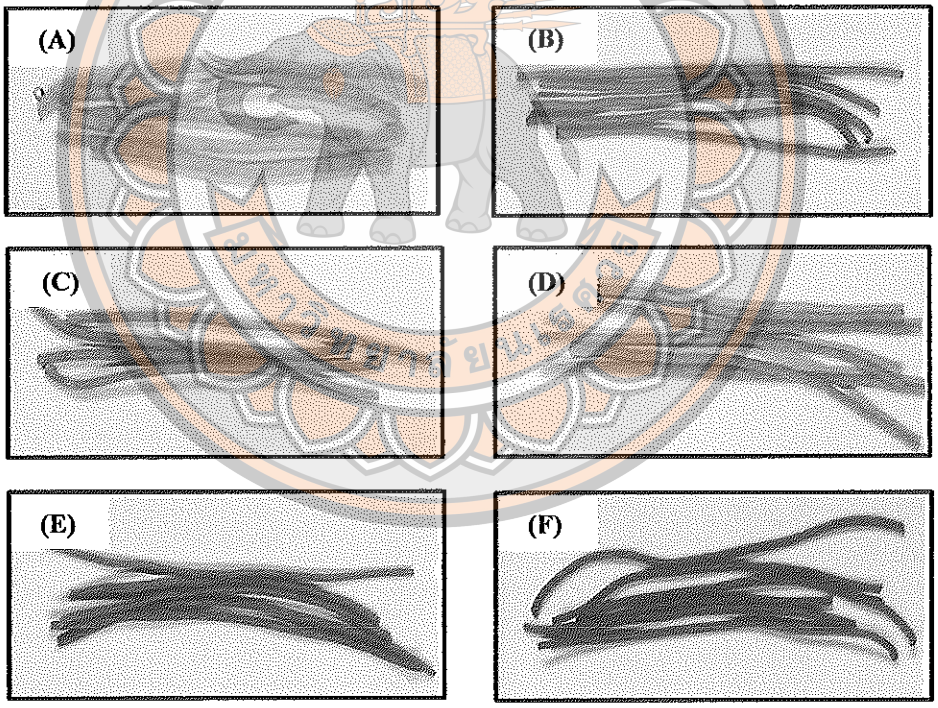


Figure 11 Pasta development from SSIR. [the pasta control (A), pasta fortified 10% SSIR (B), pasta fortified 20% SSIR (C), pasta fortified 30% SSIR (D), pasta fortified 40% SSIR (E), pasta fortified 50% SSIR (F)]

8.1 Proximate composition

The result for the proximate composition of protein-fortified pastas are presented in Table 38. The moisture content of the pasta control was also found to be a significantly highest ($p < 0.05$). Different food materials have different capacity for absorbing and retaining moisture. The decrease in moisture content with an increase in the level of substitution showed the certainty of prolonging shelf-life. The data in table 38 referred to a high fat, protein and fiber content in the T3-T5 (30-50% SSIR) while, the pasta control had the fat, protein and fiber content being 2.37, 24.70 and 1.43%. The lowest fat content in the control was due to the lipid extraction from semolina flour. For an increase in protein content was observed with SSIR. This could be attributed to a high percentage of protein content in SSIR. The protein-fortified pasta had the lowest value of ash. This may be due to the differences in the composition of pasta such as the volume of semolina or other.

8.2 Functional properties

The obtained cooking weight values are presented in Table 39. Generally, protein-fortified pasta at the studied in prepared to produce pasta with cooking weight values higher than that of the pasta control. The T3 had significantly ($p < 0.05$) the highest cooking weight values compared to the pasta control. These increases were due to the high protein content in the prepared protein products. Cooking properties are important indicators of pasta quality. A change in the recipe of pasta, particularly the addition of protein or fiber, can significantly affect its properties. Similarly, up to 8% protein (albumen) supplemented pasta weighed about 27% more water than control (Savita et al., 2013). Das, & Chattoraj (1989) assessed that in the case of pasta containing protein, a higher percentage of water was absorbed during cooking. On the other hand, Baiano et al. (2006) found that with increasing the bean flour more than 15% in prepared pasta the water absorption decreased.

Cooking loss was defined as the weight of the total solids lost in the cooking water. The obtained cooking loss data prepared in Table 39. High loss of solids resulted in poor-quality cooked spaghetti and on the contrary, very low cooking loss of 4.18% and it was only found in T3 sample. High cooking loss of pastas was influenced primarily by the protein level and entrapment of gelatinized starch in protein network could prevent the leaching of starch components. The type of ingredients in the

pasta mix influences the loss of soluble and solids during cooking, and it has been reported that a compact texture of the pasta often results in less cooking loss than the loose textured pasta (Del Nobile et al., 2005). Bergman et al. (1994) reported that the higher protein content in the pasta made from cowpea compared to wheat flour might have provided a superior framework of denaturated protein that was better able to trap starch molecules, preventing their loss during cooking and thus ultimately decreasing cooking loss. The obtained results in Table 39 presents the SI for the different prepared pasta samples of the T1-T5. The pasta control had significantly ($p < 0.05$) lower SI than that of protein-fortified pasta in the T1-T5 (10-50% SSIR). The SSIR at the level of 30% in preparation of protein-fortified pasta gave the highest SI value (9.96%) with significant difference ($p < 0.05$) compared to other protein-fortified pasta and the control sample that had a SI value 8.96-7.28%. The SI values for protein-fortified pasta prepared using the levels of 40% or 50% and 20% SSIR came in the second and third order, respectively with significant differences ($p < 0.05$). The SI of pasta expressed as gram of water absorbed per g of dry pasta depends on the type of ingredients in the pasta and their competitive ability to absorb and retain water in the cooked pasta. During the cooking process, the pasta matrix absorbs water and starch granules swell (increasing the volume). Several authors reported that the water absorption capacity depend on the protein denaturation and the function of the amylose/ amylopectin ratio.

8.3 Texture profile analysis (TPA)

Pasta samples fortified with SSIR led to an increase in hardness of cooked samples when compared to the control (Table 40). The SSIR at the level of 50% in preparation of protein-fortified pasta gave the highest hardness value (1455 N) with significant difference ($p < 0.05$) compared to other protein-fortified pasta and the control. The hardness of cooked spaghetti was affected by the protein denaturation. Starch gelatinization and protein coagulation were the major structural changes during cooking that affected the texture of pasta. The results of texture analysis indicate that the cohesiveness, springiness, gumminess and chewiness of pasta samples in SSIR at the level of 30% (T3) was higher than that of the control sample. PC and quality were considered the most important that affect cooking and texture quality. As protein content increases, cooked pasta becomes hardness and more cohesiveness, springiness, gumminess and chewiness (Marchylo et al., 2004).

Table 44 Proximate composition of pasta development from SSIR

Proximate composition (% dry wt.)	Pasta					
	C	T1	T2	T3	T4	T5
Moisture	13.68±0.04^{a}	10.57±0.11^{e}	10.57±0.19^{e}	10.46±0.18^{d}	10.63±0.05^{b}	10.64±0.08^{b}
Ash	2.40±0.01^{a}	2.24±0.08^{b}	2.13±0.05^{c}	1.92±0.03^{f}	1.98±0.06^{d}	1.95±0.03^{e}
Protein	24.70±0.42^{f}	26.13±0.05^{e}	26.57±0.11^{d}	27.28±0.16^{c}	27.31±0.02^{b}	27.38±0.05^{a}
Fat	2.37±0.14^{e}	2.64±0.07^{c}	2.62±0.08^{d}	2.69±0.08^{b}	2.69±0.21^{b}	2.72±0.06^{a}
Fiber	1.43±0.14^{e}	2.21±0.05^{d}	2.25±0.03^{c}	2.27±0.13^{b}	2.27±0.01^{b}	2.32±0.18^{a}
Carbohydrates	55.42±0.10^{d}	56.31±0.06^{a}	56.16±0.10^{b}	55.38±0.08^{e}	55.42±0.03^{d}	55.49±0.08^{c}

Note: Data are expressed as the mean ± SD (n=3)

$^{a-f}$ Values in each row for each analytical property with different are significantly different ($p<0.05$)

Table 45 Cooking weight, cooking loss, swelling index of pasta development from SSIR

Functional Properties (%)	Pasta					
	C	T1	T2	T3	T4	T5
Cooking weight	178.88±0.95^{f}	179.14±0.84^{e}	184.33±0.20^{b}	187.83±0.52^{a}	181.68±0.14^{d}	182.15±0.29^{c}
Cooking loss	5.56±0.31^{a}	4.67±0.49^{d}	4.74±0.71^{c}	4.18±0.17^{f}	4.88±0.50^{b}	4.59±0.44^{e}
Swelling index	7.28±0.01^{e}	8.32±0.01^{d}	8.81±0.01^{c}	9.96±0.01^{a}	8.96±0.01^{b}	8.96±0.00^{b}

Note: Data are expressed as the mean ± SD (n=3)

$^{a-b}$ Values in each row for each analytical property with different are significantly different ($p<0.05$)

Table 46 Texture profile analysis of pasta development from SSIR

Texture profile	Pasta					
	C	T1	T2	T3	T4	T5
Hardness (N)	1189.33±0.04^{f}	1245.00±0.51^{e}	1361.33±0.40^{d}	1406.67±0.75^{c}	1434.33±0.27^{b}	1455.67±0.72^{a}
Cohesiveness	0.53±0.02^{b}	0.50±0.05^{c}	0.50±0.05^{c}	0.54±0.09^{a}	0.46±0.04^{d}	0.39±0.02^{e}
Springiness (mm.)	10.66±0.32^{b}	10.18±0.83^{b}	10.72±0.82^{b}	11.21±1.05^{a}	9.73±0.39^{c}	10.26±0.83^{b}
Gumminess (N)	658.84±0.88^{b}	593.44±0.90^{d}	624.64±0.50^{c}	758.13±0.41^{a}	475.02±0.37^{f}	524.51±0.99b^{e}
Chewiness (N.mm)	7328.41±0.35^{b}	6746.00±0.29^{c}	6725.69±0.47^{c}	8015.90±0.15^{a}	3791.07±0.17^{e}	5219.16±0.07^{d}
Adhesiveness (N.s)	-69.13±0.86b	-42.66±0.74^{a}	-76.96±0.26^{c}	-133.47±0.11^{e}	-125.30±0.95^{d}	-121.89±0.62^{d}

Note: Data are expressed as the mean ± SD (n=3)

$^{a-b}$ Values in each row for each analytical property with different are significantly different ($p<0.05$)

Part 2 Enzymatic proteolysis process of Sacha Inchi protein

Enzymatic hydrolysis of the SIPI was carried out using five different proteases (Alcalase, Neutrase, Alcalase combined Neutrase, Pepsin, Papain and Flavourzyme) and the results are presented in Figure 12. Moreover, the enzymes have different proteolysis mode of action and would produce peptides that differ in chain length and amino acid composition, which enhances the diversity of potentially active peptides. These four enzymes are endoproteases (i.e. their cleavage action occurs within the polypeptide chain). Alcalase acts as an esterase, enabling it to catalyze stereoselective hydrolysis of some esters. Alcalase also efficiently hydrolyzes amino esters which include heterocyclic amino esters, which liberates small peptides and thus increases the numbers of terminal amino group residues in the hydrolysates (Xia et al., 2012). Trypsin preferentially hydrolyzes ester bonds whose carboxyl groups are contributed by Lys or Arg, except when either is followed by proline. The enzymatic mechanism of action is similar to other serine proteases. The aspartate residue located in the catalytic pocket of Trypsin is responsible for attracting and stabilizing positively charged lysine and/or arginine and is thus responsible for the specificity of the enzyme (Yin et al., 2008). Neutrase is neutral, zinc Metallo endo-protease from Bacillus amyloliquefaciens that randomly hydrolyzes internal peptide bonds and also facilitates the enzymatic synthesis of oligopeptides by the reverse proteolysis reaction with zinc metal as co-catalyst. Neutrase belongs to the same protease family as thermolysin, a zinc-dependent metallo endo-protease. Pepsin is endopeptidases that mainly cleave peptide bonds involving hydrophobic and aromatic amino acids (such as phenylalanine, tyrosine, tryptophan), thus they generate peptides having hydrophobic and/or aromatic amino acid as terminal groups (Boschin et al., 2014). Flavourzyme is a mixture of fungal endo- and exoproteases from Aspergillus oryzae strain with very board specificity that minimizes the bitterness that can occur in protein hydrolysates (Benjakul et al., 2014). The presence of exo-proteases in Flavourzyme which cleave at the C- or N-terminal end of hydrophobic amino acid residues led to a reduction in bitterness (O'Sullivan et al., 2017).

In recent years, there has been a continuous rise in the demand for plant protein use as functional food ingredients in the food industry. This is in part because functional properties such as emulsion and foaming capacities can contribute to the

sensory attributes of a food product (Wouters et al., 2016), and thus enormously influence its commercial success. Structure-dependent functionalities can also influence the interactions of proteins with other biomolecules as illustrated by the propensity of protein hydrophobic groups to interact with lipids and lipid-soluble compounds (Karaca et al., 2011). Alterations of the protein environment e.g. through changes in pH, temperature, ionic strength, etc. are known to often result in structure modifications and consequently, in their biological, physic-chemical and functional properties. For instance, changes in pH have been reported to affect the surface hydrophobicity, solubility, foaming capacity and foam stability of proteins isolated from Australian chia seeds and emulsification properties of flaxseed albumin and globulin fractions (Timilsena et al., 2016; Nwachukwu, & Aluko, 2018).

1. PY (%) and PC (%)

The PY (%) indicates the efficiency of the enzymatic hydrolysis process. Usually, a higher yield of peptides is the expected outcome for increased protein breakdown (Girgih et al., 2011b). From the data presented in Table 41, the results show that the yield (%) of protein hydrolysate carried using 1% pepsin was significantly higher ($p<0.05$) than that obtained by other enzymes. The PY of the hydrolysates varied considerably. This is because the enzymes used in the hydrolysis are endo-peptidases and mixture of endo- and exopeptidases, which hydrolyzed the peptide bonds, usually at specific residues, producing large peptides, in turn resulting in increased PC (Silva et al., 2014). For the PC of SIPI was determined to be 87.79%. The PY (Table 41) in this study is higher than that obtained from Sacha Inchi (Rawdkuen, Rodzi and Pinijsuwan, 2018), Citrullus lanatus seed (Arise et al., 2016b) and from South African bambara groundnut landraces (Arise et al., 2015). This might be attributed to the extraction method employed since the degree of protein yield depends on the extraction protocol used (Boye et al., 2010). Approximate costs of production of 100 grams protein hydrolysates produced with each enzyme were found to be 1,000 baht/ 100 g enzyme (Alcalase), 1,300 baht/ 100 g enzyme (Neutrase), 1,150 baht/ 100 g enzyme (Alcalase combined Neutrase), 3,210 baht/ 100 g enzyme (Pepsin), 2,033 baht/ 100 g enzyme (Papain) and 1,800 baht/ 1000 g enzyme (Flavourzyme) were 13, 20, 16, 40, 31 and 2 baht per 100 grams Sacha Inchi protein hydrolysates, respectively (Table 41).

