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RESEARCH ARTICLE

Effect of initial bacteria cells number and fermentation time on increasing nutritive value of sago flour

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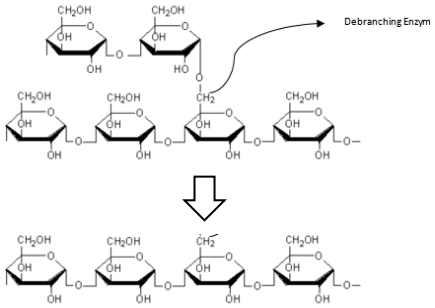
Graphical abstract



Abstract

Indonesia is the largest sago feedstock in the world. There are about 2 millions ha sago forest that approximately half of the world's sago forest is present in Indonesia. Naturally, sago spreads widely in Papua, while semi-cultivation is in Maluku, Sulawesi, Borneo, and Sumatra. The species sago (*Metroxylon sago*) was used in this study. It has a relatively high starch content (95.99%) with low amylose content (20.61%) and low protein content (1.63%). Modified sago flour is a product from sago flour that modified with fermentation to increase the nutritional value of the sago flour. It can be used as a gluten-free flour and low-calorie food products. The bacteria (*Lactobacillus plantarum*) was used in the fermentation. However, the color of the modified sago flour is off-white, if the fermentation time is too long. Therefore, it is necessary to investigate the effect of fermentation time and initial bacteria cells number on increasing nutritive value of sago flour. The variables used were fermentation times (12, 24, and 36 h) and initial bacteria cells number (7 x 10^{10}, 7 x 10^{11}, 1.05 x 10^{12}, and 3,05 10^{12} cells of *L. plantarum*). The result showed that amylose and protein content increased from 20.61% to 33,06% and from 1.41% to 4.11%, respectively, with bacterial variables of 3,5 x 10^{12} and fermentation time of 36 h.

***Keywords*:** Fermentation, *Lactobacillus plantarum, Metroxylon sago*, modified sago flour

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INTRODUCTION

Sago palms (*Metroxylon spp.*) give an economic importance in societies. They are endemic plants of the wetlands and are widely distributed in Northwestern Melanesia (such as Papua New Guinea and the Solomon Islands) and Southeast Asia (such as Thailand, Malaysia, Indonesia, and Philippines) (Rauwerdink, 1986; Mc Clatchey et al, 2006). Indonesia has more than 90% of the world's sago area, with 85% of which is found in Papua and West Papua provinces. All parts of sago palms can be processed for various end products. Their leaves, rachis, cortex of trunk and starch are used for thatching, house building, firewood, and food preparations, respectively (Flach, 1997).

Sago is a staple food in some areas of eastern Indonesia to supply the energy needs. This is because it is one of the important sources of starch to satisfy the caloric needs. However, it is considered as a low-quality raw material due to low vitamins, minerals, and proteins contents. Moreover, its starch has a low amylose content which is increasing the starch's ability to experience gelatinization (Tester and Morisson, 1990).

Proteins are one of bio-macromolecule that have an important role in living things. Their main function is forming cell structure and acting as a biocatalyst for reactions in metabolism. Another, amylose is more resistant to digestion than amylopectin. It is an important form of resistant starch. Resistant starch is a kind of starch that is not completely broken down by enzymes in our small intestine into sugar but rather turned into short-chain fatty acids and is then absorbed into the blood. This may lead to some unique health benefits for the human.

As a commodity, sago starch can be utilized to produce any kind of products, such as food (Sidaway and Balasingam, 1971; Gorinstein et al, 1994; Pontoh and Low, 1995), ethanol ((Haska and Ohta, 1993; Pranamuda et al 1995; Ratnam et al 2003, 2006), plastic (Odusanya et al, 2000), cosmetic and textile (Solichin 1986) industries.

Processing sago into modified sago flour provides a more varied development opportunities and can help the development of healthy foods. Several researchers have focused on the fermentation of sago for ethanol and glucose production. However, the enhancing and improving the quality of sago flour for food industry still remains unknown. Moreover, Gunawan et al (2015) investigated the production of modified cassava flour without any additional nutrients at a suitable microorganism (bacteria, yeasts, or filamentous fungi) for enhancing detoxification and improving the quality in a reasonably short time. It was found that modified cassava flour was successfully obtained by fermenting cassava with bacteria (*Lactobacillus plantarum*) for fermentation time of 5 days. The color of the flour is off-white if the process is too long.

