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Antioxidant activity and isoflavone content of overripe Indonesian tempe

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Abstract

The germination process and prolonged fermentation have been shown to improve tempe quality. The present study aimed to measure and compare the changes in nutritional and isoflavone content and antioxidant activity of tempe flour due to soybean germination and fermentation time treatments. Soybeans were germinated for 24 hrs before being fermented into fresh and overripe tempe for 2 and 7 days, respectively. Fresh and overripe tempe were dried using a cabinet dryer at 60°C for 8 hrs, then ground and sifted to produce flour. The nutritional contents were determined through proximate analysis, antioxidant activity was measured using the DPPH method, and isoflavone was measured using an HPLC instrument. The germination process and fermentation time were shown not to affect the nutritional contents but affect isoflavone content and IC_{50} value significantly. Overripe tempe flour made from germinated soybeans had the highest daidzein and genistein isoflavone content, at 343 and 701 μg/g, respectively. However, overripe tempe flour made from non-germinated soybeans has the highest antioxidant capacity, with an IC_{50} of 1375 ppm. This result showed that to reap the benefits of high antioxidants, tempe fermentation may be prolonged to produce overripe tempe without any requirement to germinate the soybeans beforehand.

1. Introduction

Tempe is a soybean-based traditional food that is fermented by *Rhizopus* spp. mould. Every 100 g of tempe contains 20.8 g protein, 8.8 g fat, and 1.4 g crude fibre. In addition, every 100 g of tempe contains vitamins and minerals such as 0.9 mg vitamin B1, 34 μg carotene, 155 mg calcium, 326 mg phosphorus, and 4 mg iron (Astawan, Wresdiyati, Subarna and Asyaifullah, 2020). Extended fermentation has changed the protein, fibre, vitamin, and total free amino acid contents compared to fresh tempe (Puspitasari *et al.,* 2020). The production of enzymes by the *Rhizopus* spp. mould increases during fermentation, the potential for hydrolysis of protein into simple peptides and amino acids will increase (Kadar *et al.,* 2018; Astawan, Wresdiyati, Subarna *et al.,* 2020).

Another method to increase the nutritional content of tempe is soybean germination. Germination is an applicative, relatively affordable, accessible technology and can improve the nutritional quality of tempe (Astawan, Wresdiyati, Subarna *et al.,* 2020b). The soybean germination process can encourage the activity of the enzyme protease that plays a role in protein hydrolysis and can therefore increase the production of bioactive peptides (Astawan, Rahmawati, Cahyani *et al.,* 2020). It also increases the change in carbohydrate content, fat, protein, water, ash, and minerals (González-Montoya *et al.,* 2018), increase the change in antioxidant activity and isoflavone profile (Astawan *et al.,* 2016) and increase the vitamin E content (Huang *et al.,* 2014).

Overripe tempe is fresh tempe that has undergone an extension of fermentation time for five days. Fresh tempe is obtained from the fermentation of soybeans by *Rhizopus* spp. mould (Astawan *et al.,* 2016), while in overripe tempe the fermentation process is continued by bacteria. Overripe tempe has a pungent aroma due to fatty acid hydrolysis, has a greyish color, and is less attractive than fresh tempe in appearance (Shi *et al.,* 2010). Overripe tempe is generally consumed by the people of Central and East Java Indonesia, either as a

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side dish or as a condiment in several dishes (Utami *et al.,* 2016). Meanwhile, overripe tempe is suggested to have potential as a source of isoflavones and other bioactive components.

Isoflavones are secondary metabolites with 12 types that can be classified into aglycone, glycoside, acetyl-glucoside, and malonyl-glucoside groups. The soybeans fermentation process in the production of tempe triggers the hydrolysis of isoflavone glycosides into the highly active physiological aglycone forms such as daidzein, genistein, and glycitein (Andriani *et al.,* 2014). Past studies have shown that the soybean germination process can increase the isoflavone aglycones in soybeans by 84%. The extended fermentation time can increase the isoflavone content by 71.4±8.5 mg/g (Sulistiani *et al.,* 2014; Astawan *et al.,* 2016).