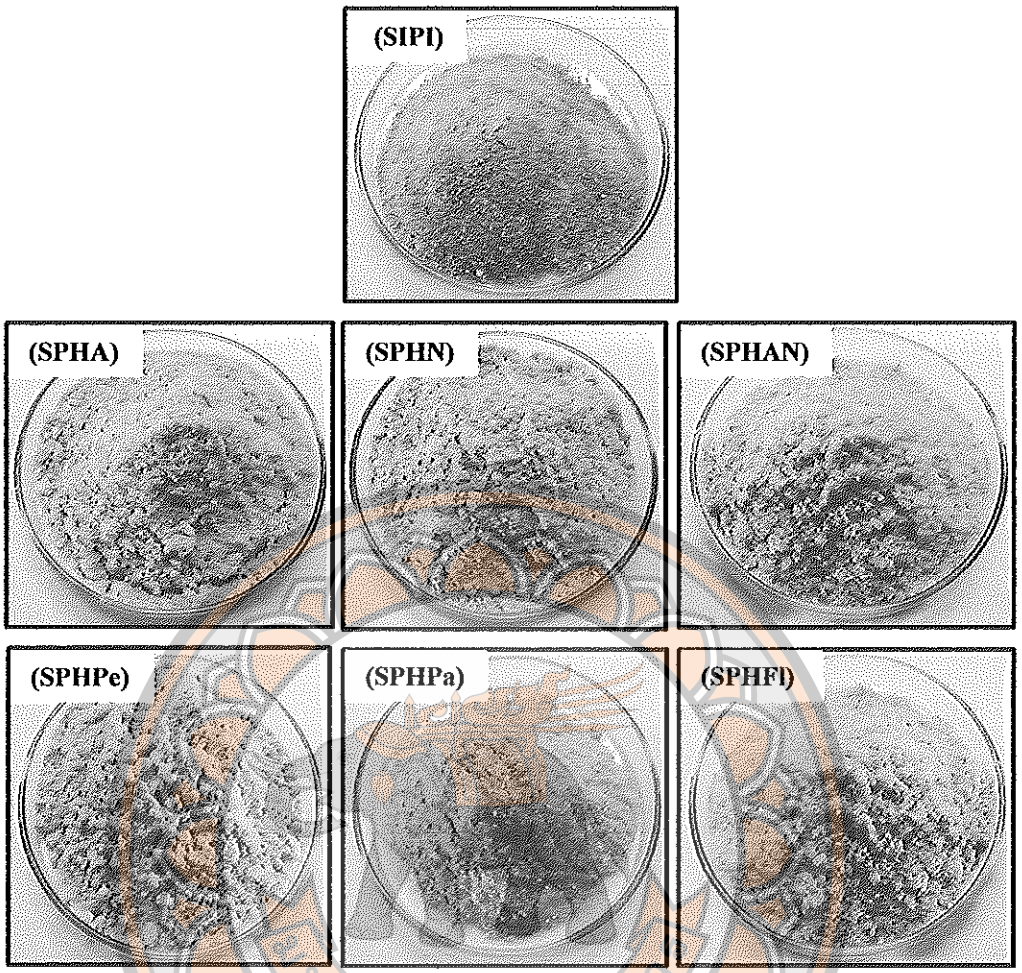


Figure 12 Protein hydrolysate of the Sacha Inchi. [Sacha Inchi protein isolate (SIPI), 1% Alcalase (SPHA), 1% Neutrase (SPHN), 0.5 % Alcalase / 0.5 % Neutrase (SPHAN), 1% Pepsin (SPHPe), 1% Papain (SPHPa) and 1% Flavourzyme (SPHFl)]

Table 47 PY (%), PC (%), DH (%) of SIPI and hydrolysate and cost of protein hydrolysate (100 g)

Enzymatic hydrolysis	PY (%)	PC (%)	DH (%)	Cost of protein hydrolysate (100 g)/baht
SPHA	75.06±0.06^{b}	90.46±1.15^{a}	28.19±0.61^{a}	13.30
SPHN	64.19±0.14^{e}	90.15±1.30^{a}	25.39±0.75^{b}	20.25
SPHAN	67.03±0.89^{c}	90.61±0.79^{a}	25.18±0.09^{b}	16.78
SPHPe	78.27±0.13^{a}	90.95±0.66^{a}	25.33±0.11^{b}	40.77
SPHPa	65.50±0.08^{d}	90.43±1.13^{a}	25.18±0.43^{b}	31.01
SPHFl	65.10±0.06^{d}	90.16±1.54^{a}	24.78±0.08^{c}	2.76
SIPI	32.17±0.51^{f}	87.79±1.06^{b}	10.02±0.42^{e}	-

Note: Results are presented as mean ± SD dev from replicate determinations (n=3) For each column, mean values that contain different letters are significantly different at $p < 0.05$

2. DH (%)

The use of protein hydrolysates for the development of new food products requires their characterization including the determination of the DH. In this study, the protein hydrolysates produced by Alcalase, Neutrase, Alcalase combined Neutrase, Pepsin, Papain, Flavourzyme and SIPI presented different DH values of 28.19%, 25.39%, 23.18% 25.33 %, 24.18%, 24.78% and 10.02%, respectively (Table 41). Sacha Inchi protein hydrolysed by Alcalase exhibited highest DH compared to other enzyme treatment. This was due to the high specific activity of an enzyme-substrate (E/S). Besides that, the extent of hydrolysis is important as excessive proteolysis reduces the Mw and could create unwanted flavours such as bitterness due to the formation of small peptides with a relatively high content of hydrophobic amino acids (Molina, & Anon, 2001), whereas different DH indicate different functionality of hydrolysates such as Mw, where low Mw hydrolysates have lower emulsifying or foaming. Higher DH values were also obtained with Alcalase enzyme similar hydrolysis of corn protein

(Jin et al., 2016), pumpkin oil cake protein isolate (Vastag et al., 2011) and canola proteins (Cumby et al., 2008).

3. Amino acid composition

According to Malomo, & Aluko (2018), antioxidant properties of peptides are greatly influenced by the amino acid composition of the peptide. Amino acid compositions of the SIR, SIPI and hydrolysates are shown in Table 37. Slight changes were observed in the amino acid composition of the protein hydrolysates when compared to the SIPI. A similar trend was also observed for hemp seed protein isolate and its protein meal (Malomo, 2014). Generally, the isolate and the hydrolysates showed high contents of Asp, Arg and Try. The apparently low content of Cys for SPHPa sample, when compared to others, might be due to the specificity of the papain for peptide bonds involving mainly lysine or arginine and the adjacent amino acid residue. The combined summary of the compositions reveals the presence of more hydrophobic amino acids such as Ala, Val, Ile, Leu, Tyr, Phe, Trp, Pro, Met, and Cys in the protein hydrolysate. Some of these amino acids, especially Tyr and Met have been shown to play specific roles in improving the antioxidant properties of peptides (Samaranayaka, & Li-chan, 2011; Udenigwe, & Aluko, 2012). Besides, aromatic amino acids with a large side group such as His (imidazole group) and Trp (indolic group) contribute to the antioxidant potency of peptides because they act as hydrogen donors (Nam et al., 2008) According to Sarmadi, & Ismail (2010), the interaction of peptides with lipids or entry into target organs can be enhanced by the hydrophobic properties, which helps in promoting the antioxidant effects of peptides. Furthermore, hydrophobicity of peptides, which helps to improve their solubility in lipid medium, has been reported to also improve their antioxidant potentials (Rajapakse et al., 2005; Shimada et al., 1992). The protein isolate and hydrolysates also show high contents of essential amino acids, which is an indication of their high nutritional values.

Table 48 Percent amino acid composition of SIR, SIPI and protein hydrolysates

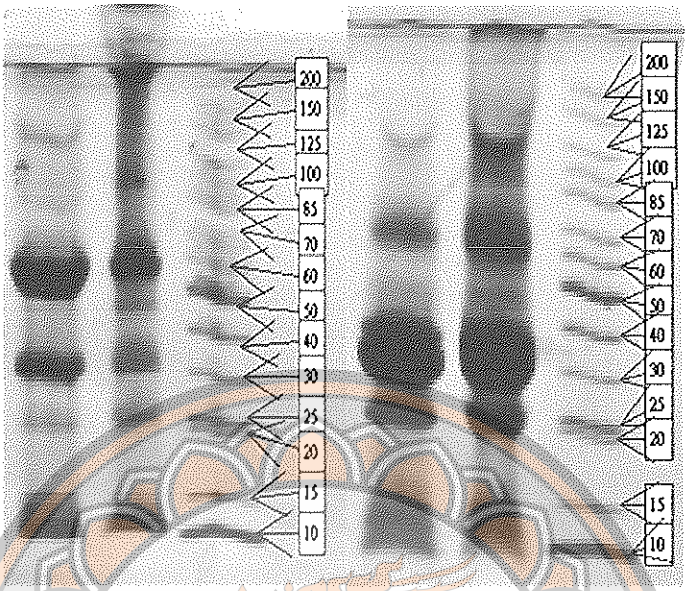
Amino acid	SIR	SIPI	SPHA	SPHN	SPHAN	SPHPe	SPHPa	SPHFl
ASP	3.31	7.61	10.95	8.53	8.10	10.91	7.94	10.04
THR	0.60	3.74	4.06	3.69	3.95	4.15	3.94	3.53
SER	0.81	3.40	4.87	4.94	5.00	5.06	3.94	5.31
GLU	6.79	9.09	10.55	10.6	10.91	11.46	10.69	10.76
PRO	1.25	2.76	3.47	3.04	3.24	3.57	3.07	3.22
GLY	2.44	3.42	3.48	3.74	3.81	4.08	3.71	4.25
ALA	0.84	2.15	3.67	3.54	2.78	3.85	2.72	3.09
CYS	3.69	1.10	1.44	1.08	1.28	1.84	1.04	1.16
VAL	2.05	3.06	4.41	4.03	4.52	4.69	3.17	4.16
MET	0.20	0.53	1.07	0.64	0.94	0.78	1.17	1.01
ILE	2.42	2.10	3.59	3.36	3.99	3.71	3.12	3.16
LEU	1.93	3.31	5.16	5.38	5.97	5.91	4.31	5.12
TYR	1.89	2.50	4.03	3.88	4.04	4.15	3.43	2.68
PHE	3.27	1.02	1.83	1.75	1.76	1.84	1.35	1.26
HIS	0.29	1.11	1.82	2.12	2.46	2.31	1.32	2.06
LYS	0.88	2.20	4.98	3.47	3.24	4.05	3.03	3.57
NH3	0.57	0.60	1.49	1.16	0.82	1.46	1.22	1.28
ARG	3.16	6.92	8.12	8.13	8.08	8.15	7.27	8.14
TRP	0.24	0.96	1.66	1.57	1.17	2.39	1.55	1.07

4. SDS-PAGE

The SIR and SIPI were analyzed by SDS-PAGE. The SDS-PAGE separation suggests that the SIR has more polypeptides than the SIPI under reducing and non-reducing conditions. The reducing and non-reducing (without β-mercaptoethanol) demonstrate a marked difference in the size of polypeptides presented in the SIR and SIPI (Figure 13). The electrophoretic profile of SIPI was divided into regions with high (125, 85 kDa), intermediate (75,60, 55, 45kDa) and low molecular weights (30, 25, 15,

10 kDa), while SIR was found at 10-200 kDa, to consist of 10, 16, 25, 30,45, 60 and between 73-200 kDa in the non-reducing gel (Figure 12A), associated with typical bands corresponding to vicilin (7S fraction) and legumin (11S fraction) subunits and polypeptides (Chel-Guerrero et al., 2007). The polypeptides of 7S and 11S fractions exhibit different susceptibilities to isolate due to their different aggregation forms, caused by the structural native conformation of globulins (Kain et al., 2009; Chel-Guerrero et al., 2011). The samples were analyzed with electrophoresis SDS-PAGE with β-mercaptoethanol or reducing gel. Soluble proteins were characterized by polypeptides 15-125 kDa range in SIR (Fig 12B). However, the presence of new polypeptide bands after the addition of mercaptoethanol was evident mainly in the SIR. These results are in accordance with other authors. The 45 kDa band corresponds to 11S globulin and the band with 73 kDa corresponds to 7S globulin. Finally, a band with 66.5 kDa can be 3S albumin. One possible reason of the different behavior between albumins and globulins, when present together, is, the protein-protein interactions. These protein-protein interactions are facilitated by different bonding forces (e.g., ionic, hydrogen, hydrophobic, and disulfide) depending on the environmental conditions permitted by the experimental conditions used (e.g., pH, ionic strength, and temperature). Such protein-protein interactions may lead to formation of large protein complexes (soluble and insoluble) thereby affecting their solubility. If globulin solubility is limited due to the presence of albumins, the insoluble globulins would then be extracted as the glutelins solubilized by alkali solution (Sathe, Kshirsagar, & Sharma, 2012). These proteins are named 7S and 11S or 2S depending on their sedimentation coefficients (Mao et al., 2014). 11S globulins are hexamers with molecular weights between 300 and 400 kDa, consisting of two opposed hexagonal rings, each containing three hydrophobically associated pairs of disulfide-linked acidic (29-35 kDa) and basic (18-28 kDa) subunits. 7S globulins are glycoproteins with molecular weights between 150 and 200 kDa (Romero-Zepeda, & Paredes-Lopez, 1996). 2S albumins have been characterized in different plant proteins, for example, amaranth, quinoa, lupin seed, soybean, and Sacha Inchi, as they are considered as allergens (Czubinski et al., 2014).

A- Non-reducing B- Reducing



SIPI SIR Std (kDa) SIPI SIR Std (kDa)

Figure 13 SDS-PAGE profiles of SIR and SIPI under (A) non-reducing and (B) reducing conditions

5. Surface hydrophobicity (S_o)

Figure 14 shows significantly higher ($p < 0.05$) S_o of the SPHFl when compared to that of all samples at each of the four pH conditions (3.0–9.0). The results suggest that a protein conformation exposed more hydrophobic patches in the SPHFl. However, all proteins decreased progressively as the pH changed from acidic through neutral to basic, which suggests the influence of protein charge. This is because the 1-anilino-8-naphthalene-sulfonate (ANS) probe also has an acidic group in addition to the main hydrophobic ring. At pH 3, the effect of the charge is smaller since the protein and ANS groups will be protonated and interaction depended mainly on hydrophobicity, hence the higher overall S_o. In contrast, at values above pH 3, there is an increased tendency for ionization of acidic groups in the ANS and protein, which will contribute to enhanced repulsion and hence reduced ANS binding (lower S_o). The results obtained in this work are similar to those reported for flaxseed albumin and globulin protein fractions where ANS hydrophobicity was highest at pH 3 but

decreased at pH 5, 7, and 9 (Nwachukwu, & Aluko, 2018). The S_o of polypeptide chains is a measure of the degree to which they adhere to one another in an aqueous environment, and has been shown to greatly affect their structure as well as functional properties (Alizadeh, & Chan, 2000).

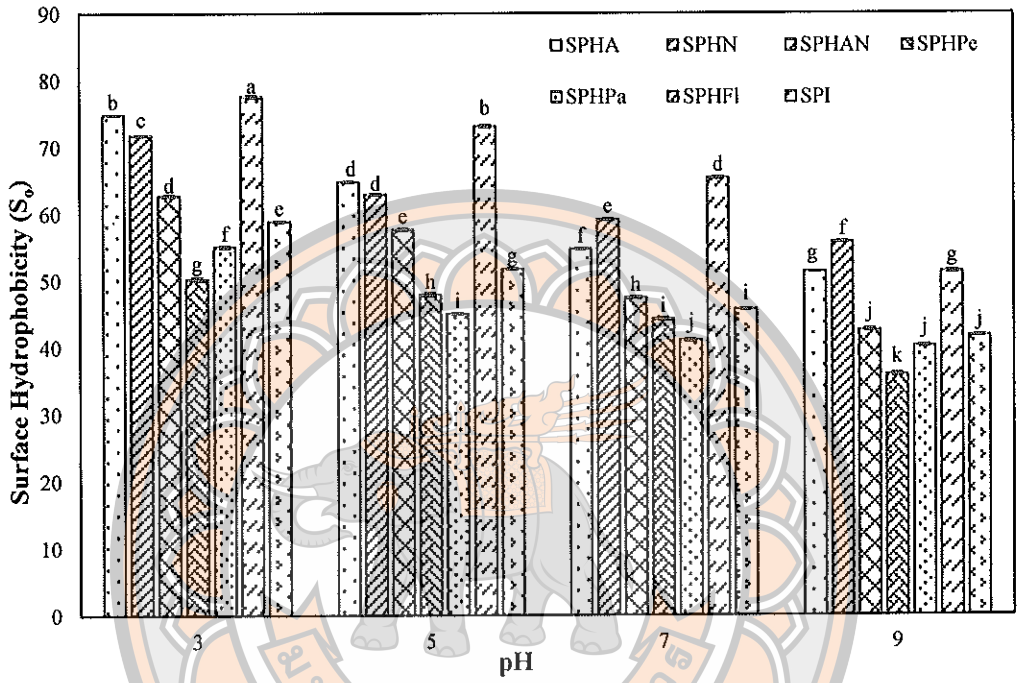


Figure 14 The surface hydrophobicity of SIPI and hydrolysates at different pH

6. Emulsion formation and stability

The effects of pH and sample concentration on the mean oil droplet size of emulsions formed and stabilized by SIPI and hydrolysates are shown in Table 42. The protein different in concentrations were used to represent low, medium, and high protein levels. In general, increased sample concentration led to improved EC at all pH values for proteins as evidenced by reduced oil droplet size as the sample concentration increased from 10 to 30 mg/ml. The results are consistent with increased protein availability within the continuous phase, which contributes to interfacial membrane formation that is necessary for oil droplet encapsulation. However, with the exception of pH 5, the poorer emulsifying ability (bigger oil droplet sizes) of protein hydrolysates when compared to SIPI. At 10 mg/mL, a progressive improvement in the

emulsifying ability of SIPI with an increase in pH was observed, while the protein hydrolysate was a more effective emulsifier at pH 3 and 9. At each pH value, the range of oil droplets sizes became narrower and moved towards the lower sizes as the protein concentration increased from 10 mg/mL to 30 mg/mL, which is consistent with the decreases in average oil droplet sizes reported in Table 41. The high ES of the protein hydrolysates at pH 3, 7, and 9.0 for the 10 and 20 mg/mL sample concentrations were dramatically reversed when protein concentration was increased to 30 mg/mL (Table 42). The results suggest protein crowding at the 30 mg/mL concentration, which may have led to increased protein charge and viscosity of the continuous phase. The high level of charges would increase protein-protein repulsions while increased viscosity reduces the rate of polypeptide translocation to the oil-water interface, both of which will reduce interfacial membrane strength and cause an increased potential for rupture and oil droplet coalescence. From the relatively low stability of the protein hydrolysates emulsion at 30 mg/mL (pH 7.0 and 9.0), the samples generally demonstrated high ES (over 80%), thus highlighting their potential for use as ingredients in the formulation of food emulsions where short-term stability is required (Nwachukwu, & Aluko, 2018). Figure 15A–15D illustrates the oil droplet size distribution of emulsions formed with SIPI and protein hydrolysates. The size distributions were mainly monomodal, which indicates uniform distribution and better emulsion quality than lentil protein isolate samples which exhibited largely bimodal droplet sizes (Primozic et al., 2017).

