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The production process of tempe protein isolate from germinated soybeans and its potential as an antidiabetic

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Abstract

Physical activity limitations during the COVID-19 pandemic have the potential to be a community-wide risk factor for type 2 diabetic mellitus (DM). Tempe, an indigenous Indonesian food, can be developed into protein isolate for DM treatment. The protein isolates that were used in this study were tempe protein isolate from germinated soybeans (GTPI), tempe protein isolate from non-germinated soybeans (NGTPI), and commercial soybean protein isolate (CSPI) as a control. This study aimed to evaluate the effect of the soybean germination process on the production of tempe protein isolate (TPI) and antidiabetic potency of TPI by assessing amino acid composition, aglycone isoflavone level, and *in vivo* hypoglycaemic assays. The results showed that the production of GTPI requires a higher extraction pH (11.6-12.6) and a lower pI (3.6-4.2) than the manufacture of NGTPI. In *vivo* tests showed that GTPI has a better hypoglycaemic ability than NGTPI and CSPI. The hypoglycaemic ability of GTPI is supported by the higher content of arginine and isoflavone aglycones (daidzein and genistein) in GTPI compared to NGTPI and CSPI. These findings indicated that the germination process might increase bioactive compounds in soybean, which had a strong association with tempe protein isolate's antidiabetic effectiveness.

1. Introduction

COVID-19 is a severe acute respiratory disease caused by the 2019-nCoV or often called SARS-CoV-2 (Singhal, 2020). In 2020, WHO declared that COVID-19 is a pandemic and advised people to stay at home. This regulation limited people's physical activity, which became a potential risk factor for type 2 diabetes mellitus, significantly impacting mortality (Silva *et al.,* 2019; ADA, 2021). Moreover, diabetes mellitus (DM) and blood glucose levels are the independent predictors for mortality and morbidity of COVID patients, which increased glucose levels boost SARS-CoV-2 replication (Muniyappa and Gubbi, 2020; Lim *et al.,* 2021). Otherwise, SARS-CoV-2 was associated with a higher risk of incident diabetes (Wander *et al.,* 2022). COVID-19 survivors had an increased risk and burden of diabetes, as well as an elevated risk of antihyperglycemic therapy (Xie and Al-Aly, 2022). A total of 537 million persons were diagnosed with diabetes in 2021 and expected to rise to 643 million by 2030 and 784 million by 2045. Thus, DM is considered a global problem (IDF, 2021).

Lately, plant protein-based products have been used to prevent and treat DM. Soy protein isolate is a protein-based product that is referred to the most functional soy protein because of its excellent nutritional values, acting as an emulsifier, and enhanced texture quality (Singh *et al.,* 2008). Besides, soy protein isolates consumption reduced glycemia, and consumption for six months significantly reduced fasting blood glucose of DM type 2 patients (Balasasirekha and Chandrasekar, 2012; Mendes *et al.*, 2014). Furthermore, soy isolate consumption on an oral glucose tolerance test resulted in lower peak blood glucose and more robust insulin responses (Konya *et al.,* 2019). Tempe, an indigenous Indonesian protein-based foodstuff, has been highly consumed and extensively researched for its health benefits. Fermentation during tempe production can improve nutritional value and bioactive compounds

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beneficial for health (Astawan *et al*., 2016).

The nutritional value and bioactive components of tempe can be enhanced by the germination process of soybean as the main ingredient. Soybean germination reduced the anti-nutrition compound, increased saponin, estrogenic substance, and phytosterol (Bau *et al.*, 2000; Murugkar, 2014). In addition, isoflavone content increased over the first two days of germination, and soluble protein content increased until 72 hrs of germination time (Mora-Escobedo *et al.*, 2009; Paucar-Menacho *et al.*, 2009). The fat and carbohydrate level of tempe prepared from germinated soybeans was higher, but the protein amount was lower (Kadar *et al.*, 2020). Moreover, tempe flour made from germinated soybean had more excellent antioxidant activity than non-germinated one (Astawan *et al.,* 2020).

Three types of protein isolates were used in this study: tempe protein isolate from germinated soybeans (GTPI), tempe protein isolate from non-germinated soybeans (NGTPI), and comparative, commercial soybean protein isolate (CSPI). The objectives of this research were to determine the impact of the soybean germination process on protein extraction pH and precipitation pH (pI) in the production of tempe protein isolate (TPI) and assess TPI's potential as an antidiabetic by measuring aglycone isoflavone levels, amino acid composition, and *in vivo* hypoglycemic assays.