The main physiological activity of isoflavones is as an antioxidant. Antioxidants can be defined as compounds that can prevent and alleviate the adverse effects of free radicals. Free radicals are reactive compounds that can oxidize biomolecules, causing mutagenic changes, tissue damage, and cell mortality. Antioxidant compounds operate by donating electrons to the radical compounds and thus inhibiting the compounds' activity (Astawan *et al.,* 2012; Riyanto and Hartati, 2017). The balance between oxidants and antioxidants is essential because it is related to the function of the immune system. The role of isoflavones in tempe could inhibit insulin resistance in diabetes mellitus type 2 patients, improve the systolic blood pressure, decrease oxidative stress, and prevent hypertension (Hidayat and Wiboworini, 2021; Kurian *et al.,* 2022).

Studies related to the nutritional content and functional properties of overripe tempe are still rare. The present study was aimed to measure and compare the nutritional content, antioxidant activity, and isoflavone content of fresh tempe and overripe tempe flour made from germinated and non-germinated soybeans. In its application as a functional food, this effort is expected to positively contribute to improving the antioxidant activity and isoflavone content of tempe.

2. Materials and methods

This study had an experimental design with two phases. The first phase included producing germinated soybeans, fresh tempe, and overripe tempe from germinated and non-germinated soybeans and making flour from these materials. The second phase included the analyses of nutritional content, antioxidant activity, the aglycone isoflavones daidzein and genistein contents.

2.1 Materials

The main ingredient in producing fresh tempe and overripe tempe was non-GMO soybeans obtained from the Indonesian Tempe and Tofu Producer Cooperation (KOPTI) in Bogor. Other materials used in this study were tempe yeast (RAPRIMA, LIPI Bandung), polypropylene plastic wrapping, a genistein standard (HPLC suitable assay > 97%, Sigma Aldrich, USA), DPPH reactant (2,2-diphenyl-1-picrylhydrazyl) (Sigma Aldrich, USA), L-ascorbic acid 99% (Sigma Aldrich, USA) and other chemicals.

2.2 Production of germinated soybeans

Soybeans were sorted, then soaked for three hrs in water, and drained. Then the soybeans were doused with clean water every 3 hrs. The production of germinated soybeans was conducted without light for 24 hrs (Hashim *et al.,* 2018).

2.3 Production of overripe tempe

The process of making fresh and overripe tempe from germinated and non-germinated soybeans has been carried out according to Indonesian custom. The steps in making the overripe tempe began with making fresh tempe using the non-GMO soybeans referring to the production process employed by the Rumah Tempe Indonesia, Bogor. The length of fermentation for fresh tempe was two days (48 hrs) at room temperature (approximately 28–30^{o}C). In order to produce overripe tempe, the fresh tempe was subjected to an extended fermentation time of five days (120 hrs) at room temperature (approximately 28–30^{o}C).

2.4 Production of tempe flour

The production of tempe flour started with slicing the tempe using a slicer to make tempe slices approximately 0.5 cm thick. The sliced tempe was then blanched with steam for two minutes. The blanched slices of tempe were then dried in a cabinet dryer at a temperature of 60^{o}C for 8 hrs. The dried tempe slices were then ground using a disc mill and sifted using an 80 mesh sieve (Astawan, Rahmawati, Cahyani *et al.,* 2020).

2.5 Yield and nutritional content analysis

The overripe tempe flour yield from germinated and non-germinated soybeans was analysed by comparing the weight of the flour obtained with the overripe tempe used then multiplied by 100%. The nutritional content analysis included moisture, ash, protein, fat, crude fibre, and carbohydrate content referred to AOAC (2012) method.

2.5.1 Determination of moisture content

The gravimetric method was used to estimate moisture content (AOAC, 2012). The samples (1-2 g) were dried in an air oven at 105°C for hrs. The dish was then weighed after cooling in a desicator.

2.5.2 Determination of ash content

The ash content was determined using gravimetric method by measuring the weight of the sample before and after ashing in the furnace at 550°C (AOAC, 2012).

2.5.3 Determination of protein content

Kjeldahl method was used to determine the protein content, which was then calculated by multiplying the nitrogen content by 6.25 (AOAC, 2012).

2.5.4 Determination of fat content

Fat content was evaluated by using Soxhlet extraction method (AOAC, 2012). Fat content was determined by weight loss of the sample or the weight of the fat removed.

