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ORIGINAL RESEARCH PAPER

Increase in the production of β-carotene in recombinant *Escherichia coli* cultured in a chemically defined medium supplemented with amino acids

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Abstract *Escherichia coli* DH5α strain was selected as the recombinant host, and a chemically defined medium supplemented with amino acids was used instead of a complex medium for the efficient production of β-carotene. In a fed-batch culture using glycerol with a chemically defined medium supplemented with amino acids, the concentration, specific content, and productivity of β-carotene were 2,470 mg/l, 72 mg/g cells, and 77 mg/l h after 32 h, respectively. These values were, respectively, 43, 33, and 26 % higher than those obtained using the complex medium. This is the highest β-carotene production that has been reported among the recombinant cells to date.

Keywords β-Carotene · Chemically defined medium · Fed-batch culture · Recombinant *Escherichia coli*

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Introduction

β-Carotene occurs in plants, fruits and microorganisms. It has been widely used in the pharmaceutical, nutraceutical, cosmetic, and food industries as a provitamin, functional cosmetic agent, food colorant, and feed supplement (Spears 1988; Nelis and Leenheer 1991) because of its beneficial effects in humans, including anti-cancer (Albanes et al. 1996) and antioxidant activities (Sies and Stahl 1995).

β-Carotene has been produced by *Rhodotorula glutinis* (Bhosale and Gadre 2001), *Blakeslea trispora* (Mantzouridou et al. 2002; Nanou et al. 2007), *Sphingomonas* sp. (Silva et al. 2004), and recombinant *Escherichia coli* (Kim et al. 2006, 2009a; Yoon et al. 2007). Lycopene is converted to β-carotene by lycopene cyclase, and it has been produced by *Blakeslea trispora* (Choudhari et al. 2008), recombinant *Pichia pastoris* (Bhataya et al. 2009), and recombinant *E. coli* (Kim et al. 2009b, 2011). β-Carotene has been produced in a complex medium instead of a chemically defined medium. However, its production levels are insufficient for industrial applications. The complex medium makes it difficult to achieve high cell concentrations, whereas the chemically defined medium involves the appropriate nutrients and achieves high cell concentrations (Zhang et al. 1996). Supplementation with amino acids to the chemically defined medium has been used as a potential method to increase effectively cell density and microbial products because amino acids compensated for nitrogen

deficiency in the chemically defined medium (Kim et al. 2011).

In the present study, *E. coli* DH5α strain was selected as the recombinant host, and the type and concentrations of amino acids in a chemically defined medium were determined for efficient β-carotene production. Increase of β-carotene production was attempted using a chemically defined medium containing amino acids in a fed-batch culture of glycerol.

Materials and methods

Bacterial strains, plasmids, and media

Four different strains of *E. coli* BL21(DE3), DH5α, ER2566, and K-12(W3110) were used in this study. The pT-DHB plasmid was constructed by cloning the genes containing downstream genes for production of β-carotene, such as *dxs*, *ipiHP1*, *crtE*, *crtB*, *crtI*, and *crtY* genes, into pTrc99A (Yoon et al. 2009). The pS-NA plasmid was constructed by cloning the genes encoding the enzymes of the entire mevalonate pathway, namely, *mvaE* (the bifunctional gene of *mvaA* and *phaA*) *mvaS*, *mvaK1*, *mvaK2*, *mvaD*, and *idi* genes into pSTV28 and it was used for production of the universal isoprenoid building blocks isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) (Kim et al. 2011). The two plasmids were transformed into each strain by electroporation for β-carotene production. The complex medium was 2YT medium (16 g tryptone/l, 10 g yeast extract/l, and 5 g NaCl/l) supplemented 10 g glycerol/l. The chemically defined medium consisted of 10 g glycerol/l, 11.2 g KH_2PO_4/l, 3 g $(NH_4)_2HPO_4$/l, 0.3 g NaCl/l, 1 g $MgSO_4.7H_2O$/l, trace elements (2.5 mg $CoCl_2{\cdot}6H_2O$/l, 15 mg $MnCl_2{\cdot}4H_2O$/l, 1.5 mg $CuCl_2{\cdot}2H_2O$/l, 3 mg H_3BO_3/l, 2.5 mg $Na_2MoO_4{\cdot}2H_2O$/l, 13 mg $Zn(CH_3COO)_2{\cdot}2H_2O$/l, and 12.5 mg Fe(III)citrate/l (Kim et al. 2011). To increase β-carotene production, amino acids (2 g glutamine/l, 0.5 g isoleucine/l, 0.5 g leucine/l, 2 g lysine/l, 2 g methionine/l, 2 g phenylalanine/l, 0.5 g threonine/l, and 1 g valine/l) were added to the chemically defined medium. Antibiotics (50 mg ampicillin/l, and 25 mg chloramphenicol/l) were added to all the media.