2. Materials and methods

2.1 Materials

The primary materials utilized for TPI production were local Grobogan soybean variety (Grobogan, Central Java, Indonesia), commercial tempe culture starter (Raprima, PT. Aneka Fermentasi Industri, Bandung, Indonesia) obtained from KOPTI, Bogor, Indonesia, hexane, distilled water, NaOH, and HCl. Commercial soybean protein isolate (CSPI) was utilized as a comparative.

2.2 Tempe protein isolate production

The steps of TPI production were germinated soybean preparation, tempe production, tempe flour defatting, and tempe protein isolation. Germinated soybean preparation was adopted from Astawan *et al*. (2020). Any dirt or impurities from soybeans were removed through the sortation and rinsing process. The sorted soybeans were then soaked for 4 hrs in water. The soybeans were then incubated in dark conditions and watered every 4 hrs. The incubation was done until the radicle was 2.5-5.0 mm long.

The tempe production steps was according to Astawan *et al.* (2020). Non-germinated soybeans were steeped for 4 hrs, while germinated soybeans were directly boiled at 100^{o}C for 30 mins. The soybeans were then immersed in the previously heated water for 30 hrs for lactic acid bacteria fermentation, which resulted in a pH drop into 4.0-5.0. After the lactic acid fermentation, the soybeans were dehulled and rinsed with 100^{o}C hot water. Because of the bitter taste, the hilum was removed from non-germinated soybeans. Then, the soybeans were drained and allowed to cool until they reached room temperature. The cooled soybeans were inoculated with a 0.2% (w/w) commercial tempe culture starter and incubated for 40 hrs at 28-30^{o}C and 80% humidity.

Soybean tempe, both germinated and non-germinated, were sliced into 5 mm thick slices and blanched for 2 mins with steam. Tempe was then dried at 60°C for 8 hrs before being pulverized in a disc mill with a 60-mesh sieve. The defatting procedure was conducted twice with hexane as the solvent in a 1:3 (w/v) ratio. The hexane and tempe flour were mixed at room temperature for 2 hrs before being separated. The cakes were dried for 2 hrs at 50°C in a drying oven and sieved using a 60-mesh sieve (Puteri *et al.,* 2018; Astawan *et al.,* 2020).

Defatted tempe flour was then used for protein isolation. According to Astawan *et al.* (2020), the protein isolation was made through two main steps. The first step was protein extraction, and the latter was protein precipitation. Protein extraction was performed at the optimal extraction pH and protein precipitation was done at the pI. First, defatted tempe flour was dissolved in distilled water at a ratio of 1:10 (w / v). Then, sodium hydroxide solution (2M) was added to the optimum pH for extraction. The extraction process was carried out for 2 hrs. The mixture was then centrifuged at 3000×*g* for 10 mins at 4^{o}C. The supernatant obtained was then precipitated by hydrochloric acid solution (2M) until it reached the pI. After that, the precipitation process was conducted for 2 hrs, followed by centrifugation at 3000×*g* for 10 mins at 4^{o}C. The supernatant was discarded, and the precipitate was washed twice with distilled water in a 1:1 (w/v) ratio. The precipitate was then added by sodium hydroxide solution (2M) for the neutralization process, then freeze-dried (Lyovapor L200, Büchi, Switzerland) at -40^{o}C. The tempe protein isolate was then used for further analysis.

2.3 Assessment of tempe protein isolate potential as antidiabetic

Tempe protein isolate potential as an antidiabetic was analysed by measuring amino acid composition, aglycone isoflavone levels, and *in vivo* hypoglycemic assays. Free and total amino acids analyses were conducted using HPLC and the o-phthalaldehyde (OPA)

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method. For the total amino acids, protein isolate was reacted with hydrochloric acid first, while free amino acid analysis used distilled water. As much as 5 mL of sample was injected into RP-HPLC with a fluorescence detector (Shimadzu CT-20) with Hypersil ™ ODS-2 C-18 4.0×125 mm column (Thermo Scientific, USA). The excitation and emission wavelengths of the fluorescence detector were adjusted to 350 nm and 450 nm, respectively.