2.5.5 Determination of crude fibre

Crude fibre was estimated by using acid and alkaline hydolysis method (AOAC, 2012). Crude fibre was determined gravimetrically as the loss of the dried residue remaining after digestion of the sample with 1.25% H_2SO_4 and 1.25% NaOH solutions under specific conditions.

2.5.6 Determination of carbohydrate

The carbohydrate content was determined by substracting the differential weights of all components. The value was calculated by deducting the percentages of all other components, including moisture, protein, fat, and ash.

2.6 Sample extraction for antioxidant activity analysis

The flour samples weighing 50 g were extracted using 250 mL methanol solvent (1:5 w/v). The extraction process was conducted in a shaker for three hrs. The extraction results were then filtered using Whatman's No. 42 filter paper. The flour extract was collected and thickened in a rotary evaporator (AOAC, 2012).

2.7 Antioxidant activity

Analysis of the overripe tempe antioxidant activity was conducted using the DPPH method. This analysis was aimed to determine the antioxidant compound inhibition effect in the samples against the DPPH (2,2-diphenyl-1-picrylhydrazyl) free radicals by observing the change in color of each sample after incubation. The free electrons in DPPH will bind with the electrons in the extract samples and result in a change of color in the samples from dark purple to light yellow. An amount of 100 µL of the solutions of the sample in various concentrations was placed in a test tube, then 400 µL of DPPH solution was added. The solution was then incubated in the dark for 30 mins at room temperature then the absorbance was measured using a spectrophotometer at a 517 nm wavelength. Ascorbic acid was used as the standard (Soetjipto *et al.,* 2018).

2.8 Isoflavone content analysis

Analysis of the total isoflavone began with weighing 2 g of each sample then adding 30 mL of HCl : acetonitrile (1 : 4) solution. After that, the samples were incubated in a water bath shaker for one hr. The samples were then filtered and moved quantitatively to a volumetric flask. An amount of 20 µL of the filtrate was injected into the HPLC with a C-18 reverse phase 15 cm × 4.6 mm i.d., 5 µm column (LiChrospher, Merck Millipore, USA). The moving phase used was methanol: ammonium acetate 1 mM with a ratio of 6:4 with a rate of 1 mL/minute. The detector used was a UV Detector at a wavelength of 265 nm (Puteri *et al.,* 2018).

2.9 Data processing and analysis

This study was conducted using a randomized factorial design with two factors: soybean germination (germination and non-germination) and fermentation length (fresh tempe and overripe tempe). Data from the four types of tempe flour in this study were analyzed using ANOVA (Analysis of variance) followed by DMRT (Duncan's Multiple Range Test) using the SPSS version 23 program.

3. Results and discussion

3.1 Yield and nutritional content

In the present study, overripe tempe was obtained by extending the fermentation time of fresh tempe by five days at room temperature (approximately 28–30°C). The overripe tempe produced had greyish mycelium with blackish-grey flecks on the surface of the tempe (which are a result of the increased *Rhizopus* spp. biomass) and had a strong signature aroma.

Data in Table 1 presents the yield and nutritional content analysis results of fresh tempe flour made from germinated soybeans (FG), fresh tempe flour made from non-germinated soybeans (FNG), overripe tempe flour made from germinated soybeans (OG), and overripe tempe flour made from non-germinated soybeans (ONG). The data in Table 1 shows that the type of tempe flour did not have a significant effect ($p>0.05$) on the

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Table 1. Proximate composition of overripe tempe

Analysis	Tempe flour			
	FG	FNG	OG	ONG
Yield (%)	35.5±8.6[a]	38.2±7.9[a]	53.2±15.2[a]	56.9±13.1[a]
Moisture (% wb)	2.9±1.1[a]	3.0±1.9[a]	5.0±0.1[a]	4.8±0.2[a]
Ash (% db)	2.3±0.0[a]	2.1±0.2[a]	2.2±0.0[a]	2.2±0.0[a]
Lipid (% db)	26.2±1.1[b]	27.9±1.4[b]	25.2±1.3[a]	24.3±0.1[a]
Protein (% db)	43.6±1.6[a]	41.2±1.6[a]	42.7±0.2[a]	43.0±1.2[a]
Carbohydrates (% db)	26.8±0.1[a]	27.4±1.2[a]	30.7±0.2[b]	31.1±1.2[b]
Crude Fiber (% db)	2.30± 0.1[a]	2.1±0.2[a]	3.0±0.4[a]	2.6±0.2[a]

