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ScienceDirect
Food Chemistry 101 (2007) 351–356
Food Chemistry
www.elsevier.com/locate/foodchem

Histamine and other biogenic amines and histamine-forming bacteria in miso products

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Received 5 September 2005; accepted 21 December 2005

Abstract

Twenty-seven miso products sold in supermarkets and 13 products sold in retail markets were purchased from southern Taiwan, and tested to determine the occurrence of histamine and histamine-forming bacteria. The levels of pH, salt content, and aerobic plate count (APC) in all samples ranged from 5.1 to 5.8, 6.1% to 13.8%, and 2.1 to 9.1 log CFU/g, respectively. Only one of the supermarket miso products contained 100 MPN/g total coliform. None of these samples contained *Escherichia coli*. Although the average content for each of the nine biogenic amines in all samples was less than 5 mg/100 g, two supermarket samples (22.1 and 11.9 mg/100 g) and one retail market sample had histamine content (10.2 mg/100 g) greater than the 5.0 mg/100 g allowable limit suggested by the US Food and Drug Administration. Eight histamine-producing bacterial strains, capable of producing 10.4–39.4 ppm of histamine in trypticase soy broth (TSB) supplemented with 1.0% L-histidine (TSBH), were identified as *Staphylococcus pasteuri* (one strain), *Bacillus* sp. (one strain), *B. amyloliquefaciens* (two strains), *B. subtilis* (two strains) and *B. megaterium* (two strains), by 16S rDNA sequencing with PCR amplification.

Keywords: Histamine; Histamine-forming bacteria; *Bacillus* spp.; Miso products; 16S rDNA

1. Introduction

Miso, an important and popular fermented soybean food in the Orient, is a semi-solid soy paste, produced through fermentation similar to that of soy sauce (Fukushima, 1981). It is made from steamed cereal grain, cooked soybeans and salt, through the action of moulds, yeast and bacteria (Wang & Hesseltine, 1979). The salt content of miso generally ranges from 5% to 13% (Ebine, 1971). The starter cultures used in the manufacture of miso consists of the moulds *Asperillus oryzae* or *Asperillus soyae,* the yeasts *Saccharomyces rouxii* and *Torulopsis* spp., and lactic acid bacteria such as *Pediococcus halophilus*, *P. cerevisiae*, and *Streptococcus faecalis* (Kirschbaum, Rebscher, & Bruckner, 2000). By varying the ratios of the raw materials, different types of miso are produced.

Biogenic amines are basic nitrogenous compounds occurring in many foods, especially fermented foods such as cheese, sauerkraut, wine and fermented meat, due to amino acid decarboxylation activities of certain microbes during fermentation (Arnold & Brown, 1978). High levels of histamine in foods can have important vasoactive effects in humans (Lehane & Olley, 2000; Taylor, 1985). Although no miso has been incriminated in an incident of histamine poisoning, many cases in Japan have involved fish that had been seasoned or eaten with miso (Taylor, 1985). Even though the incriminated fish were undoubtedly the source of the majority of the histamine, the other biogenic amines present in the miso might have contributed to the toxicity of the fish. Cadaverine and putrescine are known to enhance histamine toxicity (Arnold & Brown, 1978;

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doi:10.1016/j.foodchem.2005.12.057

Bjeldanes, Schutz, & Morris, 1978; Lehane & Olley, 2000) and miso has been reported to contain these two biogenic amines (Shalaby, 1996).

Histamine is formed mainly through the decarboxylation of histidine by exogenous decarboxylase released by many bacterial species. These bacteria have been isolated not only from fish and other seafood products, but also from other types of foods such as cheese, fermented sausage, and wine (Taylor, 1986). In these fermented foods, several species of histamine-producing lactic acid bacteria belonging to the *Lactobacillus*, *Leuconostoc*, and *Pediococcus* genera have been isolated (Guerrini, Mangani, Granchi, & Vincenzini, 2002; Kung, Tsai, et al., 2005; Roig-Sagues, Hernandez-Herrero, Lopez-Sabater, Rodriguez-Jerez, & Mora-Ventura, 1996; Stratton, Hutkins, Summer, & Taylor, 1992; Stratton, Hutkins, & Taylor, 1991; Tsai et al., 2004b). Recently, our research group isolated histamine-formers *Staphylococcus* spp., *Enterobacter cloacae*, and *Candida* spp. from mustard pickle products in Taiwan (Kung, Lee et al., in press).