7. foam formation and stability

The ability of SIPI to form foams was dependent ($p < 0.05$) on pH and protein concentration as shown in Figure 16A-16C. The FC was lowest at pH 5.0, which indicates that protein aggregation at the isoelectric point had a negative effect on the ability to unfold and entrap air particles. In contrast, at pH 3.0 and 7.0 where the proteins would have gained net charges, the higher foaming capacity values may be due to a higher level of protein unfolding at the air-water interface, which enhanced polypeptide flexibility. Since the FC values were higher at pH 7.0, the results suggest more net charge and greater protein flexibility when compared to pH 3.0 and 5.0. The significantly ($p < 0.05$) positive relationship between protein concentration and FC suggests that the increased availability of polypeptide chains contributed to the

efficiency of protein hydrolysate as a foaming agent. The results suggest that FC is correlated to exposure of polar groups onto the protein surface (Nicorescu et al., 2011). Foam stability was less mainly at pH 3.0 but not at pH 5.0 and 7.0. Reduced foam stability at pH 3.0 (10 mg/mL protein concentration) is an indication that the protein molecules were unable to form strong interfacial films or did not possess adequate net charge, hence increased rupture of the entrapped air bubbles. However, the increased foam stability at pH 5.0 and 7.0, especially at 30 mg/mL protein concentration as shown in Figure 17D-F, suggests the presence of polypeptides with increased net charge or formation of strong interfacial films through increased hydrogen bond facilitated protein-protein interactions (Lawal et al., 2005). The results suggest once the foams were formed, the hydrophilic interfacial membranes reduced foam rupture tendency and hence better foam stability values of foam formed. Fuhrmeister, & Meuser (2003) showed that the foam-forming properties of pea protein isolate were best at pH 5 and 7. The stability of the foam showed to be much lower than that of egg white. In a study done by Fernandez-Quintela et al. (1997) the foam-forming of pea protein showed to be around 15 % and the foam stability around 94 %. The FC was greater in acid and alkaline regions. The foam stability increased with pea protein concentration and ionic strength (Akintayo, et al., 1999). Another study showed, however, that the foam stability of the pea protein was around 76-79%. The foam stability of pea protein hydrolysate was better than pea protein isolate at pH 5.0 but minor in other pH values (Toews, & Wang, 2013). Boye et al. (2010) reported that pea protein isolate showed a FC of 41.8% and foam stability of 93 %. Different proteins have different abilities to form and stabilize foams, and just as in the case of proteins and their different emulsifying properties, this is related to different physical properties of the proteins. For a protein to have superior foaming properties, it must possess high solubility in the liquid phase as well as the ability to quickly form a film around the air bubbles in the food system (Kinsella, 1981). The extrinsic factors that affect the foaming properties are e.g. pH, temperature and ionic strength. Foam stability and the protein's ability to form foams are also of big importance. In order for a protein to form stable foams the interfacial film should be rigid and not let the entrapped air escape (i.e. it should be almost impermeable).

Table 49 Mean oil droplet sizes ($d_{3,2}$) of emulsions formed by SIPI and hydrolysates at different pH values and PC

Samples	pH 3			pH 5			pH 7			pH 9		
	10 mg/ml	20 mg/ml	30 mg/ml	10 mg/ml	20 mg/ml	30 mg/ml	10 mg/ml	20 mg/ml	30 mg/ml	10 mg/ml	20 mg/ml	30 mg/ml
SIPI	11.16 ± 0.70^{a}	11.92 ± 0.20^{a}	12.96 ± 0.72^{a}	7.95 ± 0.79^{c}	8.33 ± 0.61^{b}	8.88 ± 0.05^{a}	9.65 ± 0.13^{a}	10.09 ± 0.05^{a}	11.09 ± 0.51^{a}	9.89 ± 0.19^{a}	10.28 ± 0.46^{a}	11.10 ± 0.38^{a}
SPHA	9.39 ± 0.82^{b}	7.80 ± 0.83^{c}	5.46 ± 0.92^{c}	8.69 ± 0.29^{b}	8.90 ± 0.17^{b}	8.58 ± 0.54^{a}	8.26 ± 0.15^{b}	7.02 ± 0.23^{b}	5.75 ± 0.06^{b}	4.41 ± 0.52^{c}	4.14 ± 0.91^{b}	3.98 ± 0.29^{c}
SPHN	8.20 ± 0.16^{c}	5.91 ± 0.09^{e}	5.88 ± 0.52^{c}	9.20 ± 0.74^{a}	8.54 ± 0.55^{b}	7.75 ± 0.81^{b}	6.05 ± 0.52^{d}	5.78 ± 0.86^{d}	4.49 ± 0.28^{c}	5.33 ± 0.30^{b}	4.98 ± 1.46^{b}	4.74 ± 0.55^{b}
SPHAN	8.33 ± 0.27^{c}	5.70 ± 0.38^{e}	5.04 ± 0.89^{c}	9.02 ± 0.57^{a}	8.56 ± 0.53^{b}	7.09 ± 0.32^{b}	6.76 ± 0.03^{d}	5.35 ± 0.71^{d}	3.39 ± 0.51^{d}	4.11 ± 0.45^{c}	4.44 ± 0.34^{b}	4.04 ± 0.08^{b}
SPHPe	7.81 ± 0.11^{d}	6.29 ± 0.14^{d}	5.13 ± 0.06^{c}	8.82 ± 0.19^{b}	8.98 ± 0.25^{b}	7.55 ± 0.43^{b}	6.37 ± 0.02^{d}	4.56 ± 0.18^{e}	3.65 ± 0.32^{d}	3.15 ± 0.18^{d}	3.06 ± 0.12^{c}	2.76 ± 0.20^{d}
SPHPa	8.38 ± 0.01^{c}	8.09 ± 0.77^{b}	6.83 ± 0.28^{b}	8.06 ± 0.09^{b}	8.81 ± 0.23^{b}	7.94 ± 0.16^{b}	7.53 ± 0.26^{c}	6.79 ± 0.52^{c}	4.69 ± 0.77^{c}	4.51 ± 0.15^{c}	4.34 ± 0.08^{b}	4.24 ± 0.76^{b}
SPHFl	7.27 ± 0.68^{d}	6.28 ± 0.63^{d}	5.10 ± 0.75^{c}	8.13 ± 0.43^{b}	9.03 ± 0.16^{a}	7.43 ± 0.32^{b}	6.27 ± 0.95^{d}	4.31 ± 0.25^{c}	3.10 ± 0.29^{d}	3.15 ± 0.06^{d}	3.01 ± 0.37^{c}	2.18 ± 0.11^{d}

Note: Results are presented as mean ± SD dev from replicate determinations (n=3). For each column, mean values that contain different letters are significantly different at $p < 0.05$

Table 50 The ES (percent increase in oil droplet size) of SIPI and hydrolysates at different concentrations with varying pH

Samples	pH 3			pH 5			pH 7			pH 9		
	10 mg/ml	20 mg/ml	30 mg/ml	10 mg/ml	20 mg/ml	30 mg/ml	10 mg/ml	20 mg/ml	30 mg/ml	10 mg/ml	20 mg/ml	30 mg/ml
SIPI	55.77 ± 0.55^{e}	54.18 ± 0.09^{g}	51.42 ± 0.75^{g}	56.18 ± 0.74^{c}	50.58 ± 0.36^{f}	44.47 ± 0.46^{d}	53.65 ± 0.05^{g}	59.76 ± 0.45^{f}	50.60 ± 0.12^{d}	56.39 ± 0.75^{g}	64.50 ± 0.42^{e}	54.32 ± 0.86^{g}
SPHA	65.76 ± 0.43^{c}	77.51 ± 0.39^{d}	58.95 ± 0.15^{f}	56.06 ± 0.64^{c}	51.58 ± 0.33^{g}	58.00 ± 0.16^{a}	62.20 ± 0.13^{f}	85.01 ± 0.11^{b}	50.09 ± 0.13^{d}	90.11 ± 0.09^{d}	94.71 ± 0.10^{c}	65.72 ± 0.97^{c}
SPHN	84.77 ± 0.53^{b}	64.69 ± 0.04^{e}	71.27 ± 0.96^{b}	56.55 ± 0.53^{c}	80.80 ± 0.36^{c}	51.46 ± 0.14^{c}	76.69 ± 0.05^{d}	61.89 ± 0.18^{e}	65.71 ± 0.42^{b}	76.92 ± 0.75^{e}	74.14 ± 0.31^{d}	71.47 ± 0.15^{d}
SPHAN	64.76 ± 0.36^{d}	62.90 ± 0.55^{f}	59.25 ± 0.03^{e}	57.52 ± 0.14^{b}	86.02 ± 0.15^{b}	57.74 ± 0.48^{b}	79.65 ± 0.47^{c}	75.25 ± 0.33^{d}	51.24 ± 0.36^{c}	70.52 ± 0.85^{f}	71.51 ± 0.75^{d}	60.11 ± 0.96^{g}
SPHPe	65.48 ± 0.17^{c}	78.08 ± 0.54^{c}	69.48 ± 0.25^{c}	55.98 ± 0.14^{d}	78.39 ± 0.37^{d}	58.61 ± 0.18^{e}	89.21 ± 0.29^{b}	85.13 ± 0.26^{b}	64.43 ± 0.19^{b}	95.27 ± 0.95^{b}	97.48 ± 0.08^{a}	82.76 ± 0.14^{c}
SPHPa	64.29 ± 0.11^{d}	79.47 ± 0.09^{b}	66.41 ± 0.13^{d}	50.16 ± 0.18^{e}	72.13 ± 0.11^{e}	57.08 ± 0.17^{b}	68.14 ± 0.31^{e}	78.11 ± 0.43^{c}	64.27 ± 0.17^{b}	94.38 ± 0.08^{c}	96.61 ± 0.48^{b}	84.14 ± 0.04^{b}
SPHFl	95.14 ± 0.03^{a}	99.47 ± 0.09^{a}	76.60 ± 0.75^{a}	70.59 ± 0.18^{a}	87.60 ± 0.25^{a}	58.48 ± 0.31^{a}	93.25 ± 0.81^{a}	95.16 ± 0.88^{a}	71.76 ± 0.26^{a}	96.27 ± 0.63^{a}	97.93 ± 0.16^{a}	87.14 ± 0.14^{a}

Note: Results are presented as mean ± SD dev from replicate determinations (n=3). For each column, mean values that contain different letters are significantly different at $p < 0.05$

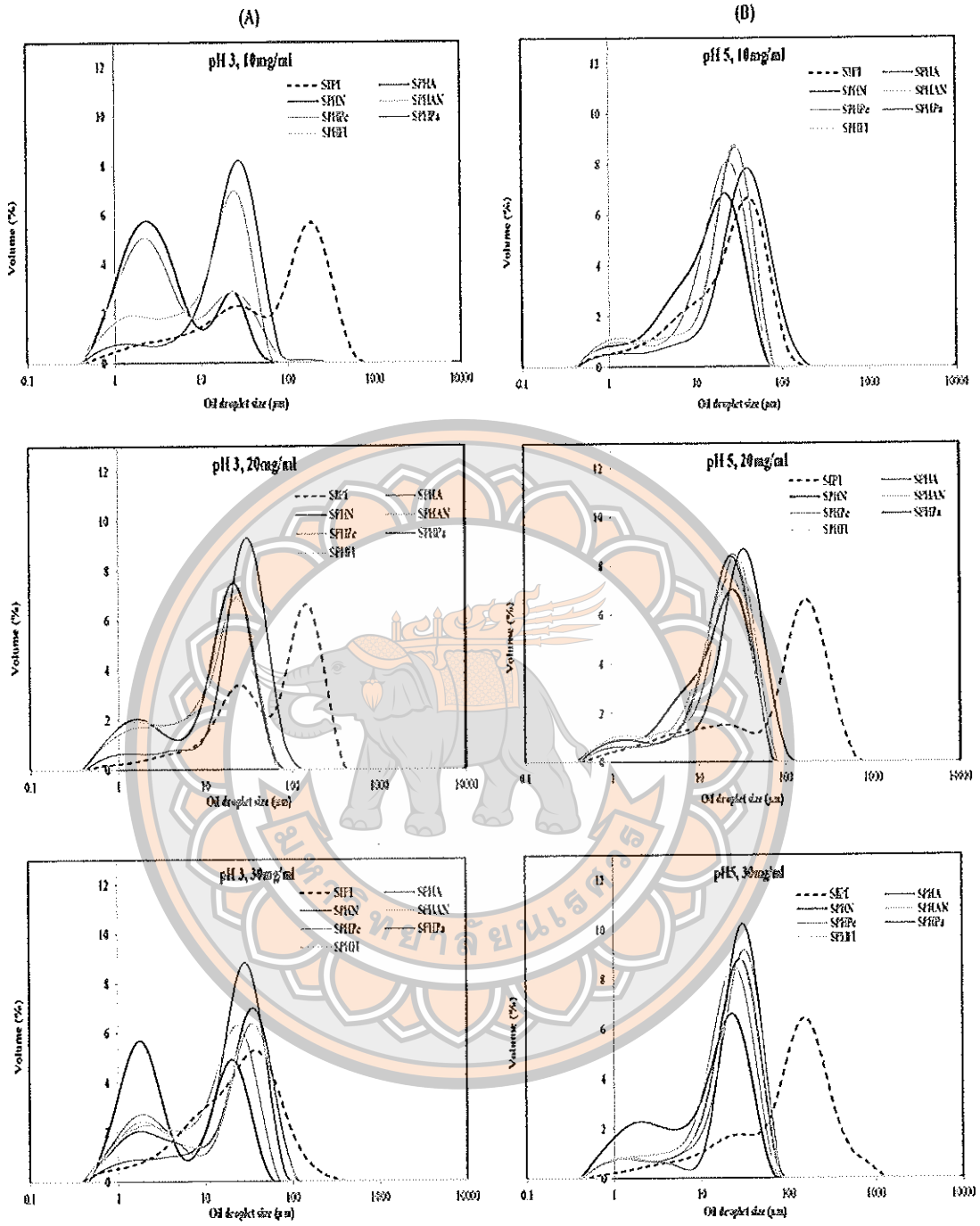


Figure 15 Oil droplet size distribution of ES by SIPI and Sacha Inchi protein hydrolysate at (A) pH 3 and 10, 20 or 30 mg/mL (B) pH 5 and 10, 20 or 30 mg/mL protein concentrations