Culture conditions

To prepare a seed culture, a single colony of the recombinant *E. coli* DH5 was inoculated into a 20 ml test tube containing 5 ml complex medium and cultured at 37 °C and 250 rpm for 12 h to maximize cell growth. In the flask culture, 2 ml seed culture was transferred into a 250 ml baffled flask containing 50 ml complex medium, the chemically defined medium, or the chemically defined medium supplemented with amino acid, and cultured at 30 °C and 250 rpm for 40 h to maximize β-carotene production. In the fermentor culture, 4 ml seed culture was transferred to a 500 ml baffled flask containing 100 ml complex medium and cultured at 37 °C and 200 rpm for 5 h until mid-log phase (cell mass of 2.6 g/l). The pre-culture was used to inoculate a 7 l fermentor (Biotron, Bucheon, Korea) initially containing 2 l complex medium, the chemically defined medium, or the chemically defined medium supplemented with amino acid to a starting cell mass of 0.13 g/l, and then cultured at 30 °C and pH 7.0 for 36 h. The aeration rate was fixed at 1 vvm, and the agitation speed was adjusted from 300 to 1,200 rpm to maintain the level of dissolved O_2 above 20 %. In the fed-batch culture of glycerol, a feeding solution containing 900 g glycerol/l was added to the fermentor with a peristaltic pump at 0.3 to 0.5 ml/min and the reaction volume increased from 2 l to 2.6 l. The total added glycerol in the fermentor was approx. 460–490 g.

Analytical methods

The cell dry weight was determined from the OD_{600} value and correlated to the dry wt determined separately. β-Carotene was extracted from *E. coli* with acetone at 55 °C for 15 min, as previously described (Yoon et al. 2009). The concentration of β-carotene was determined using an HPLC system and a Zorbax SIL column, which was eluted with hexane/*tert*-butyl methyl ether (97:3, v/v) at 1 ml/min; detection was at 460 nm. The concentration of glycerol was determined using the same HPLC system with an RI detector and a BP-100 carbohydrate Ca^{2+} column, which was eluted with water at 80 °C at 0.4 ml/min.

Results and discussion

Selection of a host strain of recombinant *E. coli* strain as a host based on β-carotene production

To select a host strain of recombinant *E. coli* for β-carotene production, *E. coli* BL21 (DE3), DH5α,

ER2566, and K-12 as hosts were cultured in a 7 l fermentor in a complex medium (Fig. 1). Each *E. coli* strain harbored the plasmids driving expression of both mevalonate pathway genes and β-carotene pathway genes. *E. coli* DH5α showed the highest cell mass, β-carotene concentration, and specific β-carotene content among the strains, and these values were 12.7 g/l, 735 mg/l, and 58 mg/g cells, respectively. Thus, the *E. coli* DH5α strain was selected as the host for β-carotene production.

Development of a chemically defined medium supplemented with amino acids for efficient β-carotene production in recombinant *E. coli* DH5α

We presumed that the relatively poor β-carotene yield was due to nitrogen deficiency as a result of the use of dibasic ammonium phosphate alone as a nitrogen source. To compensate for nitrogen deficiency, each amino acid was added to the chemically defined medium and its concentration varied from 0.2 to 4 g/l into separate flasks (Table 1). The optimal concentrations of glutamine, methionine, leucine, lysine, phenylalanine, isoleucine, threonine, and valine for β-carotene production were 2, 0.5, 0.5, 2, 2, 2, 0.5, and 0.5 g/l, respectively. Supplementation with optimal concentrations of glutamine, isoleucine, leucine, lysine, phenylalanine, and threonine increased the cell mass, β-carotene production, and specific β-carotene content. Supplementation with optimal concentrations of methionine and valine decreased the cell mass and β-carotene production but increased the specific β-carotene content.