Although Yen (1986) studied the biogenic amines in miso products in Taiwan, no information was available concerning the hygienic quality and histamine-forming bacteria in this product. In this paper 40 miso products sold in both the supermarkets and retail markets in Taiwan have been tested: the contents of total coliform, *Escherichia coli*, and histamine have been measured.

2. Materials and methods

2.1. Materials

Twenty-seven miso products sold in supermarkets and 13 other miso products sold in retail markets were purchased from southern Taiwan between July and September, 2004. The retail market miso products were unpackaged and were displayed at room temperature (27–32 °C), while the supermarket miso products were packaged in plastic bags and stored at refrigerator temperature. After purchase, all miso samples were kept at 4 °C and immediately transported to the laboratory for analysis.

Biogenic amines, including tryptamine hydrochloride (Trp), 2-phenylethylamine hydrochloride (Phe), putrescine dihydrochloride (Put), cadaverine dihydrochloride (Cad), spermidine trihydrochloride (Spd), spermine tetrahydrochloride (Spm), histamine dihydrochloride (Him), tyramine hydrochloride (Tyr), and agmatine sulfate (Agm), were obtained from Sigma (St. Louis, MO, USA). Trp (61.4 mg), Phe (65.1 mg), Put (91.5 mg), Cad (85.7 mg), Spd (87.7 mg), Spm (86.0 mg), Him (82.8 mg), Tyr (63.2 mg), and Agm (87.7 mg) were dissolved in 50 ml of 0.1 M HCl and used as the standard stock solution (each at 1.0 mg/ml). A series of diluted standard solutions were prepared from the standard stock solutions and used to obtain the standard curve for each biogenic amine.

2.2. pH value and salt content

Samples of miso product (10 g) were homogenized in sterile blenders with 10 ml of distilled water to make a thick slurry. The pH of this slurry was then measured using a Corning 145 pH meter (Corning Glass Works, Medfield, MA, USA). The salt content in each sample was determined according to AOAC procedures (1995).

2.3. Microbial analysis and isolation of histamine-forming bacteria

A 25-g portion of the miso was removed from each sample and homogenized at high speed for 2 min in a sterile blender with 225 ml of sterile potassium phosphate buffer (0.05 M, pH 7.0). The sterile blender was prepared by autoclaving for 15 min at 121 °C. The homogenates were serially diluted with a sterile phosphate buffer, and 1.0-ml aliquots of the dilutions were inoculated into aerobic plate count (APC) agar (Difco, Detroit, MI, USA) containing 0.5% NaCl. Bacterial colonies were counted after the plates were incubated at 35 °C for 48 h. The bacterial numbers in the miso samples were expressed as $\log_{10}$ colony forming units (CFU)/g.

To isolate histamine-forming bacteria, a 0.1-ml aliquot of the sample dilute was spread on histamine-forming bacterium isolation agar (HBI agar) fortified with L-histidine (Niven, Jeffreg, & Corlett, 1981). Following incubation of the differential agar plates for 4 days at 35 °C, colonies with blue or purple colour on the plates were picked and further streaked on trypticase soy agar (TSA) (Difco) to obtain pure cultures. Their ability to produce biogenic amines was determined by inoculating the isolates in trypticase soy broth (TSB) (Difco) supplemented with 1% L-histidine (TSBH) and incubated without shaking at 35 °C for 24 h. Two millilitres of the culture broth were taken for quantitation of biogenic amines.

Analyses of total coliform and *E. coli* in these miso samples were conducted using the methods described by FDA (1992).