Values are presented as mean±standard deviations. Values with different superscript letters along the same row are significantly different (p<0.05). FG (fresh tempe from germinated soybean), FNG (fresh tempe from non-germinated soybean), OG (overripe tempe from germinated soybean), ONG (overripe tempe from non-germinated soybean).

yield, moisture, ash, protein, and crude fibre content. The moisture content of tempe flour can be affected by several factors, such as the watering process during the soybean germination process, the soaking of the soybeans, and the drying temperature (Utami *et al.,* 2016; Uwem *et al.,* 2017).

The ash content of tempe flour tended to undergo no significant change due to the soybean germination process and tempe fermentation length. Previous research recorded a mere 1% decrease in ash content on days three and four of the soybean fermentation (Warle *et al.,* 2015). The dousing and soaking of soybeans during the germination process could decrease the ash content and cause the dissolution of some minerals into the water (Babalola and Giwa, 2012). The fermentation of soybeans could increase the activity of phytase and amylase enzymes in breaking down the food matrix. This event causes an increase in the bioavailability of a few essential minerals such as calcium, phosphorus, and iron (Samtiya *et al.,* 2021).

The fat content of tempe flour demonstrates a significant difference (p<0.05) due to the soybean germination and length of tempe fermentation treatments. There is a tendency to use fat as an energy source during the soybean germination process. Extending the fermentation time could also decrease the fat content of tempe flour because it is hydrolyzed by the lipase enzyme produced by the *Rhizopus* spp. mould (Nkhata *et al.,* 2018).

The carbohydrate content of tempe flour demonstrated a significant difference (p<0.05) tended to increase due to the fermentation length treatment. Overripe tempe flour (OG and ONG) had a significantly higher carbohydrate content than fresh tempe flour (FG and FNG). Carbohydrate in soybeans consists of simple and complex carbohydrates. Fermentation increases the activity of the α-amylase enzyme, which can break down polysaccharides (complex carbohydrates) into simple carbohydrates (Khosravi and Razavi, 2021).

The protein contents of FG, FNG, OG, and ONG were not significantly different (p>0.05) but tended to increase due to the soybean germination process in the fresh tempe flour production process. This increase could be because of the synthesis of amino acids and the decrease in the content of other components during the process of washing and boiling the soybeans, or these components might have been used by microorganisms for growth during the fermentation process (Kim *et al.,* 2013; Kaczmarska *et al.,* 2017). The increase in protein content during the germination process is probably caused by the mobilization of the nitrogen stores for producing nutrients needed during radicula formation (Joshi and Varma, 2016).

The change in crude fibre content in tempe flour could also be caused by the change in the cell wall structure. The crude fibre content originates from the epidermis, hypodermis, and parenchyma layers in the soybeans, containing cellulose, pectin, galactomannan, and glycoprotein (Krisnawati and Adie, 2008). The length of fermentation in tempe correlates with the increased growth of *Rhizopus* spp. that produces mycelia on the surface of the soybeans. An increase in the number of mycelia formed during the tempe fermentation process indicates increased crude fibre content in the tempe. The mycelium components consist of the hyphal cell walls, which contain cellulose and chitin; these two components are known as fibre components (Widoyo *et al.,* 2015). This is why overripe tempe flour tends to have higher crude fibre content than fresh tempe flour, even though it is not statistically different.

3.2 Isoflavone content

Isoflavone is a flavonoid compound beneficial as an antioxidant and can bind free radicals and prevent chain reactions. Isoflavone acts as a phytoestrogen which has pseudohormonal activity that binds on the ER (estrogen receptor). Isoflavone has antioxidant, anticancer, antimicrobial, and anti-inflammatory activities

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(Rodríguez-Roque *et al.,* 2013).