Therefore, the purpose of this study was to produce modified sago flour using fermentation without the addition of nutrients in a reasonably short time. The variability of initial cells culture addition and fermentation time were systematically investigated.

EXPERIMENTAL

Materials

Sago flour was obtained from a traditional market, namely Terong Market (Makassar, Indonesia). It was already in the form of half-wet powder with a water content of 13.29% and stored at 3-4ºC before use to maintain the quality. *Lactobacillus plantarum* was obtained from

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Microbiology Laboratory of Institut Teknologi Sepuluh Nopember (Surabaya, Indonesia). All solvents and reagents were obtained from commercial sources and of either high-performance liquid chromatography grade or analytical grade, such as Nelson reagent, 95% ethanol, NaOH 0.1 N, H_2SO_4 0.1 N, acetic acid 1 N, iodine solution, acetonitrile, CH_3CN (solvent A), and 0.01 M Na_2HPO_4.

Starter preparation

The *L .plantarum* inoculum was prepared in an Erlenmeyer flask containing 15 ml of nutrient broth (NB) (Sigma, USA) and 135 mL of distilled water. The mixture was incubated for 16 h to achieve bacterial growth in log phase. The starter volume used for fermentation was adjusted by the variable number of *L. plantarum* bacteria cells. All the tools used for experiments were sterilized previously. The sterilization was carried out in an autoclave at 121 ºC for 15 min.

Fermentation

Sago fermentation process was done using submerged fermentation method as described by Gunawan *et al*. (2015). Briefly, 150 g of sago was added to the fermentation bottle. The starter amount of *L. plantarum* cells of $7x10^{10}$, $7x10^{11}$, $1.05x10^{12}$ and $3.5x10^{12}$ were added. The fermentation was performed for 12, 24 and 36 h. The temperature was kept constant at 32 ºC during fermentation. Then, sago was washed using a 1% NaCl solution to stop the activity of *L. plantarum*.

Starch content analysis

The starch content was analyzed using AOAC 2005. The samples were weighed (2 g) and poured in 250 mL erlenmeyer. 50 mL of distilled water was added and stirred for 1 h. The suspension was filtered with filter paper and washed with distilled water. The residue was transferred quantitatively in the filter paper into the erlenmeyer, then washed with 200 mL of distilled water. 20 mL of 25% HCl was added. After that, it was closed with a cooling back and heated over a boiling water handler for 2.5 h. After cooling, it was neutralized with 45% NaOH solution and diluted to 500 mL volume. Then, it was filtered and obtained by the final filtrate. 1 mL of the final filtrate was put into a test tube. 1 mL of Nelson reagent was added and heated at 100 ºC for 20 min. Afterwards, it was cooled to room temperature. 1 mL of arsenomolibdat was added and diluted with 7 mL of aquadest. Absorbance was measured at 540 nm.

Amylose content analysis

Analysis of amylose content was performed according to the method used by Ni'maturohmah and Yunianta (2015). Briefly, 100 mg of samples were weighed and poured into the test tube. 1 mL of 95% ethanol and 9 mL of 1N NaOH were added and heated in boiling water for 10 min to form gel and cooled. The gel was put into a 100 mL poultice flask and the volume was adjusted with aquadest. 5 mL of solution, 1 N acetic acid, and 2 ml iodine solution were added. The volumetric flask was shaken until homogenized and allowed to stand for 20 min. The intensity of the formed blue color was measured by a spectrophotometer with a wavelength of 625 nm. Absorbance at 625 nm used to determine the amylose content (percent amylose) with λmax vs percent amylose content curves.

Amylopectin content analysis

Analysis of amylopectin content was determined by a different method from previous analysis of starch and amylose.

$$\text{Amylopectin content (\% w/ w)} = \text{starch content (\%)} - \text{amylose content (\%)} \quad (1)$$

Protein content analysis

Analysis of protein content was determined by AOAC 2005. 0.5 g samples were fed into Kjeldahl flasks. Then, Kjeldahl tablets of ¼ parts and 10 mL of concentrated H_2SO_4 were added. After that, the flask was heated with a Kjeldahl flask heater in the acid chamber. The heating was stopped if the solution was not smoky and the color of the solution becomes greenish/yellowish. Then the Kjeldahl flask was left to cool down.