Aglycone isoflavone analysis followed the research of Kim *et al.* (2014). As much as 2 g of each protein isolate was extracted for 2 hrs using 2 mL of hydrochloric acid solution (0.1 N) and 10 mL of acetonitrile. The extract was filtered using Whatman paper (No. 42) and evaporated using a rotary evaporator at 40°C. The sample was then added with 10 mL of methanol solution (80%) and filtered again using a nylon membrane. Finally, 20 µL of sample was injected into HPLC with a UV detector (Agilent 1200 Series, Agilent Technologies, USA) with C-18 5 mm × 10 cm column (LiChrospher, Merck Millipore, USA). The UV detector was set at 265 nm.

In vivo hypoglycemic assay for protein isolate was performed using the oral glucose tolerance test (OGTT). The approach described by Sikarwar and Patil (2010) was used in this investigation. This study complied with the European Union guidelines for animal care and protection and was approved by the Ethics Committee of the Bogor Agricultural University (Bogor, Indonesia) with approval number 146-2019 IPB.

A total of twenty-five male *Sprague* Dawley rats (obtained from the National Agency of Drug and Food Control, Jakarta, Indonesia) were individually caged and acclimated for seven days (22-24°C, 50-60% humidity) and allowed free access to food and water *ad libitum* (Astawan *et al*., 1994). Casein served as the rat's protein source. Normal and healthy rats were fasted for 16 hrs and divided into five groups for the OGTT (Table 1). First, 900 mg/kg BW protein isolate solution was orally administered, followed by 2 g/kg BW glucose solution after 30 min. Then, using a glucometer (GlucoDr, Allmedicus Co. Ltd, Korea), blood glucose was measured after 30, 60, 90, and 120 mins of glucose solution administration.

2.4 Data analysis

To establish the significance of the treatments, the analysis of variance (ANOVA) at a 95% significance level was done using SPSS 24 (IBM® SPSS Statistics). In addition, Duncan's Multiple Range Test (DMRT) was used to evaluate whether treatments significantly differed at a 95% significance level among the treatments with significant findings.

3. Results and discussion

3.1 The production of tempe protein isolate

This study used alkaline extraction and protein precipitation on isoelectric pH or pI for protein extraction. The pI is where all protein net charges are zero, implying that protein solubility is minimal (Garba and Kaur, 2014; Lee, 2017). According to Vilg and Undeland (2017), soluble protein has a negative net charge, which causes substantial repulsion and dilutes the protein during alkaline extraction. The extraction pH and pI for isolation protein of tempe are shown in Table 2. Germinated tempe protein isolate (GTPI) and non-germinated tempe protein isolate (NGTPI) have different extraction protein pH ranges and pI than commercial soy protein isolate (CSPI). CSPI is commonly made at 8-9 pH conditions and pI of 4.5. This finding indicated that seed germination and tempe fermentation could affect the extraction pH and pI while producing TPI.

Table 2. Extraction pH and pI range of tempe protein isolation

Tempe protein isolate	Maximum protein extraction pH range	pI range
NGTPI	11.2-12.2	4.0-4.6
GTPI	11.6-12.6	3.6-4.2

NGTPI: non-germinated tempe protein isolate, GTPI: germinated tempe protein isolate.

The extraction pH and pI swift of GTPI and NGTPI from the condition of production CSPI was suggested because of the new protein sub-unit that was produced during the germination and fermentation process. The net charges of the new protein subunit were different, which could affect protein extraction and precipitation (Cao *et al.,* 2014). Dobhal and Raghuvanshi (2018) reported that

Table 1. *Sprague Dawley* rats grouping for OGTT

Group of treatment	Number of rats	Consumption on minutes	
		0	30
NC	5	Water	Water
PC	5	Water	Glucose
CSPI	5	900 mg/kg BB commercial soy protein isolate	Glucose
NGTPI	5	900 mg/kg BB non-germinated tempe protein isolate	Glucose
GTPI	5	900 mg/kg BB germinated tempe protein isolate	Glucose

NC: negative control, PC: positive control, CSPI: commercial soy protein isolate, NGTPI: non-germinated tempe protein isolate, GTPI: germinated tempe protein isolate.

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germinated black soybean had higher solubility than non-germinated ones. Germination enhanced proteolytic enzyme activity to hydrolyze reserved protein into the shorter protein chain that is more soluble (Nnadozie *et al.*, 2015).