Table 2 shows that the daidzein and genistein content in tempe flour increased significantly as the fermentation time increased in the tempe-making process ($p<0.05$). This is why the overripe tempe flour (OG and ONG) had significantly higher daidzein and genistein content ($p<0.05$) than fresh tempe flour (FG and FNG). This study showed that the total aglycone isoflavone (daidzein + genistein) increased with increasing tempe fermentation time. Aglycone is an isoflavone compound with higher bioavailability than other forms (Ferreira *et al.,* 2011).

Table 2. Isoflavone content of overripe tempe

Parameter	Tempe flour			
	FG	FNG	OG	ONG
Daidzein (μg/g)	316.8±2.4[a]	290.2±0.6[b]	343.3±3.3[c]	343.0±2.6[c]
Genistein (μg/g)	422.1±2.6[a]	368.2±1.3[a]	701.0±44.8[c]	587.1±2.4[b]

Values are presented as mean±standard deviations. Values with different superscript letters along the same row are significantly different ($p<0.05$). FG (fresh tempe from germinated soybean), FNG (fresh tempe from non-germinated soybean), OG (overripe tempe from germinated soybean), ONG (overripe tempe from non-germinated soybean).

The aglycone isoflavone content (daidzein and genistein) in tempe increases because of the activity of microorganisms during the fermentation process producing the enzyme β-glucosidase (Li *et al.,* 2021). This enzyme can hydrolyze the glycoside bond, which results in increased availability of isoflavones in the form of aglycones (Haron *et al.,* 2009; Fawwaz *et al.,* 2017). In addition to microorganism activity, the increased aglycone isoflavone content in tempe could also be due to the decrease in the glucoside isoflavone content.

Table 2 also shows a tendency of the soybean germination process to increase daidzein and genistein isoflavone content in tempe flour. This result caused FG to have a higher daidzein and genistein content than FNG, as with OG, which had a higher content than ONG. The germination process in soybeans can significantly increase the genistein content, but not the daidzein content (Júnior and Ida, 2015).

3.3 Antioxidant activity

Evaluation of the antioxidant activity in tempe flour can be done using the DPPH (2,2-diphenyl-1-picrylhydrazyl) method. The antioxidant activity of foodstuff can be measured using the DPPH free radical inhibition method with the IC_{50} parameter. IC_{50} is the effective concentration required to deactivate 50% of the free radicals, which means that the lower the IC_{50}, the higher the antioxidant activity is (Firdaus *et al.,* 2010; Prvulović *et al.,* 2016).

An antioxidant is a compound that inhibits or prevents oxidation due to free radicals in a substrate at a low concentration. The body can naturally produce antioxidants due to cellular metabolism known as endogenous antioxidants (Yadav *et al.,* 2016). Endogenous antioxidants consist of superoxide dismutase (SOD), catalase, and glutathione peroxidase. In a pathological condition such as diabetes mellitus, there is an increased number of free radicals in the body, requiring exogenous antioxidants in large numbers to neutralize the effects of the free radicals (Firdaus *et al.,* 2010; Wresdiyati *et al.,* 2018). Figure 1 presents the IC_{50} of the tempe flours (FG, FNG, OG, and ONG). The soybean germination treatments and tempe fermentation time in Figure 1 significantly affected the IC_{50} ($p<0.05$). The lowest IC_{50} was found in ONG at 1375 ppm. This value showed that the antioxidant activity of overripe tempe flour made from non-germinated soybeans (ONG) was significantly higher than that of the other tempe flours.

Figure 1 also shows that the germination process tended to decrease antioxidant activity (increasing the IC_{50}). The IC_{50} for FG was higher than that of FNG, and the IC_{50} for OG was higher than ONG. This result is suggested because of the younger soybeans, the type of seed, and the growing conditions (Eshraq *et al.,* 2016). According to a prior study, young soybeans have lower antioxidant activity than older soybeans, but their viability is better than that of older soybeans (Carvalho *et al.,* 2014).