2.4. Identification of histamine-forming isolates

The presumptive histamine-forming isolates were identified on the basis of morphology, Gram stain, endospore stain, catalase and oxidase reaction. The identity of histamine-forming isolates was further confirmed by amplifying and sequencing approximately 1400 basepairs of the 16S ribosomal DNA (rDNA) for bacteria (Kuhnert, Capaul, Nicolet, & Frey, 1996; Kuhnert, Heyberger-Meyer, Nicolet, & Frey, 2000). Amplification of histamine-forming bacteria was performed using the universal primers UNI-L (5′-AGAGTTTGATCATGGCTCAG-3′) and UNI-R (5′-GTGTGACGGGCGGTGTGTAC-3′) (Kuhnert et al., 1996, 2000). Bacterial cells were cultured overnight in 2 ml of TSB at 35 °C and then centrifuged at 8000 rpm for 10 min. The cell pellet was washed and then resuspended

in 0.5 ml of TE-buffer (10 mM Tris–HCl, 1 mM EDTA; pH 8.0). After the cells in the suspension were lysed by adding 38 μl of 20% sodium dodecyl sulfate (SDS), the solution was boiled for 20 min and the cellular debris discarded following centrifugation at 13,000*g* for 3 min. Total DNA in the supernatant was precipitated with 70% ethanol and used as template DNA for PCR. PCR amplification was performed on a 20 μl reaction mixture containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 20 pmol of each primer, a 0.2 mM concentration of each of the four deoxynucleotide triphosphates, 0.5 U of *Taq* DNA polymerase (Applied Biosystems, Foster City, CA, USA), and template DNA (10 ng). Amplifications were carried out for 35 cycles (94 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s) in a GeneAmp PCR 2400 Thermal Cycler (Applied Biosystems) with an initial denaturation at 94 °C for 4 min and a final extension at 72 °C for 7 min (Kuhnert et al., 1996, 2000). Amplicons were detected by electrophoresis on a 1.5% agarose gel staining with ethidium bromide. Amplicons were purified using a QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA) eluted in Tris–HCl (10 mM, pH 8.5) prior to sequencing. The amplified DNA was directly sequenced with the ABI TaqDye Deoxy Terminator Cycle sequencing kit and ABI Model 377 automated DNA sequencer (Applied Biosystems). The sequences were analyzed with BLAST software (NCBI) for the identification of histamine-forming bacteria.

2.5. Biogenic amine analysis

Each miso sample was ground in a waring blender for 3 min. The ground samples (5 g) were transferred to 50-ml centrifuge tubes and homogenized with 20 ml of 6% trichloroacetic acid (TCA) for 3 min. The homogenates were centrifuged (10,000*g*, 10 min, 4 °C) and filtered through Whatman No. 2 filter paper (Whatman, Maidstone, England). The filtrates were then placed in volumetric flasks, and TCA was added to bring to a final volume of 50 ml. Samples of standard biogenic amine solutions and 2-ml aliquots of the miso extracts were derivatized with benzoyl chloride according to the previously described method (Hwang, Chang, Shiau, & Chai, 1997). Two millilitres of each bacterial culture broth were also benzoylated using the same procedures for miso extracts. The benzoyl derivatives were dissolved in 1 ml of methanol, and 20 μl aliquots were used for HPLC analysis.

The contents of biogenic amines in the test samples were determined with a Hitachi liquid chromatograph (Hitachi, Tokyo, Japan) consisting of a Model L-7100 pump, a Rheodyne Model 7125 syringe loading sample injector, a Model L-4000 UV–vis detector (set at 254 nm), and a Model D-2500 Chromato-integrator. A LiChrospher 100 RP-18 reversed-phase column (5 μm, 125 × 4.6 mm, E. Merck, Damstadt, Germany) was used for chromatographic separation. The gradient elution program began with 50:50 (v/v) methanol:water at a flow rate of 0.8 ml/min for the first 0.5 min, followed by a linear increase to 85:15 methanol:water (0.8 ml/min) during the next 6.5 min. The methanol:water mix was held constant at 85:15 (0.8 ml/min) for 5 min, and then decreased to 50:50 (0.8 ml/min) during the next 2 min.

A set of biogenic amine standards and their mixtures were analyzed together with test samples. During analysis, a standard solution was also injected intermittently between test samples to check chromatographic consistency. Each sample was injected twice. The peak heights of the biogenic amine standard solutions were used to prepare standard curves, and then for determination of the amine concentrations in test samples.