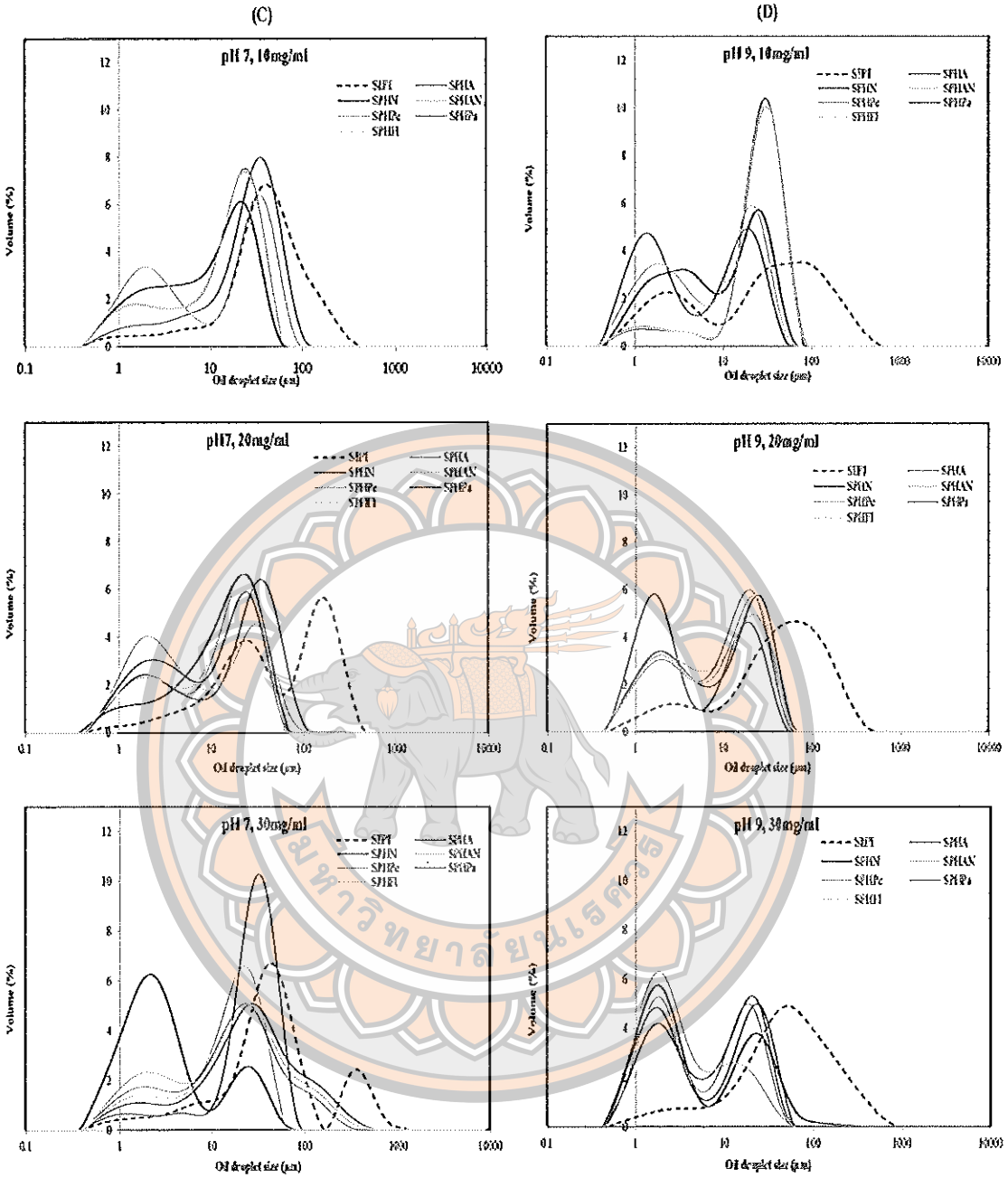


Figure 15 (cont.)

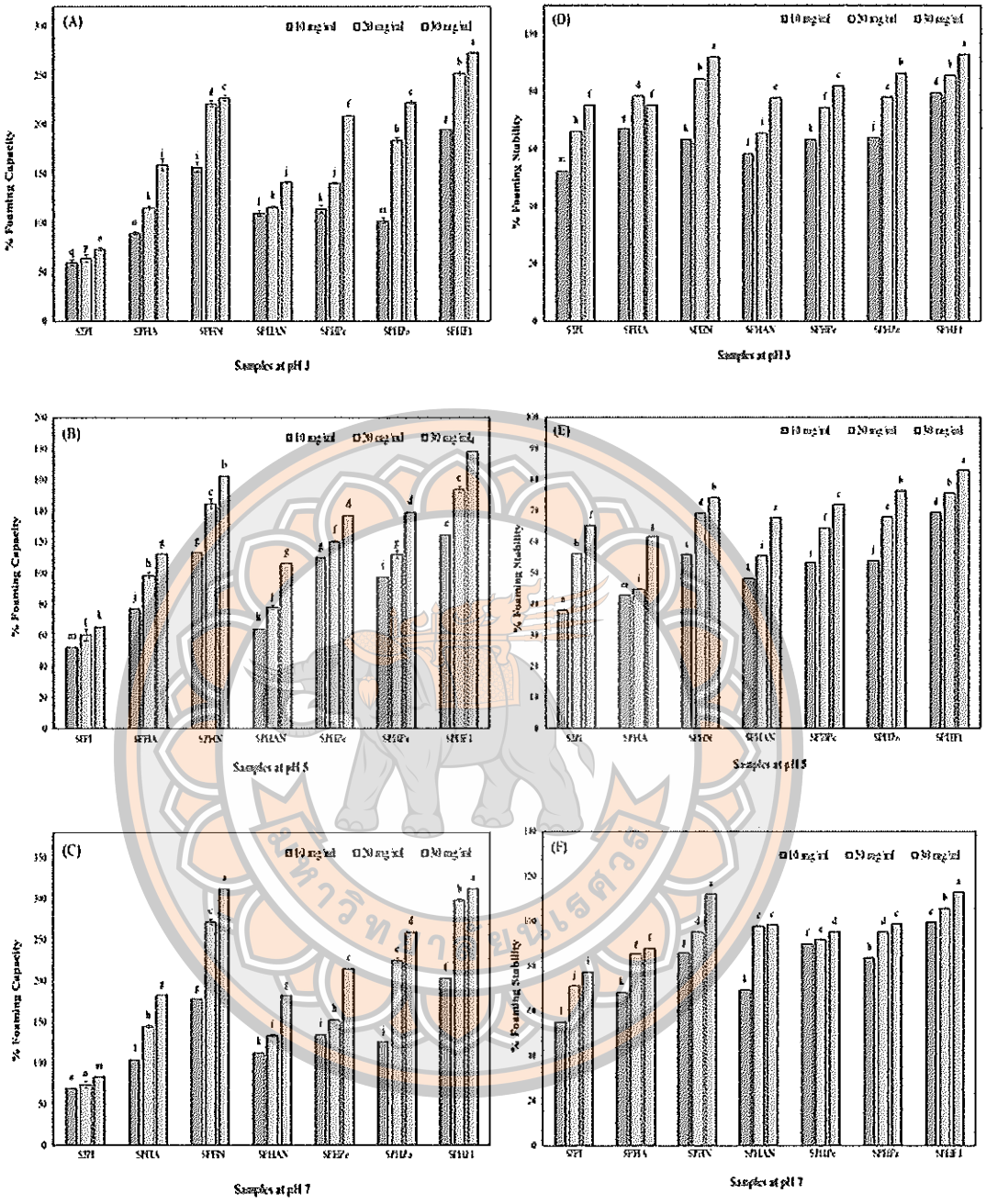


Figure 16 The FC of SIPI and Sacha Inchi protein hydrolysate (A) pH 3.0; (B) pH 5.0; (C) pH 7.0 and Foam stability (D) pH 3.0; (E) pH 5.0; (F) pH 7.0

8. Biological activity

Enzymatic protein hydrolysis consists of the cleavage of protein molecules into small peptides of various sizes, and eventually amino acids. Peptides are specific protein fragments that can present biological activity, such as antioxidant, antimicrobial and antihypertensive properties, positively influencing human health (Cumby et al., 2008; Pazinatto et al., 2013; Ranamukhaarachchi et al., 2013; Magana et al., 2015). According to Elias et al. (2008), the antioxidant activity of proteins has been related to their amino acid composition. However, such property is limited by the tertiary structure, because many amino acids with antioxidant potential can be buried within the protein core where they are inaccessible to prooxidants. Therefore, enzymatic hydrolysis favors the exposure of antioxidant amino acids in proteins, increasing the antioxidant activity of peptides. Several factors affect the biological activity of protein hydrolysates, and depend on: type of protein and enzyme, degree of hydrolysis and pretreatment of the substrate (Elias et al., 2008; Pazinatto et al., 2013; Polanco-Lugo et al., 2014). This activity occurs by specific mechanisms including the capacity of amino acid residues to donate electrons to free radicals. Bioactive peptides have a great ability to reduce the reactivity of free radicals due to the exposure of amino acids that react more effectively with these radicals (Elias et al., 2008). Some authors have observed antioxidant activity in hydrolyzed soybean proteins due to the exposure of amino acids, resulting in high free radical scavenging activity, reducing power, inhibition of lipid oxidation and metal chelation capacity (Sanjukta et al., 2015; Zhao et al., 2013; Zhu et al., 2008b).

8.1 Antihypertensive properties

Table 43 shows the differential inhibition of ACE by five enzymatic of protein hydrolysates. The highest inhibitory IC_{50} value of 0.64 mg/mL was recorded for the SPHPe. This level of ACE inhibition was significantly ($p < 0.05$) higher than the values obtained for SIPI. Similarly, the IC_{50} for Mung-bean protein hydrolysates (0.321 mg/mL) was in the range of ACE-inhibition reported for enzymatic hydrolysates from different plant sources (IC_{50} 0.180-5.4 mg/mL) (Yust et al., 2003; Ruiz-Ruiz et al., 2013b). In this regard, the IC_{50} of protein hydrolysate was not dependent on higher DH; rather it depended on the peptide mixture resulting from the application of sequential hydrolysis, whose structural patterns allowed for interaction with active

ACE sites (Li et al., 2005). Therefore, the potential inhibition of ACE in protein hydrolysate was promoted by the amino acid composition of the residues (Phe, Tyr, Pro), which are preferably associated with the last three positions of the C-terminal region (Torruco-Uco et al., 2009; Cheung et al., 1980) and N-terminal (Val, Ile), contributing significantly to ACE inhibitory activity. ACE inhibitory ability of a peptide is significantly dependent on molecular weight, amino acid composition, and their primary structure (Contreras et al., 011). Bhaskar et al., (2019) identified two ACE inhibitory peptides derived from horse gram flour after hydrolysis with alcalase. The amino acid sequences of the peptides were TVGMTAKF and QLLLQQ, with IC_{50} value of 75.0 ± 4.2 μM and 30.3 ± 2.3 μM. Even though the structure-activity relationship of ACE inhibitory peptides derived from food protein has not been fully identified, several studies have suggested that the binding ability to ACE of these peptides were related to the first three amino acids at C-terminus (Iwaniak et al., 2014) The positively charged amino acids (Arg, & Lys) and the hydrophobic amino acid residues (Phe, Pro, Try, Val, Tyr, Ala, Leu, and Ile) have been revealed to increase ACE inhibitory activity (Daskaya-Dikmen et al., 2017). Structure-activity relationship analysis also implied that amino acids Ile, Leu, Ala, and Val were preferred at the N-terminus, whereas Phe, Trp, and Tyr were preferred at the C-terminus had a positive effect on potency of ACE inhibitory peptides. This could explain the high ACE inhibitory ability of peptide FPAIGMK (Phe-Pro-Ala-Ile-Gly-Met-Lys) which consists of an aliphatic amino acid (alanine, A) and an aromatic amino acid (phenylalanine, F) at the N-terminus, a positively charged amino acid (lysine, K) at the C-terminal. Ashok and Aparna (2017) reported that some longer peptides showed high ACE inhibitory activity while some more hydrophilic ones with possessed low inhibitory activities.

8.2 DPP-IV inhibitory activity

Control of DPP-IV activity is an insulin regulatory strategy for the treatment of type II diabetes. To date, a number of peptidie components similar in structure to the incretin hormones, insulinotropic polypeptide (GIP) and glucagon-like peptide 1 (GLP-1), have shown promising results as DPP-IV inhibitory components. A limited number of these components are food protein-derived peptides (Nongonierma and FitzGerald 2013). However, to the best of our knowledge, there appears to be no information in the literature in relation to the inhibition of DPP-IV of Sacha Inchi

protein hydrolysates. In this study, The SPHPe showed an inhibitory value of 64.37% (IC_{50} value = 0.82 mg/mL), the highest DPP-IV inhibitory activity, whereas the SIPI was the least effective at inhibiting the activity of the enzyme (38.5%, IC_{50}=1.28 mg/mL), when present at a concentration of 1 mg/mL (see in table 43). A similar trend in the DPP-IV IC_{50} values ranged from 0.73 to 5.71 mg of hemp, pea, rice, and soy were hydrolyzed (Nongoniermal, & Fitz Gerald, 20115). Numerous DPP-IV substrate specificity studies using synthetic di- tri- and oligopeptides have yielded information in relation to the structural characteristics of DPP-IV inhibitors (Lacroix, & Li-Chan 2012b). Several of these inhibitory peptides contain proline and/or hydrophobic residues in their sequence. Furthermore, the position of these residues appears to significantly affect the potency of the peptides (Lacroix, & Li-Chan 2012b; Li-Chan et al., 2012).

Table 51 In vitro inhibitory activity (ACE-inhibitory and DPP-IV inhibitory activities) of SIPI and hydrolysate

Samples	ACE-inhibitory (1mg/ml)	IC_{50} value (mg/ml)	DPP-IV inhibitory (1mg/ml)	IC_{50} value (mg/ml)
SIPI	39.93 ± 0.56^{g}	1.91 ± 0.93^{a}	38.51 ± 0.77^{g}	1.28 ± 0.07^{a}
SPHA	74.45 ± 0.31^{b}	0.65 ± 0.16^{f}	59.93 ± 0.29^{c}	0.87 ± 0.16^{e}
SPHN	53.72 ± 0.14^{f}	0.91 ± 0.22^{b}	57.85 ± 0.07^{d}	0.90 ± 0.22^{d}
SPHAN	55.67 ± 0.63^{e}	0.93 ± 0.09^{c}	53.21 ± 0.10^{f}	0.95 ± 0.09^{b}
SPHPe	74.92 ± 0.51^{b}	0.64 ± 0.48^{f}	64.42 ± 0.31^{b}	0.82 ± 0.48^{f}
SPHPa	58.11 ± 0.11^{d}	0.87 ± 0.36^{d}	56.30 ± 0.74^{e}	0.91 ± 0.36^{c}
SPHFl	61.46 ± 0.73^{c}	0.73 ± 0.11^{e}	56.65 ± 0.08^{e}	0.91 ± 0.11^{c}
Captopril (10 ug/mL)	93.86 ± 0.28^{a}	0.04 ± 0.29^{g}	97.02 ± 0.05^{a}	0.23 ± 0.12^{g}

Note: IC_{50} values are reported as the mean ± SD from triplicate determinations and expressed at final assay concentration. Within the same column, values with different lowercase letters are significantly different ($p < 0.05$)

9. Antioxidant activity

Enzymatic food protein-derived peptides, in comparison to synthetic compounds, are believed to be safer natural antioxidants that can be used as protective agents to help the human body reduce oxidative damage and associated diseases (Guo et al., 2009). In general, the antioxidant properties of these hydrolysates largely depend on enzyme specificity, degree of hydrolysis, and the nature of the peptides released including molecular weight, amino acid composition, and hydrophobicity (Kim et al., 2007). The antioxidant properties of peptides include their ability to scavenge such as free radicals (Tang et al., 2009).

9.1 Free radical scavenging properties (RSA)

The DPPH radical is relatively stable and has been widely used to test the ability of natural compounds to act as free radical scavengers or hydrogen donors as a means of evaluating their antioxidant potentials (Zhu et al., 2006). In the present work, EC_{50} value (mg/mL; meaning the concentration that causes a decrease in initial DPPH concentration by 50%) is applied as an indication to evaluate the scavenging activity. The lower the EC_{50} value, the higher the free radical scavenging ability. Table 44 shows the ability of SIPI and hydrolysate to scavenge DPPH radicals. The highest inhibitory EC_{50} value of 3.2 mg/mL was recorded for the SPHFl and this level of DPPH and hydroxyl radical's inhibition was significantly ($p < 0.05$) higher than the values obtained for SIPI and another hydrolysate. Distinct differences in DPPH radical scavenging ability were observed for the hydrolysates obtained by different proteases, which is in agreement with similar findings obtained for hemp protein hydrolysate by Flavourzyme had highest radical scavenging ability (EC_{50} 3.5 mg/mL). Chang et al. (2007), who reported that the highest DPPH radical scavenging ability (EC_{50} 2.3 mg/mL) of hemp protein hydrolysates (e.g., obtained by Neutrase) was close to the highest value of hemoglobin hydrolysate obtained by Alcalase, but much lower than that of wheat germ protein hydrolysate (EC_{50} 1.3 mg/mL; Zhu et al., 2006) and chickpea protein hydrolysate (EC_{50} about 1.0 mg/mL; Li et al., 2008) obtained by Alcalase. The hydrolysates obtained by Alcalase had much lower DPPH radical scavenging ability, relative to those hydrolysates from hemoglobin, wheat germ protein, and chickpea protein. The differences may be attributed to differences in the type and nature of protein and hydrolysis pattern of protease. Previous studies have

pointed out that high DPPH or other radical scavenging activities for the protein hydrolysates or peptides are usually associated with high hydrophobic amino acid or hydrophobicity (Li et al., 2008; Rajapakse et al., 2005).