3.2 Total and free amino acids of tempe protein isolate

Total amino acids of GTPI were higher than NGTPI and CSPI (Table 3). During germination, hydrolytic enzyme activity degrades reserved carbohydrate, fat, and protein for amino acid synthesis used for growth (Joshi and Varma, 2016). Apart from aspartic acid, leucine, arginine, and lysine, glutamic acid was the most abundant amino acid in CSPI, NGTPI, and GTPI. A similar result was reported by Mukherjee *et al.* (2016). Glutamic acid was the highest amino acid found in soybean meals. Peptides containing glutamic acid residue showed anti-diabetic benefits (González-Montoya *et al.*, 2018; Tamam *et al.*, 2019)

Alanyl glutamate (MW = 219.10 g/mol) and glutamyl proline (MW = 245.11 g/mol) found on tempe had the inhibitory activity of dipeptidyl peptidase-IV (DPP-IV). DPP-IV inhibitory activity was discovered in alanyl glutamate (MW = 219.10 g/mol) and glutamyl proline (MW = 245.11 g/mol) detected on tempe (Tamam *et al.,* 2019). DPP-IV is a cell surface enzyme that can inactivate the glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide (GLP). Both GIP and GLP affect insulin secretion and postprandial glucagon (Almagthali, 2019).

Alanine, arginine, phenylalanine, isoleucine, leucine, and lysine are insulinotropic amino acids, which improve insulin secretion (Kanetro *et al*., 2018). Arginine was 7.8-8.2% of total amino acids on GTPI, NGTPI, and CSPI. Aside from its role as insulinotropic amino acid, arginine is considered hypoglycaemic amino acid because of its ability to produce nitrite oxides. Nitrite oxides induce insulin secretion by increasing glucose transport, reducing glucose synthesis, and increasing pancreatic islet blood flow (Utari *et al.,* 2011; Nyström *et al*., 2012).

The free amino acids glycine, alanine, arginine, histidine, lysine, and serine were identified in this investigation; however, their amounts were minimal (Table 3). This finding indicated that small peptides, rather than free amino acids, were produced throughout the germination and fermentation process. Storage proteins are hydrolysed, and vacuoles are merged during seed imbibition and germination. Peptide transporters (PTRs) transport free amino acids and oligopeptides to the cytosol when proteins are hydrolysed. PTRs cotransport protons (H+) and a wide range of nitrogen (N)-containing substrates, such as nitrate, amino acids, and di- and tripeptides (Choi *et al*., 2020). Peptide transport plays an essential role in protein mobilization, such as N redistribution, protein deposition during seed development, and storage protein hydrolysis during germination. The efficiency of N transport may be increased by direct uptake of peptides (Miranda *et al.,* 2003).

Table 3. Amino acid composition of protein isolate

Amino acid	mg amino acid/g protein isolate			mg free amino acid/g protein isolate			pI*
	CSPI	NGTPI	GTPI	CSPI	NGTPI	GTPI	
Glycine	27.62	25.64	26.67	0.71	0.79	0.93	5.97
L-Alanine	29.74	29.04	30.32	n.d.	1.43	1.31	6.00
L-Arginine	53.49	51.56	53.50	0.64	n.d.	n.d.	10.76
L-Aspartic acid	79.44	83.00	83.36	n.d.	n.d.	n.d.	2.77
L-Glutamic acid	140.64	135.21	138.29	n.d.	n.d.	n.d.	3.22
L-Phenylalanine	38.65	42.16	42.64	n.d.	n.d.	n.d.	5.48
L-Histidine	18.25	16.16	17.04	0.88	0.90	0.76	7.59
L-Isoleucine	32.06	35.15	35.31	n.d.	n.d.	n.d.	6.02
L-Leucine	55.69	58.77	59.03	n.d.	n.d.	n.d.	5.98
L-Lysine	45.97	44.03	42.00	3.18	2.09	2.47	9.74
L-Methionine	9.35	9.70	9.90	n.d.	n.d.	n.d.	5.74
L-Serine	35.37	35.65	37.09	1.01	1.39	1.14	5.68
L-Threonine	27.54	25.92	27.79	n.d.	n.d.	n.d.	5.60
L-Tyrosine	26.24	26.58	26.82	n.d.	n.d.	n.d.	5.66
L-Valine	34.39	34.91	35.09	n.d.	n.d.	n.d.	5.96
Total	654.44	653.49	664.84	6.42	6.60	6.60	

n.d.: not detected, indicate result was lower than the instrument detection limit, CSPI: commercial soy protein isolate, NGTPI: non-germinated tempe protein isolate, GTPI: germinated tempe protein isolate.
*cited from Haynes (2017)