Next, 50 mL of distilled water was poured into a distillation flask filled with boiling stones and then pour the solution present in the Kjeldahl flask into the distillation flask. The Kjeldahl flask was rinsed with 50 mL of distilled water. Next, 30 mL of 40% NaOH solution was added gradually and then close with rubber stoppers and shake slowly (keep no steam out of the distillation flask). Then, 25 mL of H_2SO_4 0.1 N and 3 drops of methyl red indicator were added into the erlenmeyer.

After that, the solution in the flask was distilled until the solution in the distillation flask remained 1/3 of a part. The exit NH_3 vapor was collected in an erlenmeyer containing the H_2SO_4 solution that has been spilled on the indicator. Then the distillation result was titrated using NaOH 0.1 N until the color change from pink to orange.

A blank consisting of 25 mL H_2SO_4 0.1 N solution and 3 drops of methyl red indicator then was made and was titrated with 0.1 N NaOH solution until the color change.

$$\%\ crude\ protein = 6.25\ x\ \%\ N \quad (2)$$

$$\%N = \frac{titer\ blanko - titer\ sample\ x\ N\ x\ 0.014}{Berat\ Sample}\ x\ 100\% \quad (3)$$

N: Normality of NaOH

Amino acid analysis

The HPLC system consisted of a Varian 5060 pump, Rheodyne 7120 injector (50-ul loop), one or two Waters Associates Bondapak C-18 columns (each 30 cm x 3.9 mm i d.) connected in series, a WISP 710 A autosampler, and a Turner Designs model 10 filter fluorometer. The fluorometer used a Corning 7-60 excitation filter (open between 310 and 390 nm) and two emission filters, a wratten 2A and a Corning 4-96 (clear above 410 nm), as suggested by Turner Designs. The detector response was displayed and peak areas were calculated by a Hewlett-Packard 3388A printer plotter automation system. The five-step multilinear gradient used was a modification of that reported by Lookhart et al (1984) and was composed of acetonitrile, CH_3CN (solvent A), and 0.01 M Na_2HPO_4 at pH 7.4 (solvent B). The flow rate was 1.0 mL/min, and the back pressure was 2,300 psi. The method resolved all the common α-amino found in protein. Successive samples could be injected every 65 min.

RESULTS AND DISCUSSION

Microbial growth

The growth curve of *L. plantarum* was applied to determine the time needed to cult bacteria (the number of cells corresponding to the variables in the fermentation) as shown in Fig. 1.

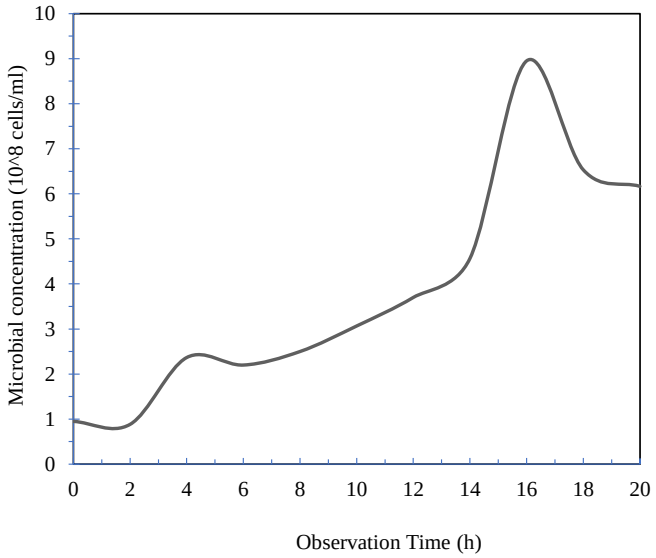

Fig. 1 Growth curve of *L. plantarum*.

The phases of bacterial growth are a phase of cleavage of bacteria through several phases ie, lag, exponential, stationary and death phases.