2.6. Statistical analysis

Pearson correlation was carried out to determine relationships between pH, salt content, APC and histamine contents in the 40 samples collected from southern Taiwan. All statistical analyses were performed using the Statistical Package for Social Sciences, SPSS Version 9.0 for windows (SPSS Inc., Chicago, IL, USA). Value of $P < 0.05$ was used to indicate significant differences.

3. Results and discussion

Values of the pH, salt content, aerobic plate count (APC), total coliform, and *E. coli* in the miso products are presented in Table 1. The levels of pH, salt content, and APC in all samples ranged from 5.1 to 5.8, 6.1% to 13.8%, and 2.1 to 9.1 log CFU/g, respectively. The average salt content in miso products obtained from retail markets (9.3%) were significantly lower than those samples from supermarkets (11.4%) ($P < 0.05$). In contrast, the average level of APC in miso products obtained from retail markets (6.8 log CFU/g) were significantly higher than those samples from supermarkets (4.3 log CFU/g) ($P < 0.05$). The rates of unacceptable supermarket and retail market miso products were 22.2% (6/27) and 84.6% (11/13), respectively, based on the regulatory level for APC (5.0 log CFU/g) in Taiwan. Only one of the supermarket miso products contained 100 MPN/g total coliform. None of

Table 1
Values of the pH, salt content, aerobic plate count (APC), total coliform (TC), and *E. coli* in tested miso products

Source and number of samples	pH	Salt content (%)	APC (log CFU/g)	TC (MPN/g)	*E. coli* (MPN/g)
Supermarket, 27	5.1–5.8 (5.5 ± 0.17)[aA]	6.5–13.8 (11.4 ± 1.4)[A]	2.1–7.1 (4.3 ± 1.3)[B]	<3–100	<3
Retail market, 13	5.1–5.7 (5.5 ± 0.16)[A]	6.1–11.3 (9.3 ± 1.5)[B]	4.7–9.1 (6.8 ± 1.3)[A]	<3	<3

Values in the same column with different letters are statistically different ($P < 0.05$).
[a] Mean ± SD.

these samples contained *E. coli* (see Table 1). Based on the Taiwanese regulatory standard of 10 MPN/g for total coliform, 2.5% (1/40) of the samples was unacceptable in all miso products. In general, no correlation existed among the pH values, salt contents, APC, and histamine contents in the tested 40 samples. However, a negative correlation ($r = -0.64$, $P < 0.05$) was noted between the salt contents and APC values in the tested samples. The higher salt contents in these samples apparently had some inhibitory effect on bacterial growth.

None of the 40 tested miso samples contained 2-phenylethylamine and spermidine (see Table 2). Although the average content for each of the remaining seven biogenic amines in all samples was lower than 5.0 mg/100 g, two of the supermarket samples (22.1 and 11.9 mg/100 g) and one of the retail market sample had histamine content (10.2 mg/100 g) greater than the 5.0 mg/100 g allowable limit suggested by the US Food and Drug Administration (USFDA, 2001, Chap. 7). Therefore, based on the content of histamine in the test products, a 7.5% (3/40) unacceptable rate was obtained with these miso samples. Yen (1986) reported similar findings with the levels of biogenic amines in Taiwanese miso products. However, Yamamoto, Wakabayashi, and Makita (1980) reported that miso products contained 0.21–169.5 ppm tyramine. Shalaby (1996) reported that fermented soybean products (miso) contained high levels of histamine (462 mg/100 g), putrescine (1234 mg/100 g), cadaverine (634 mg/100 g), and tyramine (3568 mg/100 g). Unlike those samples tested by Shalaby (1996), the miso products tested in this study did not contain high levels of biogenic amines. Variations in the contents of biogenic amines in these commercial miso products could be attributed to variability in the ratio of soybean to other seeds used, the microbiological composition, and the conditions and duration of fermentation (Chin & Koehler, 1983; Nout, Ruiker, & Bouwmeester, 1993).