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3.3 Aglycone isoflavone content of tempe protein isolate

Soybeans and soybean derivatives are well known for their high isoflavone content. Aglycones, glycosides, acetylglycosides, and malonylglycosides are several types of isoflavones present in soybeans (Wang *et al*., 2013). Isoflavone glycosides are the most common form of isoflavone found in plant-based foods. According to Nurrahman (2015), the primary components of isoflavones that can act as phytoestrogens and antioxidants are genistein and daidzein (aglycone isoflavone). The amounts of daidzein and genistein in different protein isolates were significantly different ($p<0.05$) (Table 4).

GTPI had the highest quantities of daidzein and genistein, which were significantly different ($p<0.05$) compared to other protein isolates (Table 4). Compared to other samples, CSPI contained the lowest concentrations of daidzein and genistein. It may suggest that isoflavone found in CSPI was daidzin and genistin, which both are glycosides. The fermentation and germination processes in GTPI production result in a high amount of isoflavones. In tempe fermentation, the activity of glucosidase and glucuronidase enzymes can hydrolyse phenolic chemicals still bonded in the form of glycosides into aglycones. The body will more easily metabolize antioxidant aglycone isoflavones (Yang *et al.,* 2011; Bavia *et al.,* 2012).

Table 4. Aglycone isoflavone of protein isolate

Protein isolate	Daidzein content (mg/g)	Genistein content (mg/g)	Aglycone isoflavone total (mg/g)
CSPI	11.16±3.50[a]	40.28±2.71[a]	51.44±0.79[a]
NGTPI	345.48±6.97[b]	329.71±1.66[b]	675.19±5.30[b]
GTPI	384.66±16.15[c]	373.62±7.45[c]	758.28±8.70[c]

Values are presented as mean±SD. Values with different superscripts within the same column are statistically significant different ($p<0.05$) using the Duncan's Multiple Range Test (DMRT). CSPI: commercial soy protein isolate, NGTPI: non-germinated tempe protein isolate, GTPI: germinated tempe protein isolate.

As a self-defence mechanism, germination can create oxidative stress in plants, forcing them to produce more phenolic chemicals. Most phenolic compounds in plants form complexes with plant cell wall fibres. The germination process can cause the links between phenolic chemicals and fibre to break down, resulting in free phenolic compounds (Mahmoud *et al.,* 2016; Gunenc *et al.,* 2017). Isoflavone metabolism begins with hydrolysis using the beta-glucosidase enzyme produced by the small intestine or intestinal microflora (Alkhalidy *et al.,* 2018). The flavonoid component forms conjugates with glucuronic acid in the small intestine, which then conjugates with sulphate or methyl in the liver. The intestinal bacteria subsequently hydrolyse the flavonoid conjugates to produce phenolic acids, which the body can easily absorb. Isoflavone aglycones can boost pancreatic beta-cell survival while inhibiting alpha-amylase and alpha-glucosidase enzyme activity (Mahmoud *et al.,* 2016).

3.4 In vivo hypoglycaemic assay for protein isolate

The ability of the body to stabilize blood glucose after 2 hrs of glucose consumption is measured by the OGTT. The blood glucose levels of rats given GTPI had steady drops and were close to those of negative control (NC) rats (Figure 1). This finding suggests that GTPI can suppress glucose absorption in the intestine to maintain blood glucose levels. Table 5 shows the changes in blood glucose from 30 to 150 mins of glucose consumption in

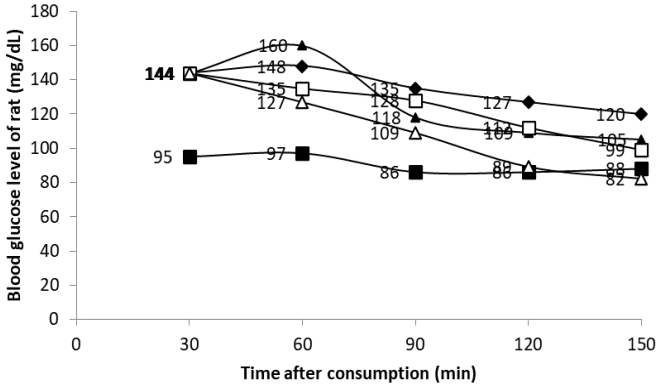

Figure 1. Blood glucose lever during OGTT test. NC: negative control rat, PC: positive control rat, CSPI: rat that consumed 900 mg/kg BW commercial soy protein isolate, NGTPI: rat that consumed 900 mg/kg BW non-germinated tempe protein isolate, GTPI: rat that consumed 900 mg/kg BW germinated tempe protein isolate.