The lag phase is a bacterial adjustment phase with the new environment and occurs at 0 to 14 h. Then, the cell begins to divide until it reaches its maximum population that was occurred at 14 to 16 h. The static phase occurred at 16 until 18 h. In this phase, there is no population increase, the cell undergoes changes in chemical composition and increases in size and the intracellular substance. Then, the bacteria enters the stationary phase when the rate of bacterial growth is equal to the rate of death. So the total bacterial bacteria remains constant.

The results were comparable with previous works. Longhi *et al*. (2013) reported that adaptation phase occurs at 0 to 12 h. Another, Gunawan *et al.* (2015) reported that *L. plantarum* has a fairly high growth rate within 24 h. This can be occurred because of difference in media used. In previous research, the media used was deMann Rogosa Sharpe Broth. While in this research media used was Nutrient Broth.

Initial proximate composition of sago

The initial proximate composition of sago is shown in Table 1. It can be seen that the sago has relatively high of starch (95.99%) and low of protein content (1.63%). The other components, such as fiber, lipids, and ash contents were 0.49%, 0.30%, and 1.59%, respectively.

Table 1 Proximate Composition of Sago (dry basis)

Composition	Content (wt %)
Starch	95.99
Protein	1.63
Fibre	0.49
Lipid	0.30
Ash	1.59

Starch is a complex carbohydrate that contains two kinds of polymers, amylose, and amylopectin, in different compositions (Gunawan *et al*. 2017). Amylose is a polysaccharide and a non-branched polymer that composed of glucose as its monomer. Each monomer is connected to a (1,4) glycosidic bond. Amylopectin is a polysaccharide composed of α-glucose monomers. Structurally, it is formed from glucose chains bonded with (1-6) glycosidic bonds, this is the same as that found in amylose. However, it forms in the branches (about every 20 links of glucose) with a (1,4) glycosidic bond.

The functional properties of starches in flour are varied depending on the variety, natural condition, and place of the plant. The tendency for retrogradation leads to crystallization accompanied by small amylose molecules and length of the amylopectin chain (Peroni *et al*., 2006). Amylopectin is a component that plays an important role in gelatinization process. High levels of amylose can decrease the starch's ability to experience gelatinization (Tester and Morisson, 1990).

In this study, the content of amylose (20.61%) was lower than amylopectin which is 79.39%. This result was significantly lower than that of previous research. Ahmad and Williams (1998) reported that the ratio of amylose to amylopectin was 27/73.

Effect of fermentation on starch content

Effect of fermentation time and cell number of bacteria on starch content is shown in Fig. 2. It can be seen that the starch content decreased with increasing the fermentation time, among the number of *L. Plantarum* used. At an initial number of bacteria of $7x10^{10}$ cells, the starch content decreased from 82.9% to 76.39%, 73.62%, and 71.18% for fermentation time of 12 h, 24 h, and 36 h, respectively.

Decreasing content of starch was caused by the breakdown of starch into dextrin or maltose then became oligosaccharide which is a monosaccharide. Then, a monosaccharide was fermented into lactic acid when anaerobic conditions. Conversion of starch into dextrin, glucose and lactic acid was performed for further study. The best result was obtained in the variable of $3.5\ x10^{12}$ cells with 36 h fermentation time. It was successfully to decrease the starch content of 51%.

Effect of fermentation time and a number of *L. plantarum* on amylose content is shown in Figure 3. It was found that the longer the fermentation and the number of bacteria, the content of amylose increased. The increasing amylose occurs due to the breaking of the branches of amylopectin in the bonds α 1-6 glycosides. Therefore, the number of branches of amylopectin decrease and the number of straight chain amylose increase as the result of the breaking of the amylopectin branch bond. Termination of this chain was done by the debranching enzyme in the form of isoamylase enzyme.