The tested miso samples produced 31 purple colonies on the differential HBI agar plates. Only eight of them (25.8%) produced histamine in TSBH medium. The remaining 23 isolates were false-positive histamine-formers. Lopez-Sabater, Rodriguez-Jerez, Hernandez-Herrero, Roig-Sagues, and Mora-Ventura (1996) also found that 63.1% of the presumptive histamine-producers that were isolated from Niven's medium were actually false-positives when grown in a histidine-supplemented culture broth. Thus, our results confirm the previous observations that Niven's medium may yield false-positive isolates of histamine-producers, because other alkaline products of bacterial origin can also cause colour changes of the colonies on the agar plates (Ababouch, Afilal, Rhafiri, & Busta, 1991; Chen & Malison, 1987; Tsai, Kung, Lee, Lin, & Hwang, 2004a).

Table 3 listed the identity of these eight histamine-forming bacteria, as determined by 16S rDNA sequences, following comparison to reference strains, using NCBI database analysis. The PCR amplicons from strains M4-1 and M4-3 had a 100% homology with *Staphylococcus pasteuri* and *Bacillus* sp., respectively, while those from strains M5-1 and M5-2 aligned with *B. amyloliquefaciens* at 99.8%. The PCR amplicons from strains M6-1 and M6-2 had a 100% homology with *B. subtilis*, whereas those from strains M38-1and M29-2 had a homology with *B. megaterium* at 99.5% and 99.8%, respectively (Table 3). These eight histamine-forming isolates as *S. pasteuri*

Table 2
The levels of biogenic amines in tested miso products

Source and number of samples	Range of biogenic amine level (mg/100 g)								
	Put[a]	Cad	Try	Phe	Spd	Spm	His	Tyr	Agm
Supermarket, 27	ND[b]–1.2 (0.12 ± 0.33)[c]	ND–20.1 (3.16 ± 4.53)	ND–43.4 (2.70 ± 4.35)	ND	ND	ND–21.6 (0.80 ± 3.16)	ND–22.1 (1.64 ± 4.04)	ND–2.8 (1.21 ± 0.48)	ND–6.6 (0.24 ± 1.27)
Retail market, 13	ND–0.9 (0.11 ± 0.28)	ND–3.0 (0.91 ± 0.28)	ND–76.2 (4.90 ± 5.20)	ND	ND	ND–9.3 (0.72 ± 2.58)	ND–10.2 (0.77 ± 2.65)	ND–4.9 (1.58 ± 1.02)	ND–7.5 (0.58 ± 2.08)

[a] Put, putrescine; Cad, cadaverine; Try, tryptamine; Phe, 2-phenylethylamine; Spd, spermidine; Spm, spermine; His, histamine; Tyr, tyramine; and Agm, agmatine.
[b] ND, not detected (amine level less than 0.1 mg/100 g).
[c] Means ± SD.

Table 3
Identification of histamine-forming bacteria isolated from tested miso products by 16S rDNA, basing on the output results from NCBI database analysis

Source of miso product	Strain	Organism identified	Percentage identity (%)	Gene bank accession number
Supermarket	M4-1	*Staphylococcus pasteuri*	100	AJ717376.1
Supermarket	M4-3	*Bacillus* sp.	100	AY462199.1
Supermarket	M5-1	*B. amyloliquefaciens*	99.8	AY620954.1
Supermarket	M5-2	*B. amyloliquefaciens*	99.8	AY620954.1
Supermarket	M6-1	*B. subtilis*	100	AY881647.1
Supermarket	M6-2	*B. subtilis*	100	AY881647.1
Supermarket	M38-1	*B. megaterium*	99.5	AY030338.1
Retail market	M29-2	*B. megaterium*	99.8	AY030338.1

Table 4
Histamine and other biogenic amines (ppm) produced in culture broth by histamine-forming bacteria isolated from tested miso products

Histamine former	Histamine content in original miso sample (mg/100 g)	His[a]	Put	Cad	2-Phe	Spd
Staphylococcus pasteuri M4-1	0	28.1	ND[b]	ND	6.4	ND
Bacillus sp. M4-3	0	15.3	1.6	2.1	2.7	8.6
B. amyloliquefaciens M5-1	0.5	10.4	ND	2.6	3.1	ND
B. amyloliquefaciens M5-2	0.5	22.6	ND	1.0	ND	8.4
B. subtilis M6-1	1.3	20.4	ND	ND	ND	10.0
B. subtilis M6-2	1.3	39.4	1.0	1.2	ND	ND
B. megaterium M38-1	1.1	16.5	ND	ND	7.9	ND
B. megaterium M29-2	0.8	12.6	ND	ND	7.4	9.3

[a] His, histamine; Put, putrescine; Cad, cadaverine; 2-Phe, 2-phenylethylamine; Spd, spermidine.
[b] ND, not detected (amine level less than 1 ppm).