The hydroxyl radical (·OH) is generated from the conversion of superoxide and hydrogen peroxide as well as metal-catalyzed processes (Du, & Gebicki, 2004). Hydrogen peroxide and superoxide anion can be converted to hydroxyl radicals in vivo in the presence of metal ions or may be generated from several other radical systems leading to oxidative stress. The scavenging activities of SIPI and hydrolysate were compared to that of GSH (Table 44). Protein hydrolysate exhibited similar and low EC_{50} value of HRSAs 2.5-4.7 mg/mL. These activities were high EC_{50} than those observed for GSH (EC_{50} 0.38 mg/mL). The results obtained in this work are similar to those reported for the hemp protein hydrolysate (Girgih et al., 2011) as well as other samples reported in the literature such as kidney bean protein hydrolysates (Mundi, & Aluko, 2014) and African yam bean (Ajibola et al., 2011). The Sacha Inchi protein hydrolysate is poor HRSAs when compared to chickpea (Li et al., 2008) and wheat germ (Zhu et al., 2006) peptides that showed lower EC_{50} 1.5 mg/mL.

9.2 Iron chelating activity and FRAP

Transition metal ions are involved in many in-vivo oxidations reactions. Ferrous ions (Fe^{2+}) can catalyze the Haber-Weiss reaction and induce superoxide anions to form more hazardous hydroxyl radicals. These hydroxyl radicals react rapidly with adjacent biomolecules to cause severe tissue damage (Xie et al., 2008). Chelating agents may serve as secondary antioxidants because they reduce the redox potential, hence stabilizing the oxidized form of the metal ions. Table 44 shows the Fe^{2+} chelating effects of reduced GSH, SIPI, and protein hydrolysate. The Fe^{2+} chelating ability was estimated in this study by the decrease in the absorbance of ferrozine-Fe^{2+} complex after the addition of the test samples. Clearly, the SPHPe exhibited the strongest chelating capacity (EC_{50} =0.37 mg/mL) and significantly stronger ($p<0.05$) than that of GSH. Due to the negligible metal chelating activity of GSH, indicating that the presence of Cys may not be important for metal chelation. The observed difference among the hydrolysates might be due to the specificity of pepsin enzymes in liberating particular amino acids with metal-ion chelation ability. The presence of some side groups such as amino and carboxyl of the acidic (Glycine, & Asx) and basic (Lys, His, & Arg) in

amino acids have been reported to be involved in chelating metal ions (Saiga et al., 2003). However, GSH and SIPI showed high EC_{50} of chelating activity ranging from 3.76 mg/mL to 4.60 mg/mL compared to the moderate activity exhibited by SPHA, SPHFl, SPHAN, SPHN and SPHPa, which had 0.46 mg/mL, 0.55 mg/mL, 0.97 mg/mL, 1.12 mg/mL and 2.96 mg/mL, respectively.

FRAP values for the isolate and protein hydrolysates are shown in Table 44. FRAP evaluates the electron-donating potential of an antioxidant compound such as peptides whereby the Fe^{3+}/ferricyanide complex is reduced to the ferrous form (Yıldırım et al., 2001). SPHPe had the highest FRAP at 0.06 mmol Fe^{2+} /g while the SPHA, SPHAN, SPHFl, SPHN, SPHPa, SPHN and SIPI had 0.043, 0.027, 0.019, 0.013, 0.013 and 0.010 mmol Fe^{2+} /g respectively. The results show that only Pepsin was able to release a high amount of hydrogen or electron-donating amino acid responsible for Fe^{3+} reduction. Aderinola et al. (2018), reported that Moringa oleifera seed protein isolate (ISO) and its enzymatic protein hydrolysates. ISO was subjected to enzymatic (Alcalase, Pepsin, and Trypsin) hydrolysis to obtain Acalase isolate, Pepsin isolate and Trypsin isolate hydrolysates (AIH, PIH, TIH), PIH had the highest FRAP at 0.04 mmol Fe^{2+}/g while the isolate, AIH and TIH had 0.015, 0.013 and 0.01 mmol $Fe^{2+/}$g respectively. The antioxidant potential of a compound is directly related to its reducing ability (Oktay et al., 2003). Previous study also confirmed the direct relationship between antioxidant properties and the ferric reducing abilities of bioactive compounds (Wang et al., 2008). The FRAP involves an antioxidant probe accepting an electron from the antioxidant analysate (e.g. peptides) and converts into the reduced probe which is colored (Berker et al., 2007). Different factors such as the enzymes used for hydrolysis, the molecular weight of the peptides and amino acid composition can be responsible for the ability of protein hydrolysates to reduce ferric iron (He et al., 2013).

Table 52 Antioxidant activity of SIPI and hydrolysate

Samples	DPPH (EC_{50} mg/mL)	HRSAs (EC_{50} mg/mL)	Iron chelating activity (EC_{50} mg/mL)	FRAP (1 mg/mL)
SIPI	7.35±0.17^{a}	7.11±0.06^{a}	4.60±0.21^{a}	0.010±0.32^{g}
SPHA	3.85±0.26^{c}	2.73±0.09^{e}	0.46±0.22^{g}	0.043±0.14^{c}
SPHN	4.10±0.18^{d}	3.86±0.17^{c}	1.12±0.15^{d}	0.013±0.16^{f}
SPHAN	3.97±0.09^{e}	3.31±0.38^{c}	0.97±0.17^{e}	0.027±0.17^{d}
SPHPe	5.70±0.14^{c}	2.50±0.24^{f}	0.37±0.11^{h}	0.060±0.25^{b}
SPHPa	6.48±0.28^{b}	3.18±0.27^{d}	2.96±0.34^{c}	0.013±0.34^{f}
SPHFl	3.22±0.17^{f}	4.70±0.18^{b}	0.55±0.61^{f}	0.019±0.17^{e}
GSH	1.54±0.35^{g}	0.38±0.08^{g}	3.76±0.24^{b}	0.110±0.14^{a}

Note: EC_{50} values are reported as the mean ± SD from triplicate determinations and expressed at final assay concentration. Within the same column, figures with different lowercase letters are significantly different ($P < 0.05$)

9.3 Inhibition of linoleic acid peroxidation

Peroxidation of fatty acids causes deleterious effects in foods by forming a complex mixture of secondary metabolites of lipid peroxides. When these affected foods are consumed, they can cause some adverse effects including toxicity to cells (Li et al., 2008). The antioxidant activities of GSH, SIPI, and protein hydrolysate against peroxidation of linoleic acid were evaluated and results were obtained after 7 days of incubation. Table 45 shows that the protein hydrolysate effectively inhibited linoleic acid autoxidation at varying degrees, similar to the effect of GSH. After the 4th day, GSH and the protein hydrolysate seem to gradually lose their antioxidant effects, as evident by slight increases in absorbance from days 5-7. The present results are similar to previous work (Zhu et al., 2006), which showed that wheat germ protein hydrolysate gradually lost its effectiveness against linoleic acid oxidation after 3 days of incubation. Chen et al. (2007) found negligible inhibition of linoleic acid peroxidation at 2 mg/mL in peanut hydrolysates, but observed activity at higher

concentrations. Li et al. (2008) identified a low molecular weight fraction from chickpea protein that contained a higher amount of hydrophobic amino acids as having the strongest activity in preventing linoleic acid oxidation. Overall, protein hydrolysate showed the highest activity at day 7 in protecting linoleic acid against peroxidation, which may be due to the additive effects of all the active peptides present in the different fractions. The strong antioxidant effects of the peptide samples may have been due to the high levels of hydrophobic amino acids, which enhanced the solubility of the peptides in the lipid phase and thus facilitating better interactions with free radical species (Rajapakse et al., 2005). In addition, the presence of His in the peptides has also been reported to act against lipid peroxidation because His possesses an imidazole ring in its structure, which may be involved in hydrogen donation and lipid radical trapping ability (Moure et al., 2006). The observed rapid increase in linoleic acid oxidation in the absence of antioxidants (protein hydrolysate) up to the 4th day is similar to previous reports (Pownall et al., 2010; Jayaprakasha et al., 2001). As incubation proceeds beyond the 4th day, the linoleic acid is probably depleted and the production of reactive oxidation products (e.g., hydroperoxides) becomes limited, which is believed to be responsible for the sharp decrease in absorbance values (Jayaprakasha et al., 2001).

Table 53 Lipid oxidation measured in linoleic acid model system for 7 days in the presence of GSH, SIPI and hydrolysate

Samples	Duration (days)						
	1	2	3	4	5	6	7
Control	0.17 ± 0.15^{d}	0.23 ± 0.22^{d}	0.25 ± 0.05^{a}	0.44 ± 0.54^{a}	0.71 ± 0.13^{a}	0.75 ± 0.14^{a}	0.76 ± 0.36^{a}
GSH	0.06 ± 0.06^{f}	0.06 ± 0.15^{h}	0.06 ± 0.11^{g}	0.06 ± 0.63^{f}	0.06 ± 0.06^{g}	0.06 ± 0.22^{f}	0.09 ± 0.15^{d}
SPHA	0.17 ± 0.45^{d}	0.19 ± 0.12^{f}	0.21 ± 0.09^{e}	0.24 ± 0.32^{d}	0.27 ± 0.33^{d}	0.29 ± 0.26^{d}	0.33 ± 0.44^{c}
SPHN	0.16 ± 0.11^{e}	0.20 ± 0.34^{e}	0.21 ± 0.36^{e}	0.25 ± 0.17^{c}	0.28 ± 0.18^{c}	0.31 ± 0.06^{c}	0.34 ± 0.33^{c}
SPHAN	0.16 ± 0.09^{e}	0.21 ± 0.26^{d}	0.22 ± 0.02^{d}	0.24 ± 0.54^{d}	0.29 ± 0.05^{b}	0.30 ± 0.08^{c}	0.33 ± 0.45^{c}
SPHPe	0.15 ± 0.22^{e}	0.18 ± 0.11^{g}	0.19 ± 0.21^{f}	0.22 ± 0.08^{e}	0.25 ± 0.24^{e}	0.28 ± 0.09^{e}	0.32 ± 0.34^{c}
SPHPa	0.17 ± 0.05^{c}	0.23 ± 0.14^{c}	0.24 ± 0.07^{b}	0.25 ± 0.14^{c}	0.28 ± 0.59^{c}	0.30 ± 0.09^{c}	0.34 ± 0.30^{c}
SPHFl	0.18 ± 0.43^{b}	0.24 ± 0.08^{b}	0.24 ± 0.13^{b}	0.25 ± 0.27^{c}	0.27 ± 0.27^{d}	0.31 ± 0.17^{c}	0.36 ± 0.08^{c}
SIPI	0.26 ± 0.13^{a}	0.27 ± 0.26^{a}	0.23 ± 0.11^{c}	0.27 ± 0.17^{b}	0.33 ± 0.13^{f}	0.46 ± 0.64^{b}	0.50 ± 0.11^{b}

Note: Different lowercase letters in each column indicate significant differences among samples ($p < 0.05$).

The control contains only linoleic.

10. Effect of peptide size on the in vitro enzyme inhibition

The sample was selected from of physicochemical, bioactive and antioxidative properties. The SPHPe showed highest physicochemical, bioactive and antioxidative properties.

10.1 PY (%) and PC (%) of SPHPe and fractions.

Enzymatic proteolysis of SPHPe and subsequent fractionation of the resultant SPHPe by membrane ultrafiltration resulted in fractions rich in small size (<10 KDa) peptides. The PY of SPHPe was 78%, and approximately 12.7, 16.3, 18.4 and 13.2% of peptides in the SPHPe had molecular weights of <1, 1-3, 3-5 and 5-10 kDa, respectively. The final retentate (>10 kDa fraction), which contained large size peptides had a yield of 17.4%. Higher percent yields are more beneficial to commercial processing and marketing of new products. The observed results show that SPHPe consists of several peptides, resulting in a higher yield. while the fractionated peptides have been separated into specific molecular weights, thus their yield will be lower (Girgih, Udenigwe and Aluko, 2011). The PC were ~77, 82, 90, 93 and 91% for the <1, 1-3, 3-5,5-10 kDa, retentate and the SPHPe, respectively. The 3-5, 5-10 kDa fractions and SPHPe had much higher PC than the <1 and 1-3 kDa fractions because low MW non-protein components such as salts and soluble sugars were removed during the previous ultrafiltration process, thus the PC were increased.

10.2 Amino acid analysis

mino acid analysis of SPHPe and the peptide fractions (<1, 1-3, 3-5, and 5-10 kDa) are shown in Table 46. The sequence of specific amino acid residues in the peptide structure is a major factor determining the activity of bioactive peptides. Generally, the SPHPe as well as the peptide fractions all contained low levels of Met and Cys, which is typical of plant proteins. Conversely, all samples had high contents of Glu, Lys and Ala. The SPHPe and the peptide fractions were rich in Glu. The percentage content of hydrophobic aliphatic amino acid (Ala, Ile and Pro) as well as hydrophobic aromatic amino acid (His) were highest in the <1 kDa fraction, decreasing as the membrane MW cut-off increased from 1 kDa to 10 kDa. However, the 5-10 kDa fraction prepared using pepsin presented higher total hydrophobic amino acids content, and particularly higher Val, Phe and Trp content, but was least in the 3-5 kDa fraction. Aluko (2014) reported verified a good correlation between inhibitory

activities and antioxidant ability of protein hydrolysates and certain amino acid residues. Peptides which have high contents of Val, Ile and Leu inhibit activities of renin and ACE enzymes could help restore normal blood pressure. The report of Chen et al. (2014), peptides which have high contents of Met, Leu, His, Ala, and Val, would possess strong antioxidant capacity. Therefore, the fractionated peptides would be expected to exhibit optimal inhibitory activities and antioxidant ability due to the high content of these amino acid residues with better antioxidant activity.

Table 54 Percent amino acid composition of SPHPe and peptide fractions

Amino acid	SPHPe	<1 kDa	1-3 kDa	3-5 kDa	5-10 kDa
ASP	10.91	3.16	5.85	8.10	11.48
THR	4.15	0.97	2.53	3.33	4.76
SER	5.06	1.69	3.27	4.08	5.70
GLU	11.46	9.92	6.93	8.02	11.84
PRO	3.57	4.87	2.11	2.79	4.04
GLY	4.08	0.96	2.12	2.61	4.07
ALA	3.85	3.91	2.72	3.24	3.60
CYS	1.84	0.06	0.13	0.21	1.05
VAL	4.69	2.56	4.87	1.96	5.54
MET	0.78	0.32	0.51	0.67	0.84
ILE	3.71	4.48	2.55	3.15	4.03
LEU	5.91	3.70	5.46	6.09	5.93
TYR	4.15	2.19	3.70	4.46	4.56
PHE	1.84	2.09	1.81	1.10	2.22
HIS	2.31	2.57	1.03	1.51	2.54
LYS	4.05	4.00	2.04	2.82	4.12
NH3	1.46	0.41	0.72	1.02	1.44
ARG	8.15	2.11	4.22	5.77	8.73
TRP	2.39	1.54	1.17	0.57	2.92

10.3 Surface hydrophobicity (S_o)

The S_o indicates the number of hydrophobic groups present at the surface of the protein and in contact with the aqueous environment (Agyare, Addo, & Xiong, 2009). The S_o of peptide fractions in comparison with SPHPe is shown in Figure 17. The lower S_o of peptide fractions compared to SPHPe demonstrated that smaller peptides have had fewer hydrophobic binding sites for ANS. The affinity of ANS for hydrophobic patches increased from the <1 kDa fraction to the 5-10 kDa. This is probably because there are more larger peptides (oligopeptides include dipeptides, tripeptides, tetrapeptides, and pentapeptides) available as the size of the membranes increased with larger surface containing the exposed hydrophobic residues. Hydrophobicity is also a very important contributing factor to the activity of enzyme inhibitory and antioxidative peptides. This is because, the S_o site is partly responsible for formation and maintenance of the spatial structures as well as protein interactions, including binding to cell membranes, protein–protein recognition, and formation of complexes with biologically active compounds (Voronov et al., 2002). Quantitative structure-activity relationship studies of ACE-inhibitory and DPP-IV inhibitory peptides have shown that peptides composed of amino acids with strongly hydrophobic (or aromatic) side chains have potent DPP-IV inhibitory and ACE-inhibitory activities (Wu, Aluko, & Nakai, 2006; Ferraris et al., 2007). Wu et al. (1998) reported a decrease in the S_o of smaller soy protein peptides prepared by longer time papain hydrolysis compared to larger peptides produced shorter time hydrolysis. The same authors also observed lower S_o for the ultrafiltrates than the hydrolysates (Wu et al., 1998). On a similar note Molina Ortiz et al. (2002) also observed that smaller chain peptides had less S_o.