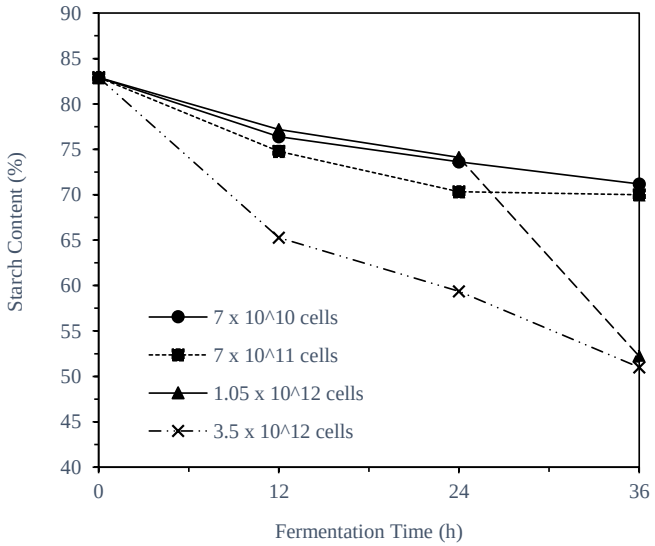

Fig. 2 Effect of fermentation time and number of microorganisms on starch content.

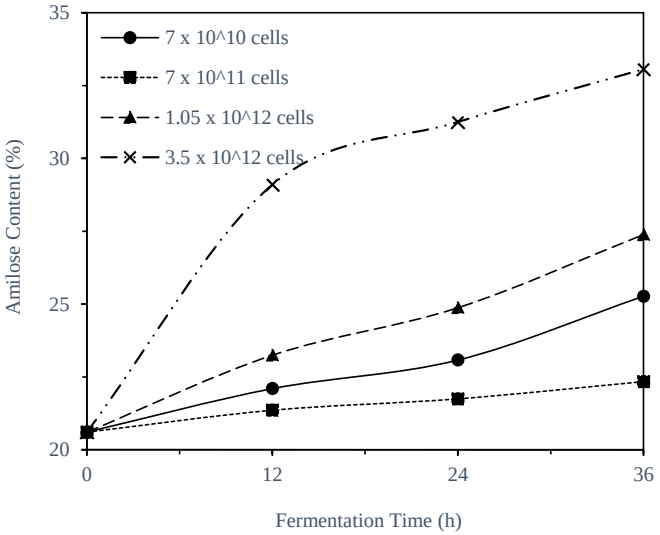

Fig. 3 Effect of fermentation time and number of microorganisms on amylose content

Starting with the initial amylose content of 20.61% increased to 25.27%, 22.34%, 27.39% and 33.06 % at 36 h for variable 7×10^{10}, 7×10^{11}, 1.05×10^{12}, and 3.5×10^{13} cells, respectively. In this study, the increasing percentage of amylose level was about 60,41%. No other study was found regarding the effect of fermenting sago on the amylose content.

Effect of fermentation on protein content

Proteins are macromolecules composed of amino acids. There are 20 kinds of amino acids that make up the protein. Amino acids are divided into 2 groups: essential and non-essential amino acids. Essential amino acids can not be produced in the body so they must be added in the form of food, while non-essential amino acids can be produced in the body.

The role and activity of proteins in biological processes, among others, as enzymatic catalysts, is that almost all chemical reactions in biological systems are catalyzed by macromolecules called enzymes that are one type of protein. Enzymes have a great catalytic power, increasing their reaction speed by millions of times.

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Effect of fermentation time and a number of *L. plantarum* on amylose content is shown in Figure 4. It can be seen that the longer fermentation time and the number of cells, the protein content increased. At an initial bacteria cells number of 7 x 10^{10} cells, the protein content increased from 1.41% to 1.72%, 1.84%, and 1.91% at 12, 24, and 36 h of fermentation time, respectively. Moreover, the effect of cells number at 7 x 10^{10}, 7 x 10^{11}, and 1.05 x 10^{12} on protein content were not significant. Another, protein content increased significantly from 1.41% to 2.42%, 3.05%, and 4.11% at 3.5 x 10^{10} cells bacteria for at 12, 24, 24 and 36 h, respectively.