(one strain), *Bacillus* sp. (one strain), *B. amyloliquefaciens* (two strains), *B. subtilis* (two strains) and *B. megaterium* (two strains) by 16S rDNA sequencing produced substantial amounts of histamine (10.4–39.4 ppm) in TSBH medium (see Table 4). Some of them also produced different amounts of putrescine, cadaverine, 2-phenylethylamine and spermidine (see Table 4). No attempt was made to determine if these histamine-formers came directly from the three miso samples that had histamine contents greater than the 5.0 mg/100 g allowable limit of the US FDA. It was therefore impossible to confirm if these histamine-formers were the initial producers that were responsible for the production of the detected histamine contents in these three miso samples. These initial histamine-forming bacteria could have been killed or inhibited during the miso making process.

Staphylococcus spp. were the most frequently reported histamine-formers in fermented salted fish, accounting for nearly 50% of histamine-forming microorganisms. They were usually shown to have powerful histamine-forming activity (Yatsunami & Echigo, 1991, 1992). For example, *S. epidermidis* and *S. capitis* isolated from salted Spanish anchovies produced more than 1000 and 400 ppm of histamine, respectively (Hernandez-Herrero, Roig-Sagues, Rodriguez-Jerez, & Mora-Ventura, 1999). The *S. pasteuri* strain M4-1 that was isolated in this study was, however, a weak histamine-former and produced only 28.1 ppm of histamine in TSBH (see Table 4). The *S. capitis* recently isolated from mustard pickle products in Taiwan was a potent histamine-former, capable of producing more than 1000 ppm of histamine (Kung, Lee et al., in press).

Although the seven *Bacillus* spp. isolates from the test samples were weak histamine-formers, they were the major histamine-producing bacteria found in this study, accounting for 87.5% (7/8) of the total histamine-forming isolates. The *Bacillus* spp. isolates from salted anchovies produced low levels of histamine at 10.5 and 12.4 ppm, respectively (Hernandez-Herrero et al., 1999; Rodriguez-Jerez, Mora-Ventura, Lopez-Sabater, & Hernandez-Herrero, 1994). The *Bacillus* spp. isolates that were most frequently detected in canned anchovies also produced negligible amounts of histamine in the culture broth (Kim et al., 2004). The recently isolated *B. coagulans* and *B. megaterium* from fermented fish products in Taiwan were also identified as weak histamine-forming bacteria (Tsai et al., 2006).

B. amyloliquefaciens and *B. subtilis*, which were reported as the dominant species of aerobic bacteria in miso (Onda, Yanagida, Shinohara, & Yokotsuka, 2003), were isolated in this study from miso products, accounting for 50% (4/8) of the total histamine-forming isolates. Specifically, *B. subtilis*, the most commonly encountered food contaminant, is usually present in miso throughout the manufacturing process. This bacterial contaminant causes off-flavour, dark-colouration and spoilage of miso during the fermentation process (Kato, Inuzuka, Kondo, & Matsuda, 2001; Onda et al., 2003). Therefore, contamination with *B. subtilis* is also a serious problem in miso production.

4. Conclusion

This study to determine the safety of 40 miso products sold in Taiwan showed that supermarket miso products had lower levels of APC than retail market miso products. The average content for each of the nine tested biogenic amines in these samples was less than 5 mg/100 g, although three of them had histamine contents at 21.6 mg/100 g, 11.9 mg/100 g and 10.2 mg/100 g. Consumption of these miso products with higher histamine content might lead to scombroid poisoning in consumers. *S. pasteuri* (one strain), *Bacillus* sp. (one strain), *B. amyloliquefaciens* (two strains), *B. subtilis* (two strains) and *B. megaterium* (two strains) were the eight weak histamine formers isolated from these samples. To our knowledge, this is first report to demonstrate the occurrence of histamine-forming bacteria in miso products.

Acknowledgement

The study was supported by a research grant provided by Tajen Institute of Technology.

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