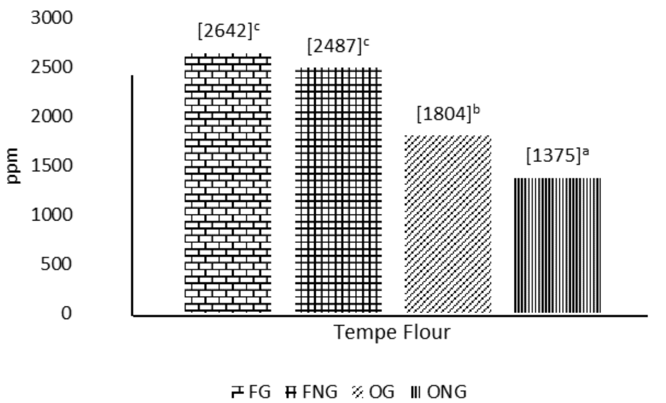

Figure 1. The IC50 value of tempe flour. FG (fresh tempe from germinated soybean), FNG (fresh tempe from non-germinated soybean), OG (overripe tempe from germinated soybean), ONG (overripe tempe from non-germinated soybean).

The extension of fermentation time in overripe tempe causes the fermentation process, which was initiated by mould to be continued by bacteria (Utami *et al.,* 2016). Fermentation of soybeans-based foods using

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bacteria could increase the antioxidants' ability to bind free radicals compared to fermentation by mould (Leonard *et al.,* 2021). The difference in the bacteria's and mould's growth rate is suggested to explain this phenomenon (Wongputtisin *et al.,* 2007). Antioxidant activity in overripe tempe can also be influenced by its isoflavone content. In the soybean germination process, isoflavones are distributed in the sprouting area and have a different distribution depending on the aglycone type (Oshima *et al.,* 2016). Isoflavone content in soybeans before germination is dominated by the glycoside group, whereas after the germination, it is dominated by the aglycone group (Murni *et al.,* 2013).

Isoflavone antioxidant activity has been reported to halt the chain reaction caused by strong oxidation peroxynitrite resulting from the reaction between nitrite oxide and superoxide. The antioxidant activity of genistein and daidzein are known to inhibit oxidation of LDL (low-density lipoprotein) and can normalize the increased superoxide anion production in rats with streptozotocin-induced type 1 diabetes (Tarasevičienė *et al.,* 2019).

The administration of tempe-based feed to hyperglycemic rats resulted in a higher antioxidant activity than that of the group fed a multivitamin (Harun *et al.,* 2017). Efforts to improve the antioxidant status in the body can be made by consuming foodstuffs rich in antioxidants such as soybeans and their products such as tempe. Consumption of tempe, which is rich in antioxidants, can increase the activity of the enzyme SOD in the blood by 56.9% (Utari *et al.,* 2011). Isoflavones demonstrated the highest antioxidant activity in the form of aglycones, especially genistein. Genistein demonstrates a stronger antioxidant activity than ascorbic acid and α-tocopherol in protecting cells against oxidative stress (Yoon and Park, 2014). Genistein is known to suppress the production of H_2O_2 by 12-O-tetradecanoylphorbol-13 acetate, which is stimulated by polymorphonuclear leukocytes in humans and HL-60 cells at a concentration range of 1–150 μM. Genistein can also increase SOD activity, thus inhibiting the dismutation of superoxide radicals (Jiang *et al.,* 2008).

Antioxidant activity in tempe flour is not only influenced by the isoflavone content. Various studies have reported that in addition to isoflavones, the amino acid and vitamin E content also have potential antioxidant activity. In addition, the non-phenolic compound content such as ascorbic acid, α-tocopherol, phytic acid, carotenoids, and saponin also play a role in increasing the antioxidant activity (Lee *et al.,* 2011; Utari *et al.,* 2011).

4. Conclusion

The soybean germination process and fermentation time did not cause any change in the nutritional content of fresh tempe flour and overripe tempe flour; however, it tended to increase the protein, fat, crude fibre, and genistein contents. Compared to fresh tempe flour made from germinated soybeans (FG), fresh tempe flour made from non-germinated soybeans (FNG), and overripe tempe flour made from germinated soybeans (OG), overripe tempe flour made from non-germinated soybeans (ONG) had a significantly higher antioxidant activity. This result showed that to reap the benefits of high antioxidants, the fermentation of tempe can be extended to produce overripe tempe without any need to germinate the soybeans beforehand.

Conflict of interest

The authors declare no conflict of interest.

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