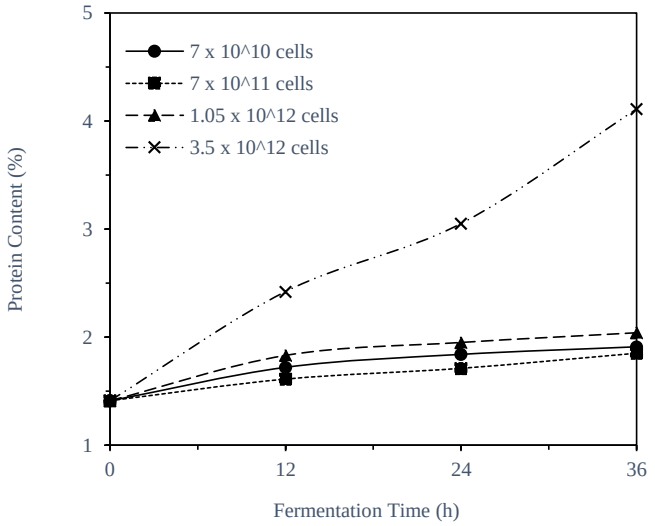

Fig. 4 Effect of fermentation time and number of microorganisms on protein content.

It can be seen that the higher the number of bacterial cells used can increase the protein content so that it can be shorter the fermentation time. It was because the ability of bacteria secretes extracellular enzymes (proteins) into sago during the fermentation process.

The increase in protein levels was also influenced by a large number of *L. plantarum* cells. Lactic acid bacteria have long been known and used by humans in the process of food processing. These bacteria contribute substantially to the flavor, texture, and fermentation product. In addition in terms of nutritional value, fermentation produces nutrient components that are more easily digested through reform and production of compounds which beneficial to the microorganisms involved.

The increasing the amount of protein was due to the increasing number of microorganisms that act as single cell protein, a protein derived from microorganisms (Ge´linas and Barrette, 2011). Another reason is that fermentation causes microorganisms to convert substrates containing carbon and nitrogen into proteins (Hu et al, 2012). *L. plantarum* was also able to produce plantarians that have antimicrobial proteins that capable of inhibiting the growth of pathogenic bacteria and decay (Atrih et al, 1993). Moreover, the acid produced by lactic acid bacteria has antibacterial properties against enteric bacterial pathogens (Ray, 1996).

The importance of essential amino acids is well known and their intake is crucial in our diet since human bodies are unable to synthesize it. The non-essential amino acids are amino acids that can be produced in our body and they are equally important as the essential amino acids in the development of our growth.

It can be seen in Table 3 that all the amino acid contents increased significantly after the fermentation. It caused by main metabolic activity during the fermentation that named proteolysis. During fermentation, protein is hydrolyzed to low molecular weight components, such as peptides and amino acids due to the action of enzymes produced by bacteria (Kiers et al., 2000).

Other researchers have also reported similar observations on the effects of amino acids content due to fermentation. According to Bujang et al. (2014), the enzymatic breakdown of proteins by *Rhizopus oligosporus* increases essential amino acids in soybean by 22% and non-essential amino acids by 16%. In groundnut, a tremendous increase of 42% was observed in essential amino acids but in nonessential amino acids, the increase obtained was by 18%. In garbanzo bean, both essential and non-essential amino acids were increased by 15 and 16%, respectively.

Table 3. Amino acid content (wt.%).

Component	Fermentation	
	Before	After
Aspartate Acid	1.846%	49.469%
Glutamate Acid	2.772%	19.23%
Alanine	0.460%	7.460%
Cysteine	0.531%	1.531%
Serine	0.268%	10.268%
Histidine	0.250%	4.501%
Arginine	0.238%	1.837%
Valine	0.311%	4.119%

Among all amino acids studied, aspartate and glutamate acids were found to be the highest. After the fermentation, aspartate and glutamate acids increased from 1,846% to 49,469% and 2,772% to 19,23%, respectively. This both amino acid is well known for the amino acid types that contribute to the pleasant umami taste or savory enhancement in foods (Chinwe et al., 2013). Thus the flavor and aroma of modified sago may be due to the production of amino acids, especially glutamic acid during the fermentation process

CONCLUSION

The content of starch and amylopectin content decreased while the content of protein increased with increasing the fermentation time and *L. plantarum* cells. Starch decomposition products of this study have the potential to be developed into low-calorie food products or commonly called gastrointestinal starch/resistance starch with an amylose content of 33.06%. The color of the modified sago flour is white due to short of the fermentation time. Moreover, modified sago flour can be used as a substitution of wheat flour in order to develop healthy foods due to the high content of protein.

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