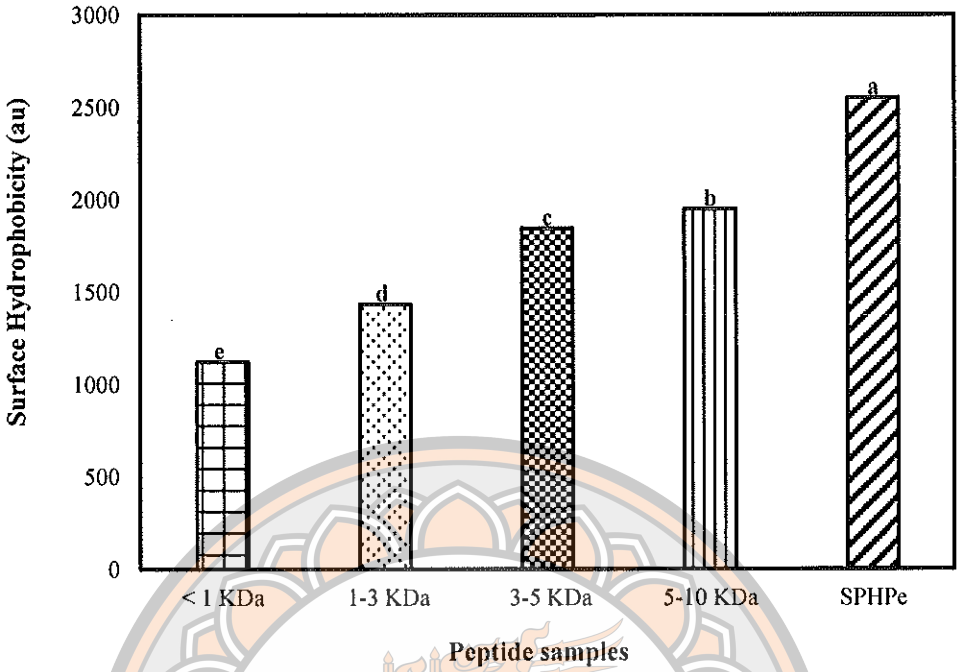


Figure 17 The S_o of SPHPe and peptide fractions (<1,1-3,3-5, and 5-10 kDa) in 10 mM phosphate buffer (pH 7.0)

10.4 ACE-inhibitory activities

Ultrafiltration separation of the SPHPe led to an increased ACE-inhibitory activities of the peptide fractions as shown in Figure 18. The ACE-inhibitory activity of SPHPe and peptide fractions were evaluated and results showed that peptide fractions exhibited significantly stronger activity ($p < 0.05$) than SPHPe. The IC_{50} values of SPHPe (0.64 mg/mL) against ACE activity was significantly higher ($p< 0.05$) than the values obtained for the membrane fractions (0.51, 0.53, 0.57 and 0.59 mg/mL, respectively, for <1, 1-3, 3-5, and 5-10 kDa peptide fractions. The reduced ACE-inhibitory activities of the ultrafiltration membrane fractions suggest that there was a synergistic effect when the peptides are present in the SPHPe. Moreover, it is widely accepted that low MW peptides are more potent in vitro ACE inhibitors than larger peptides. Girgih et al. (2011) reported that the <1 kDa peptides had lower IC_{50} value than hemp seed protein hydrolysate. The results are similar to reports obtained for camellia oleifera protein hydrolysate (IC_{50} of 1 kDa was 0.678 mg/mL) (Yao et al., 2019) and cashew nut (Yao et al., 2017), which showed an increase in ACE-inhibitory

activity with decreasing MW of peptide fractions produced by ultrafiltration. ACE prefers to bind to substrates or inhibitors containing hydrophobic (aromatic or branched-chain) such as peptides contain Tyr, Phe, Trp, Pro or Lys, while the branched-chain aliphatic amino acids such as Ile, Leu and Val are the most prevalent in highly active peptide inhibitors (Wu et al., 2006; Aluko, 2008). Thus, the higher amounts of Pro, Ile and Val in the <1 kDa fraction may have contributed to a higher ACE-inhibitory activity when compared to the high MW peptides.

10.5 DPP-IV inhibitory activity

The DPP-IV inhibitory activity of SPHPe and peptide fractions at the concentration of 1 mg/mL fractionated by ultrafiltration was show the peptide fractions of <1 kDa and 1-3 kDa had significantly different ($p<0.05$) and higher DPP-IV inhibition rates of 74.4% and 74.6% than that within the 3-5 kDa, 5-10 kDa fraction and SPHPe displaying an inhibition rate of 70.6%, 67.4% and 64.4%, respectively. The IC_{50} value of SPHPe and peptide fractions was shown in Figure 19. These results indicate that MW and size of peptide fractions play a key role in the release of potent DPP-IV inhibitory peptides. The results are similar to reports obtained rice and soy (IC_{50} <2.0 mg/mL) (Nongonierma, & Fitz Gerald, 2015) and napin of rapeseed (Xu et al., 2019), which showed an increase in DPP-IV inhibitory activity with decreasing MW weight of peptide fractions. Normally, peptide fractions of relatively small size show better DPP-IV inhibitory activity (Liu, Cheng, & Wu, 2019). Potent DPP-IV inhibitory peptides typically contain two to seven amino acid residues with Trp, Phe or Ala as the penultimate amino acid at the N-terminus. In addition, the N-terminal residue of Pro containing dipeptides was shown to influence the DPP-IV inhibitory activity (Lan et al., 2015).

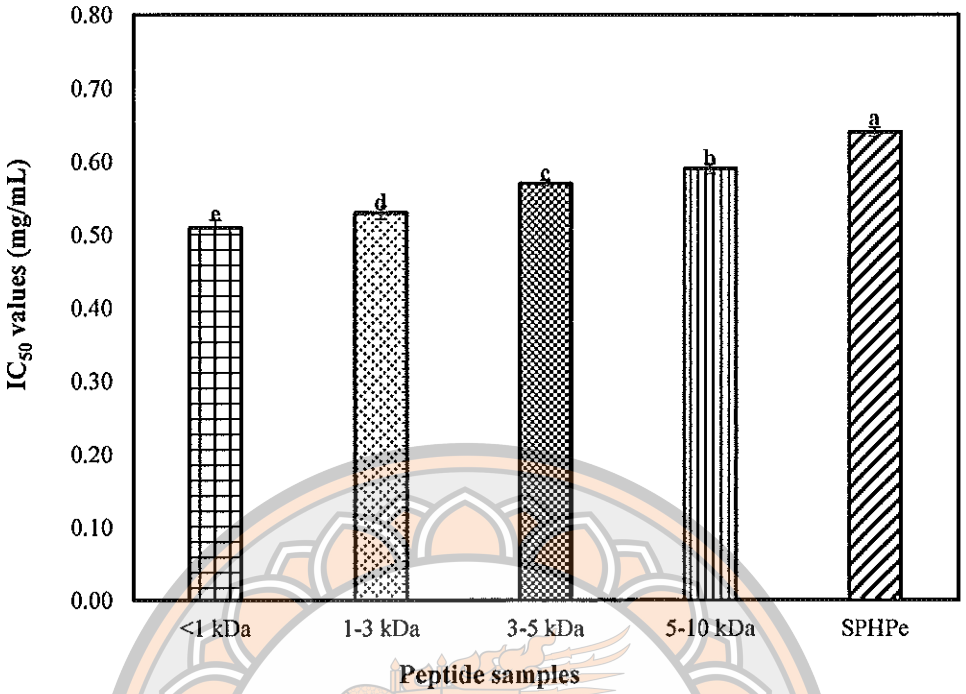


Figure 18 The SPHPe and peptide fractions (<1,1-3,3-5 and 5-10 kDa) that reduced 50% (IC_{50}) activity of angiotensin converting enzyme (ACE)

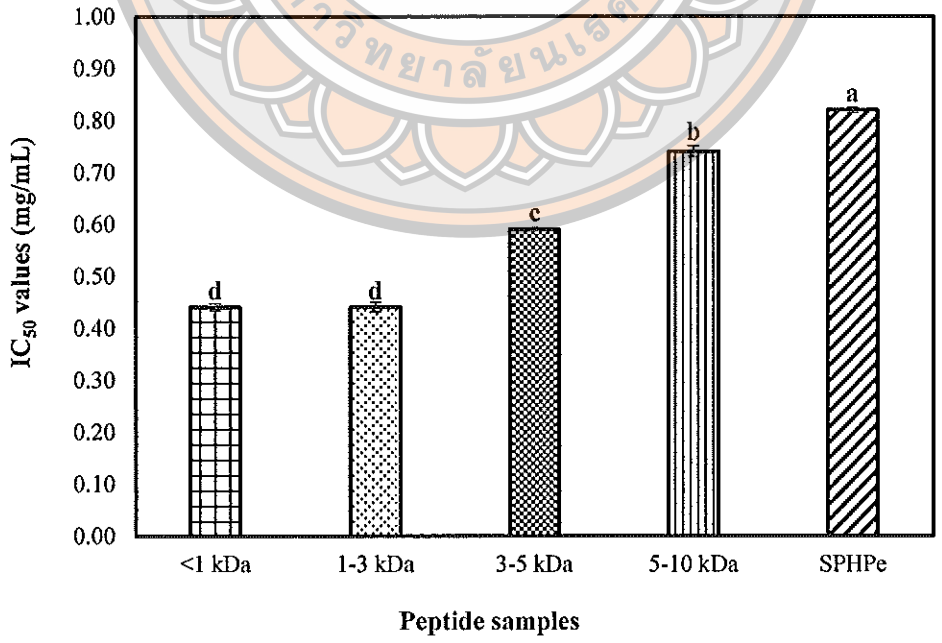


Figure 19 The SPHPe and peptide fractions (<1,1-3,3-5 and 5-10 kDa) that reduced 50% (IC_{50}) activity of DPP-IV-Inhibitory

10.6 Antioxidant activities

10.6.1 Free radical scavenging activity (FRSA)

The FRSA of SPHPe and peptide fractions were evaluated by measuring their ability to quench DPPH and HRAAs (Figure 19). DPPH radical scavenging activities of different fractions and the hydrolysate are shown in Figure 20A. The observed pattern of DPPH scavenging in this study showed no relationship with molecular size, because activity significantly decreased ($p < 0.05$) from <1 kDa to 1-3 kDa but was increased only slightly for the 3-5 kDa fraction and significantly for the 5-10 kDa fraction. The <1 kDa and 5-10 kDa fraction showed the highest inhibition (67.91%) which is statistically higher ($p < 0.05$) than the positive control, GSH (46.4%). The higher DPPH inhibitory activity shown in this study for <1 kDa and 5-10 kDa peptide fractions could have been due to higher contents of hydrophobic aliphatic (Val, Ile, & Leu) and hydrophobic aromatic (Phe and Tyr) amino acid residues in the two fractions as well as the sequence may also be significant in the ability of the hydrolysate to inhibit DPPH radicals, when compared to the 1-3 and 3-5 kDa fractions. Torres-Fuentes et al. (2015), who reported that protein hydrolyzates containing a high amount of hydrophobic amino acids such as those found in chickpea protein hydrolyzates and peptide fractions after sequential pepsin are strong free radical scavenging properties. Similarly, protein hydrolyzates containing negatively charged acidic amino acids (Glu, & Asp) could serve as potent scavengers of free radicals or reducers of metal cations due to the excess electrons that are readily donated by such amino acids (Aluko, 2012). Protein hydrolysates and peptides containing a high proportion of Ala, Leu, Pro as well as the aromatic amino acids Trp, Phe, Tyr, and His have been linke be with strong free radical scavenging activities through direct transfer of electrons (Ketnawa et al., 2018). In the same way, the increased level of amino acids such as Phe, Tyr, Val, Asp, and Glu, which are associated with strong antioxidant proper ties is thought to have contributed to the higher DPPH radical quenching ability of the high solid concentration samples in spite of their larger molecular weight distributions (Chen et al., 2018).

The HRAAs of SPHPe and its membrane fractions are shown in Figure 20B. HRAAs result from the reaction between hydrogen peroxide and superoxide. SPHPe showed low activity against the HRAAs. Considerable improvement

was observed for the peptide fraction after membrane fractionation. The 71.87% and 72.04% inhibition capacitie (<1 kDa and 5-10 kDa) which was significantly higher ($p<0.05$) than activities for the membrane fraction obtained in 62.26%, 55.91% and 53.08% (1-3 kDa, 3-5 kDa and SPHPe, respectively). The HRAAs shown in this study for <1 kDa and 5-10 kDa peptide fractions could have been due to higher contents of hydrophobic aliphatic (Ala, Leu, Pro, Val and Ile) and hydrophobic aromatic amino acid (Phe, Typ, Try and His) residues in the fractions when compared to those of 1-3 kDa and 3-5 kDa fractions. On a similar note, Aderinola et al. (2018) reported that the <1 kDa fraction of Moringa oleifera seed showed the highest inhibition (67.77%). The values obtained in this study for the membrane fractions are higher than those reported membrane fractions from kidney bean (Mundi, & Aluko, 2014). On the contrary, Mundi, & Aluko (2014) who reported that the HRAAs of kidney bean protein at 3-5 kDa (44%) which is the highest value for the membrane fraction compare to 1-3 kDa (30%). According to Yen, & Hsieh (1995), the HRAAs of bioactive peptide is due to the synergistic interaction between the reducing power, donation of hydrogen atoms and scavenging of active oxygen.

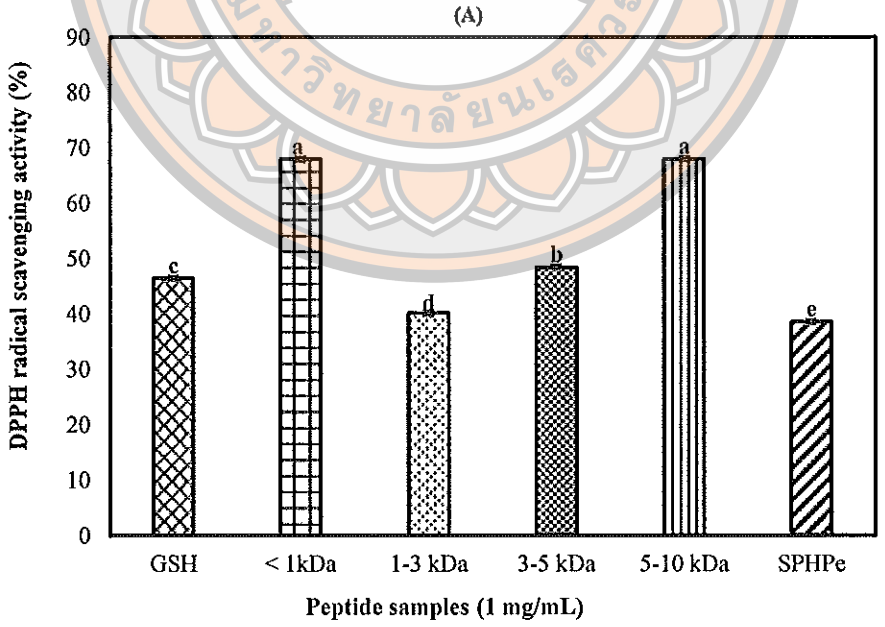


Figure 20 DPPH (A) and HRAAs (B) of SPHPe and peptide fractions (<1,1-3,3-5 and 5-10 kDa) with GSH as a positive control

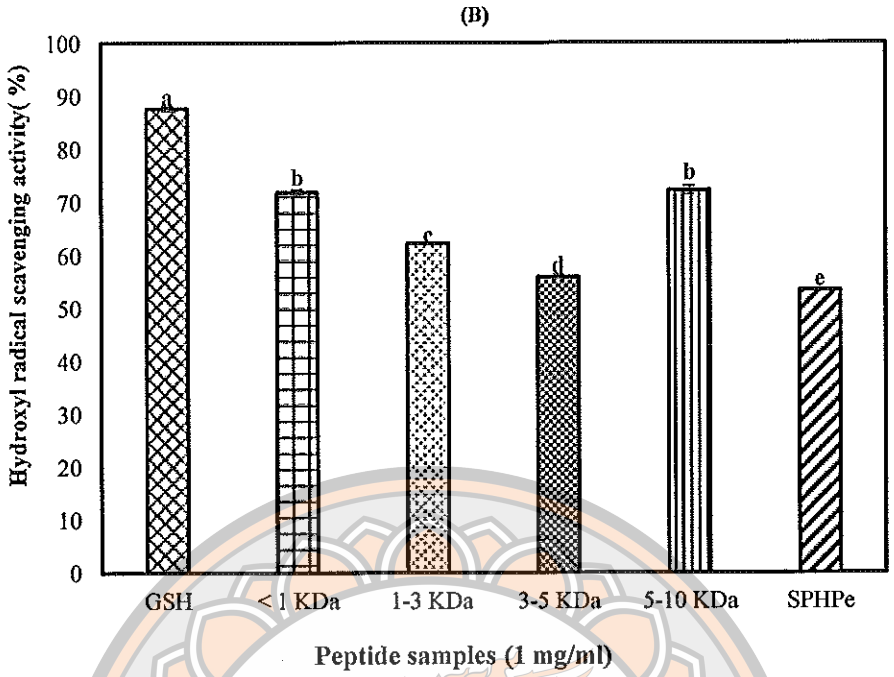


Figure 20 (cont.)