Table 5. Blood glucose level change after 2 hrs of glucose/ water administration at OGTT

Group of treatment	Blood glucose change (mg/dL)
NC	6±4[a]
PC	24±4[b]
CSPI	39±10[c]
NGTPI	45±11[c]
GTPI	62±16[d]

Values are presented as mean±SD. Values with different superscripts within the same column are statistically significant different ($p<0.05$) using the Duncan's Multiple Range Test (DMRT). NC: negative control rat, PC: positive control rat, CSPI: rat that consumed 900 mg/kg BW commercial soy protein isolate, NGTPI: rat that consumed 900 mg/kg BW non-germinated tempe protein isolate, GTPI: rat that consumed 900 mg/kg BW germinated tempe protein isolate.

the OGTT test. Again, GTPI had the most significant difference ($p<0.05$) in blood glucose change levels compared to other protein isolates.

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The increased amounts of isoflavones and insulinotropic amino acids in GTPI compared to CSPI and NGTPI are assumed to be responsible for its antidiabetic properties. The amount of genistein and daidzein GTPI was 758.28±23.61 mg/g, which was greater than NGTPI (675.19±8.63 mg/g) and CSPI (51.44±6.21 mg/g). Genistein is a tyrosine kinase enzyme inhibitor that can stimulate insulin secretion (Gilbert and Liu, 2013). In addition, according to Fu *et al.* (2010), genistein stimulates beta-cell proliferation via PKA and ERK 1/2 pathways. Thus, both systems are able to induce cell growth in the Langerhans islets. Daidzein can also activate GLUT4 via stimulating AMPK phosphorylation, which causes glucose uptake from the blood into the cells. Daidzein metabolism can also produce equol, regulating gluconeogenesis and glycogenesis (Das *et al.,* 2018).

GTPI contains more insulinotropic amino acids than CSPI and NGTPI. Total insulinotropic amino acids of GTPI, NGTPI, and CSPI were respectively 262.79, 260.72, and 255.60 mg/g protein isolate. Leucine, isoleucine, arginine, alanine, and phenylalanine exhibit intense insulinotropic action on pancreatic beta cells. Insulinotropic amino acids can depolarize the plasma membrane of pancreatic beta cells, causing the Ca^{2+} pump to activate and insulin exocytosis to occur. Furthermore, decarboxylation and allosteric stimulation of glutamate dehydrogenase by leucine can trigger insulin secretion (Manders *et al.,* 2012). Increased insulin secretion will boost glucose transport from the circulation to the cells.

Insulinotropic amino acids correlate with glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP). The protein GLP-1 can bind to the GLP-1R receptor, which is strongly expressed in pancreatic beta cells. The ligand formed between GLP-1 and its receptor can activate adenylyl cyclase to create cAMP. cAMP is a second messenger that can provide a signal for insulin synthesis. GLP-1 can also decrease stomach emptying and curb appetite by increasing insulin gene transcription, insulin production, and inhibiting gastric acid release. GLP-1 also suppresses glucagon secretion, allowing blood glucose levels to remain stable. GIP is a peptide that binds to the GIP-receptor, also expressed by beta cells in the pancreas. When GIP binds to its receptor, it raises intracellular cAMP levels, activating protein kinase A. (PKA). PKA can promote insulin exocytosis (Seino *et al.,* 2010; Cho *et al.*, 2014; Tengholm and Gylfe, 2017).

4. Conclusion

Germination of soybean on TPI production can alter the protein's maximum extraction pH and pI. GTPI had more total, and free amino acids than the other treatments and superior *in vivo* hypoglycemic capacity and was closest to the negative control. Compared to NGTPI and CSPI, GTPI has the best antidiabetic potential and delivers a better and considerably further reduction in blood glucose levels than other protein isolates.

Conflict of interest

The authors declare no conflict of interest.

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