10.6.2 Iron chelating activity and FRAP

Chelating agents may serve as secondary antioxidants because they reduce the redox potential, hence stabilizing the oxidized form of the metal ions. The capacity of the SPHPe and the peptide fractions was assessed for their ability to compete with ferrozine for ferrous ions, resulting in reduced absorbance of the ferrozine-Fe (II) complex as shown in Figure 21A. All the protein samples showed varied levels of iron-chelating activity. The samples exhibited iron-chelating potency to various extents, with SPHPe showing significantly higher ($p<0.05$) chelating activity (24.0%), which could be attributed to the additive effects of all the peptides (Glu, Asp, His) that are responsible for the activities of the hydrolysate. However, the 1-3 kDa and 3-5 kDa fractions showed lower chelating activity ranging from 8.54% to 12.09% compared to the moderate activity exhibited by the <1 kDa fractions and 5-10 kDa, which had 15.33 and 21.70% chelating activity, respectively. This trend is, however, consistent to past work (Mundi, & Aluko, 2014) that reported better activity for the kidney bean protein hydrolysate than membrane fractions due to synergistic effects of the peptides. On a similar note Girgih et al. (2011) who reported the highest iron chelating activity for unfractionated hemp seed hydrolysate, attributing the effect to additive peptide

effects. Peptides with amino acid residues containing phosphorylated hydroxyl side chain groups (Ser and Thr) and carboxyl groups (Glu, & Asp) are good metal ion binders (Mundi, & Rotimi, 2014). Increased content of charged amino acid (Glu) residues was correlated with enhanced metal ion chelating ability of mulberry leaf (Sun et al., 2018).

The FRAP assay is used to evaluate the ability of natural antioxidants to donate electrons. Figure 21B shows the FRAP assay for SPHPe and its membrane fractions. The FRAP of SPHPe and peptide fractions showed different levels of potency in reducing Fe^{3+}/ferric cyanide complex when compared to the GSH standard. An increase in absorbance indicates better reducing power of the test sample. The SPHPe and peptide fractions exhibited low absorbance values of 0.167 to 0.138 mmol Fe^{2+}/g compared to GSH which had highest absorbance value of 0.272 mmol Fe^{2+}/g. This implied that GSH had the highest reducing power when compared to SPHPe and fractionated peptides. The reducing power of the SPHPe was the highest, followed by 5-10 kDa, 3-5 kDa then <1 and 1-3 kDa, respectively (Figure 21B). This is an indication of additive effects of active groups within the peptides. Long-chain peptides will contain more reducing groups than short-chain peptides. The high reducing power of the decapeptide was linked to the terminal Met residue (Harnedy et al., 2017) because sulfur containing amino acids, such as Cys and Met are known to be highly effective in reducing the Fe^{3+} ferricyanide complex (Udenigwe, & Aluko, 2011). The trend showing higher FRAP of the SPHPe and 5-10 kDa fractions was similar Sonklin et al. (2018), the >10 kDa fraction from mungbean protein hydrolysate had higher reducing power when compared to those of the <1 kDa, 1–5 kDa, 5–10 kDa peptide fractions. In summary, protein hydrolysates had better activities compared to the membrane fraction.

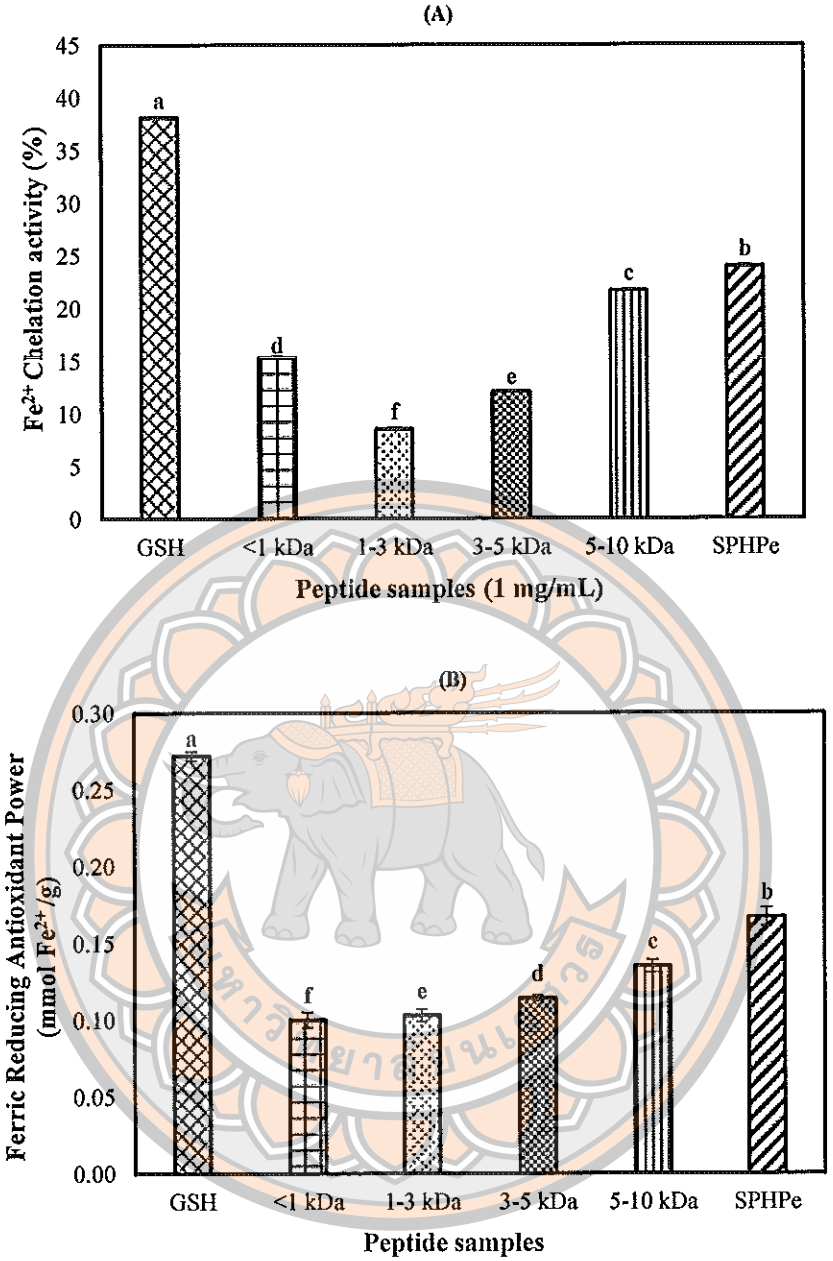


Figure 21 Percentage iron chelation ability (A) and dose dependent FRAP (B) of SPHPe and peptide fractions (<1,1-3,3-5 and 5-10 kDa)

10.6.3 Inhibition of linoleic acid peroxidation

The antioxidative activity of various fractions of SPHPe on the oxidation of linoleic acid is shown in Table 47. In all the samples, especially in the control, the absorbance increased to a maximum and then decreased gradually. The hydrolysates with higher hydrophobic amino acid residues possessed stronger

antioxidant activity because hydrophobicity of the compounds was important for interaction with and protection of the hydrophobic linoleic acid from peroxidation and the results obtained after 7 days of incubation. The 5-10 kDa fractions exhibited the highest inhibitory activity, followed by SPHPe, 3-5 kDa, <1 kDa and then 1-3 kDa. Although, the <1 kDa fraction had the highest hydrophobic amino acid residues but Tyr, Met, His, Lys, and Trp containing peptides which was highest in the 5-10 kDa fraction may have also played key role in the inhibition of linoleic acid oxidation. GSH showed the highest inhibitory activity in the first five days, after which inhibitory activity was similar to the peptide fractions. In all samples, the absorbance increased to a maximum and then decreased gradually. The decrease in absorbance after the 4th day maybe because hydroperoxides are usually unstable and they gradually decomposed into secondary metabolites as the experiment progressed. Hydrophobic amino acid residues such as Tyr, Met, His, Lys, and Trp have been shown to have strong antioxidant activity against lipid derived-radicals due to the ability of hydrophobic amino acids to interact with the lipids (Pownall et al., 2010).

Table 55 Lipid oxidation measured in linoleic acid model system for 7 days in the presence of 1 mg/ml GSH, SPHPe and peptide fractions

Samples	Duration (days)						
	1	2	3	4	5	6	7
Control	0.268 ± 0.15^{a}	0.829 ± 0.22^{a}	1.306 ± 0.05^{a}	1.546 ± 0.54^{a}	1.311 ± 0.13^{a}	0.901 ± 0.14^{a}	0.761 ± 0.36^{a}
GSH	0.059 ± 0.06^{f}	0.061 ± 0.15^{g}	0.060 ± 0.11^{f}	0.060 ± 0.63^{g}	0.059 ± 0.06^{g}	0.061 ± 0.22^{f}	0.063 ± 0.15^{f}
<1 kDa	0.253 ± 0.05^{b}	0.413 ± 0.14^{c}	0.617 ± 0.13^{b}	0.831 ± 0.36^{c}	0.623 ± 0.11^{c}	0.418 ± 0.13^{c}	0.215 ± 0.61^{c}
1-3 kDa	0.259 ± 0.13^{b}	0.474 ± 0.26^{b}	0.602 ± 0.11^{b}	0.879 ± 0.17^{b}	0.681 ± 0.13^{b}	0.465 ± 0.64^{b}	0.309 ± 0.11^{b}
3-5 kDa	0.248 ± 0.11^{c}	0.360 ± 0.34^{d}	0.525 ± 0.36^{c}	0.728 ± 0.17^{d}	0.521 ± 0.18^{d}	0.315 ± 0.06^{d}	0.212 ± 0.33^{c}

Table 54 (cont.)

Samples	Duration (days)						
	1	2	3	4	5	6	7
5-10 kDa	0.217 ± 0.45^{e}	0.328 ± 0.12^{f}	0.450 ± 0.09^{e}	0.661 ± 0.32^{f}	0.416 ± 0.33^{f}	0.210 ± 0.26^{e}	0.110 ± 0.44^{e}
SPHPe	0.238 ± 0.09^{d}	0.338 ± 0.26^{e}	0.478 ± 0.02^{d}	0.682 ± 0.54^{e}	0.477 ± 0.05^{e}	0.213 ± 0.08^{e}	0.171 ± 0.45^{d}

Note: The control contains only linoleic acid and no antioxidant compound. Different lowercase letters in each column indicate significant differences among samples ($p < 0.05$)

10.7 In vitro enzyme inhibition

10.7.1 α-Amylase Inhibition

The α-amylase is one of the major enzymes involved in the digestion of dietary starch, releasing oligosaccharides that can be further broken down into glucose, which is rapidly absorbed by the body. Therefore, α-amylase activity inhibition is regarded as an effective strategy for managing diabetes (Gropper, & Smith, 2012). The results of the α-amylase inhibition activity are shown in Table 47. The inhibitory activity of the SPHPe and fractions were conducted at concentrations of 1, 3 and 6 mg/mL. The concentration at which the samples had the highest inhibitory activity was 6 mg/mL. Inhibitory activity increased with increasing peptide concentration, indicating a dose-dependent effect. The inhibitory activity of the samples against α-amylase was much lower in comparison to the standard acarbose (acarbose = 74.94% at 1 mg/mL). This is not surprising since acarbose is a purified synthetic inhibitor of α-amylase, whereas hydrolysates and peptide fractions are crude mixtures of proteins/peptides and probably nonprotein components. Of all the samples at the highest tested concentration (6 mg/mL), the SPHPe 1-3 kDa fraction had the highest inhibitory activity of 94.13% (IC_{50} = 2.4 mg/mL), while the SPHPe had the inhibitory activity of 57.39% (IC_{50} = 5.25 mg/mL). The inhibitory activities of the <1 kDa (78.23%), 3-5 kDa (74.14 %) and 5-10 kDa (83.29%) fractions had inhibitory activity

within the SPHPe. It can be concluded that pepsin hydrolysate showed effective inhibition rate at an IC_{50} value of 1.86 mg/mL protein as compared to another enzyme hydrolysate. The MW <5 kDa showed a better inhibition rate with an IC_{50} value of 1.18mg/mL (Admassu et al., 2017). This is because, in all the groups, the low MW peptides had better mean inhibitory activity against α- amylase in comparison to the unfractionated hydrolysates. A possible reason for this observation could be that low MW peptides are smaller in size and, therefore, can easily bind to the active site of the enzyme, resulting in a higher inhibitory activity compared to the unfractionated hydrolysates that contained different and larger sizes of peptides. Thus, they are unable to bind tightly to the enzyme (Malomo, & Aluko, 2016b). Another possible explanation could be due to the fact the unfractionated hydrolysates contain peptides, which may have an antagonistic effect (Girgih et al., 2015a). However, when separated by ultrafiltration, there is a loss of the antagonistic effect. In other words, the use of ultrafiltration membrane helped to enrich specific peptide fractions that have potent inhibitory activity against α-amylase (Segura et al., 2013). This is corroborated by some studies which reported that low MW peptides (specifically, < 1 kDa and < 3 kDa) from pinto bean had the highest α-amylase inhibitory activity of 49.9% and 62.1%, respectively (Oseguera-Toledo et al., 2015; Ngoh, & Gan, 2016).

10.7.2 α-Glucosidase Inhibition

The result of the α-glucosidase inhibition activity by protein hydrolysates and fractions is shown in Table 47. The inhibitory activity of the SPHPe and fractions were conducted at concentrations of 1, 3, and 6 mg/mL and the concentration with the highest inhibitory activity obtained was at 6 mg/mL. The inhibitory activities of the samples increased with increasing concentration, indicating a dose-dependent relationship. The inhibitory activity of peptide samples against α-glucosidase was comparable to that of the standard acarbose (acarbose = 67.60% at 0.125 mg/mL). Based on the highest concentration tested (6 mg/mL) for all samples, the <1 kDa fraction had the highest inhibitory activity of 83.35% (IC_{50} =1.83 mg/mL). The 1-3 kDa, 3-5 kDa, 5-10 kDa fractions and SPHPe had inhibitory activity of 70.49%, 70.19%, 57.79% and 50.17%, respectively. The results of previous work have reported α-glucosidase inhibitory activity of 44.7% by yellow pea protein-protein hydrolysates at 1-3 kDa (Admassu et al., 2017). Similar to the α-amylase inhibition

results, the results obtained from the α-glucosidase inhibition indicate that fractionated peptides (<1 kDa, 1-3 kDa and 3-5 kDa), which are of low MW, were more potent in inhibiting α-glucosidase in comparison to the unfractionated hydrolysates. This is due to the fact that the low MW peptides are smaller in size and, therefore, can easily bind to the active site of the enzyme, resulting in a greater inhibitory effect compared to the unfractionated hydrolysates, which contain larger sizes of peptides and are unable to bind tightly to the enzyme (Malomo, & Aluko, 2016b). Moreover, it has been noted that peptides of lower MW tend to be better inhibitors of enzymes when compared to larger-sized peptides (Girgih et al., 2011a). These results are consistent with previous studies that showed low MW peptides are potent inhibitors of α-glucosidase. The <1 kDa peptide fraction derived from pinto Durango bean exhibited an inhibitory activity of 76.4% against α-glucosidase (Oseguera-Toledo et al., 2015); while the <3 kDa peptide fraction from rice bran had an inhibitory activity of 47.9 % against α-glucosidase (Uraipong, & Zhao, 2016a).

10.7.3 Trypsin inhibition

Results of the IC_{50} values for the trypsin inhibition activity of SPHPe and fractions are presented in Table 47. The results showed that the peptide fractions had lower IC_{50} values when compared to the unfractionated hydrolysates. Overall, 5-10 kDa fraction had the lowest IC_{50} value of 2.47 mg/mL, while < 1, 1-3, 3-5 kDa fraction and unfractionated hydrolysates had the IC_{50} value of 4.17, 3.22, 4.04 and 5.93 mg/mL, respectively. The results indicate that fractionation improved the inhibitory capacity of Sacha Inchi protein against trypsin activity. This may be because of the peptide antagonism within the unfractionated hydrolysates, which may have contributed to reduced trypsin inhibitory activity (Girgih et al., 2015a). Ultrafiltration separation led to reduced antagonistic effect in the fractionated peptides, hence the higher inhibitory effects of the peptide fractions. However, in comparison to the standard inhibitor of trypsin, AEBSF (IC_{50} = 0.003 mg/mL), the IC_{50} values of SPHPe and peptide fractions were significantly ($p<0.05$) higher, indicating that AEBSF is a more potent trypsin inhibitor than unfractionated hydrolysates and peptide fractions. The peptide fractions of yellow peas (pepsin-derived hydrolysate) had a lower IC_{50} in comparison to the alcalase-derived peptide fractions (Admassu et al., 2017).

10.7.4 Pancreatic Lipase Inhibition

The results of the IC_{50} values of the pancreatic lipase inhibition activity of SPHPe and fractions are presented in Table 47. The results show that SPHPe had lower IC_{50} values compared to the peptide fractions, meaning that the unfractionated hydrolysates were more potent pancreatic lipase inhibitors when compared to the fractionated peptides (SPHPe: 3.01 mg/ml; <1 kDa: 4.09 mg/mL; 1-3 kDa: 4.17 mg/mL, 3-5 kDa: 4.72 mg/mL and 5-10 kDa: 4.29 mg/mL). In other words, the results suggest that fractionation decreased the inhibitory capacity of Sacha Inchi protein against pancreatic lipase activity. This may be because of the synergistic effect of the peptides contained in the unfractionated hydrolysates. It could be that this effect was reduced or lost during the fractionation process. However, in comparison to the standard inhibitor of pancreatic lipase, Orlistat (IC_{50} = 0.067 mg/mL), the IC_{50} values of SPHPe and peptide fractions were much higher, indicating that Orlistat is a more potent pancreatic lipase inhibitor than the SPHPe and peptide fractions. The body of literature is very scarce on peptides derived from food sources that have inhibitory activity against pancreatic lipase; most of the research done on the inhibition of pancreatic lipase activity focuses on other compounds derived from food such as polyphenols, tannins, isoflavonoids, saponins, and procyanidins (Admassu et al., 2017). A few studies have shown that synthesized peptides via phage display technique can inhibit the activity of pancreatic lipase. For example, a research study synthesized a peptide called peptide D23 via phage display, which showed that this peptide inhibited pancreatic lipase activity (Lunder et al., 2005). The fractionated peptides by pepsin inhibited pancreatic lipase activity IC_{50} value of 4.75 mg/mL (Admassu et al., 2017)

Table 56 In vitro enzyme inhibition of SPHPe and fractions

Sample	α-amylase (IC_{50} mg/mL)	α-glucosidase (IC_{50} mg/mL)	Trypsin Inhibition (IC_{50} mg/mL)	Pancreatic Lipase (IC_{50} mg/mL)
< 1kDa	3.37 ± 0.36^{e}	1.83 ± 0.05^{d}	4.17 ± 0.16^{b}	4.09 ± 0.07^{d}
1-3 kDa	2.40 ± 0.14^{e}	3.12 ± 0.43^{c}	3.22 ± 0.38^{c}	4.17 ± 0.15^{c}
3-5 kDa	4.02 ± 0.38^{b}	3.11 ± 0.28^{c}	4.04 ± 0.12^{b}	4.72 ± 0.43^{a}
5-10 kDa	2.83 ± 0.54^{d}	4.84 ± 0.11^{b}	2.47 ± 0.47^{d}	4.29 ± 0.11^{b}
SPHPe	5.25 ± 0.11^{a}	5.98 ± 0.64^{a}	5.93 ± 0.67^{a}	3.01 ± 0.13^{e}

Note: Results are presented as mean ± SD (n=3). For each column, mean values that contain different letters are significantly different at $p < 0.05$

11. Development of beverage from Sacha Inchi protein hydrolysate

Given the potential health benefits of food protein, there is a growing interest in developing protein-fortified beverages. Particularly, plant-based protein beverage sales in Thailand, according to beverage marketing corporation of Thailand, have grown by more than 2.4 times and will become more entrenched in the mainstream market in the near future (Beverage in Industry, 2019). Sacha Inchi protein peptides derived from the pepsin had highest properties. So, SPHPe was selected for use in protein beverage in three formula; chocolate, coffee, and vanilla (Figure 22). The protein beverages were analyzed for basic physical properties such as proximate composition, total soluble solid, dispersibility, solubility, wettability, and in vitro digestion.

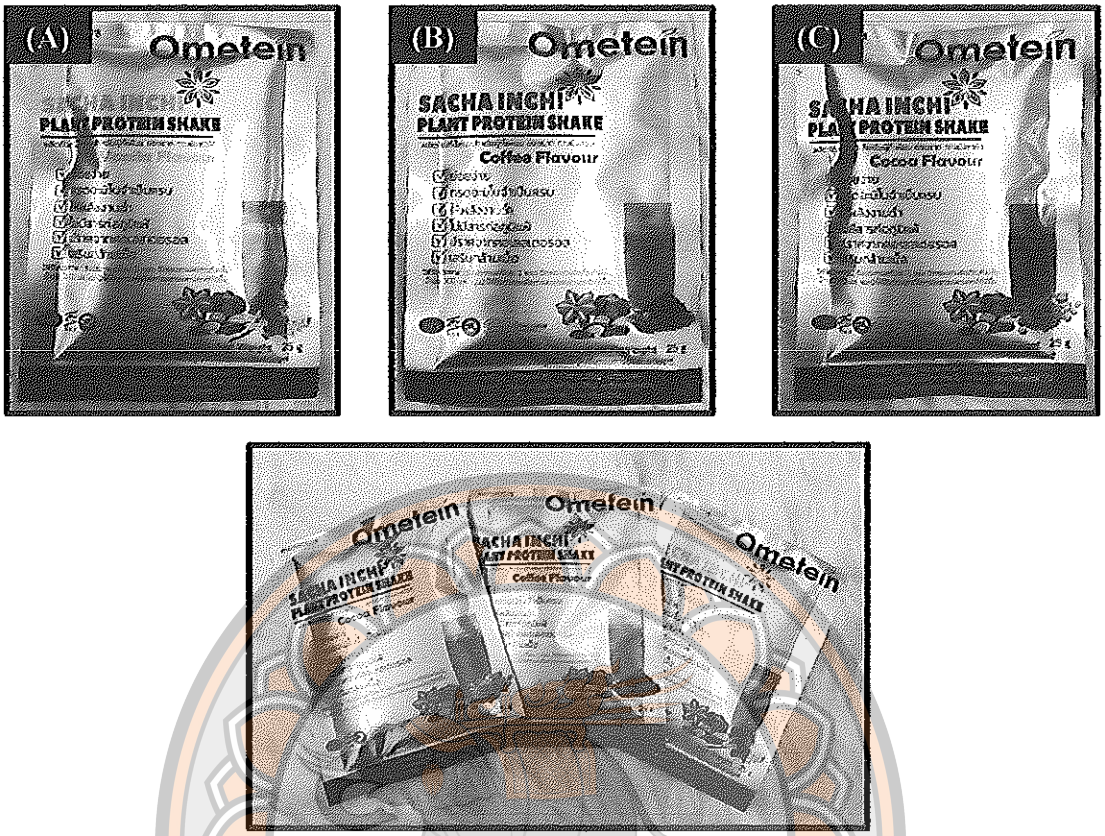


Figure 22 Beverage from Sacha Inchi protein hydrolysate (three formula; vanilla (A), coffee (B), chocolate (C))

11.1 Proximate composition

Proximate composition of the Sacha Inchi protein hydrolysate drink is presented in Table 48. The significantly ($p < 0.05$) low moisture content of protein drink makes it more likely to maintain quality during long-term storage. PC ranged between 10.8 and 11.2% of protein drink, while the content of ash and fiber was non- significant ($p > 0.05$). In 2008, the FDA approved a protein concentration of at least 4.2 % to make a label claim of a "high protein beverage" (FDA, 2008). Commonly, soy protein powder or shake formula, on the other hand, has a relatively high protein content of ~6.5% to 10.4% when formulated into a beverage following label instructions (Lee, 2011). To the best of our knowledge, there is no protein beverage in the Thailand market made with Sacha Inchi protein at greater than 11.2% protein content.

Table 57 Beverage from Sacha Inchi protein hydrolysate

Proximate composition (%)	formula		
	Chocolate	Coffee	Vanilla
Protein	10.93±0.11^{b}	10.80±0.09^{b}	11.23±0.05^{a}
Fat	0.28±0.05^{c}	0.39±0.08^{a}	0.35±0.09^{b}
Ash ^{ns}	1.56±0.11	1.57±0.02	1.57±0.39
Moisture	2.96±0.05^{a}	2.52±0.03^{b}	2.28±0.09^{c}
Fiber ^{ns}	2.33±0.06	2.35±0.11	2.32±0.06
Carbohydrates	81.94±0.12^{b}	82.37±0.09^{a}	82.25±0.11^{a}

Note: Results are presented as mean ± SD (n=3). For each row, mean values that contain different letters are significantly different at $p < 0.05$; ^{ns} not significant at $p > 0.05$

11.2 Total soluble solid, dispersibility, solubility, and wettability

The Sacha Inchi protein hydrolysate drinks had pH about pH 7.78-8.26. The °brix values of the protein drink were adjusted to a range of 10.6 to 11.5 according to the water proportions in the formulations. Fruit juice drinks containing hydrolyzed had °brix values between 10.4-13.5 (Bilek, & Bayram, 2015). Protein drink has properties such as wettability, dispersibility and solubility ranging from 137 to 146 s, 48.85% to 48.87% and 37.22% to 38.57%, respectively (in Table 49). The products were low in solubility and dispersibility due to the protein isolate in the formula being an insoluble, hydrophobic powder, which is hard to disperse in water. However, protein powders and drink are different from ready-to-drink beverages. The dried powder has to be mixed with and dispersed in water, fruit juices, or milk before serving. This is because the proteins are not stable enough to be held in a solution at such high protein percentages. They fall out and precipitate during storage (Lee, 2011). Product attributes in beverages such as wettability, dispersibility, and solubility have often been used to characterize instant powdered food (Picesky, 1986). Dispersibility is the ease with which the powder becomes distributed as a single particle in the bulk liquid phase. During use, products not consumed immediately after reconstitution, i.e. allowed to stay awhile between ingestion, have a tendency to form sediments at the

bottom of the container. This reduces the convenience in use, as it would require some intermittent stirring to ensure uniform mouth feel and probably the taste of the reconstituted food. Reconstituted foods that have low dispersibility often have high sedimentation volume, as both are opposite instant properties pertinent to instant powdered food (Park et al., 2001).

Table 58 Physical properties of beverage from Sacha Inchi protein hydrolysate

Shake formula	Physical Properties					
	pH	°Brix values	Wettability (s)	Solubility (%)	Dispersibility (%) ^{ns}	protein digestion (%)
Chocolate	8.27±0.35^{a}	10.6±0.11^{b}	141±0.01^{b}	37.22±0.12^{c}	48.83±0.03	87.16±0.07^{a}
Coffee	7.78±0.28^{b}	11.5±0.16^{a}	146±0.04^{a}	38.57±0.78^{a}	48.87±0.28	87.56±0.08^{a}
Vanilla	8.26±0.14^{a}	11.0±0.15^{a}	137±0.01^{c}	37.33±0.71^{b}	48.85±0.17	85.69±0.09^{b}

Note: Results are presented as mean ± SD (n=3). For each column, mean values that contain different letters are significantly different at $p < 0.05$; ^{ns} not significant at $p > 0.05$

11.3 In vitro protein digestion

Table 49 summarizes the in vitro protein digestibility for a protein drink (chocolate, coffee and vanilla formula). The coffee and chocolate formula were significantly high protein digestibility ($p<0.05$) in comparison to the vanilla formula. In vitro protein digestions were 87.16%, 87.56% and 85.69% for the chocolate, coffee and vanilla formula, respectively. Digestibility is used to assess the nutritional quality of proteins and is an important food attribute (Tinus et al., 2012). Although in vivo digestibility studies are preferable, in vitro digestibility studies can be used to provide useful data on protein digestibility with less cost, shorter analysis times, greater ease of analysis, and minimal to no ethical concerns. The production of beverages from protein hydrolysates and isolate could be used for biological activity, to maximize the range of potential food products, and boost the commercialization of value-added with the additional incentive of greater digestibility (Clemente, 2000).

CHAPTER V

CONCLUSION

Conclusion

The stabilization process could improve physico-chemical properties and antioxidant activities of SIR. Extrusion process at barrel temperature of 100 °C and in-barrel moisture level of 61.8% possessed highest PC and mostly significant enhancement of functional properties (EC, ES, FC, OBC, SP and WSI). A combination of autoclaving (121 °C for 60 mins) and extrusion (150 °C and moisture level of 61.8%) was the most efficient stabilization process to inhibit microbial growth, antinutrients (phytic acid and tannin), oxidation and longest shelf life of more than 12 weeks at of 4°C to 50 °C storage temperatures. Stabilized Sacha Inchi obtained from combined process was used to substitute semolina in pasta formulations at 0-50% (w/w) levels. Results indicate that utilizing of stabilized Sacha Inchi up to 30% significantly improved the nutritional quality, textural quality, cooking weight values and SI of pasta.

Hydrolysis of Sacha Inchi protein with various proteases (Alcalase, Neutrase, Alcalase combined Neutrase, Pepsin, Papain and Flavourzyme) were used to produce Sacha Inchi protein hydrolysate represented as SPHA, SPHN, SPHAN, SPHPe, SPHPa and SPHFl, respectively. The SPHFl possessed highest emulsion and foaming formation and stability. However, the SPHPe sample possessed highest PY, ACE-inhibitory, DPP-IV inhibitory activities and antioxidant activities, probably due to their higher contents of hydrophobic amino acids. The present study shows that hydrophobic amino acids of the hydrolysates correlated with the antioxidant activity, antihypertensive, antidiabetes of the Sacha Inchi protein hydrolysates obtained using different proteases. The <1 kDa fraction of SPHPe exhibited highest the ability to scavenge DPPH free radical, HRSAs and inhibit peroxidation of linoleic acid, ACE-inhibition, DPP-IV inhibition and α-glucosidase inhibition ability. Beverage products based on Sacha Inchi protein hydrolysate were successfully developed. With the results obtained in this study, it can be concluded that enzymatic proteolysis process of Sacha Inchi protein produced peptides with antioxidative, antihypertensive, DPP-IV

properties and enzyme inhibition which can be utilized in the development of functional foods and nutraceuticals such as beverage product from Sacha Inchi protein hydrolysate with blood pressure lowering ability and the potential to improve blood glucose regulation (antidiabetes).

Recommendations

Food protein-derived hydrolysates and bioactive peptides are produced from natural food sources, which have been regularly consumed for ages by humans without adverse effects, it is vital to conduct safety/toxicity tests prior to the commencement of human clinical trials in order to allay all possible peptide safety concerns.

Such tests would not only be relevant to human clinical trials conducted to test the validity of the beneficial health promoting properties, but also to peptide transport studies, given the reported hemolytic and membranolytic properties linked with certain cell-penetrating peptides.

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