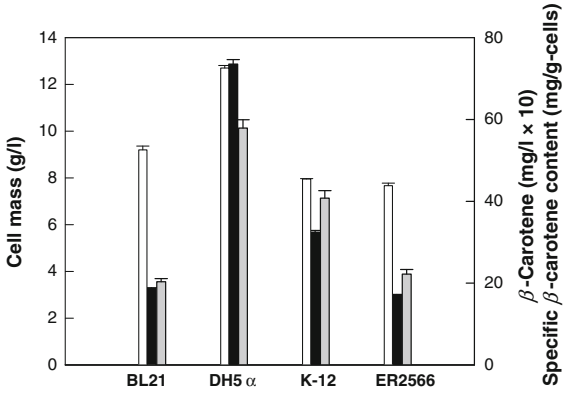

Fig. 1 Cell growth and β-carotene production by recombinant *E. coli* strains as hosts in a 7 l fermentor. Cell mass (*white bar*), β-carotene (*black bar*), and specific β-carotene content (*gray bar*). Recombinant *E. coli* was cultured at 30 °C, pH 7, and 300−1,200 rpm for 36 h. Data represent the means of three experiments and *error bars* represent standard deviation

β-Carotene production in the chemically defined medium containing optimal concentrations of all the 8 amino acids tested in a flask culture was 990 mg/l. This was double the concentrations in the chemically defined medium without amino acids (500 mg/l) and in the complex medium (490 mg/l). Moreover, β-carotene production in the chemically defined medium containing all the amino acids was 6, 38, and 41 % higher than in the chemically defined medium containing all the amino acids without valine, without methionine, and without valine and methionine, respectively (data not shown). Therefore, all 8 amino acids at optimal concentrations were added to the chemically defined medium to increase β-carotene production.

Comparison of the media for β-carotene production in recombinant *E. coli* DH5α by batch and fed-batch cultures

β-Carotene production in a complex medium, a chemically defined medium, and a chemically defined medium supplemented with amino acids using recombinant *E. coli* DH5α was compared in batch cultures using a 7 l fermentor (Fig. 2). Cell mass and β-carotene production using the chemically defined medium in a flask culture were 14.9 g/l and 500 mg/l at 40 h of the time of glycerol exhaustion, respectively, whereas those in a fermentor culture were, respectively, 12.8 g/l and 720 mg/l at 36 h of the time of glycerol exhaustion. The higher productivity in the fermentor culture may be due to the control of pH and dissolved O_2. Cell mass in the chemically defined medium in the fermentor was lower than that in the complex medium while β-carotene production was similar in both the media. Consequently, specific β-carotene content in the chemically defined medium was higher than that in the complex medium. Cell mass, β-carotene production, and specific β-carotene content in the chemically defined medium supplemented with amino acids, which were 14 g/l, 1,200 mg/l, and 87 mg/g cells, respectively, were 7, 67, and 56 % higher, respectively, than those in the complex medium and were 37, 56, and 14 % higher, respectively, than in unsupplemented chemically defined medium.

Table 1 Effects of the type and concentration of amino acid on cell growth and β-carotene production in flask cultures

Amino acid (g/l)	0	0.2	0.5	1	2	3	4
Control							
X	14.9 ± 0.05						
P	500 ± 1.1						
P/X	32.9 ± 0.01						
Glutamine							
X			15.1 ± 0.07	15.6 ± 0.02	16.7 ± 0.08	16.3 ± 0.06	14.1 ± 0.08
P			513 ± 3.0	541 ± 2.3	627 ± 2.0	564 ± 1.3	471 ± 1.9
P/X			34.0 ± 0.16	34.7 ± 0.12	37.4 ± 0.25	34.6 ± 0.12	33.4 ± 0.60
Isoleucine							
X		15.9 ± 0.03	17.9 ± 0.07	16.0 ± 0.18	15.9 ± 0.03	10.5 ± 0.01	10.2 ± 0.04
P		572 ± 1.3	667.2 ± 1.50	558 ± 1.2	572 ± 2.1	379 ± 0.4	366 ± 2.1
P/X		35.9 ± 0.55	37.2 ± 0.02	36.9 ± 0.10	36.0 ± 0.41	36.1 ± 0.11	35.9 ± 1.10
Leucine							
X		15.5 ± 0.03	16.2 ± 0.12	16.0 ± 0.13	14.3 ± 0.02	13.7 ± 0.01	12.1 ± 0.03
P		507 ± 1.8	559 ± 1.0	548 ± 1.3	465 ± 1.0	442 ± 0.4	403 ± 1.8
P/X		32.6 ± 0.45	34.5 ± 0.26	34.3 ± 0.20	32.5 ± 0.09	32.0 ± 0.28	33.4 ± 0.62
Lysine							
X			15.2 ± 0.25	15.8 ± 0.04	16.9 ± 0.02	15.4 ± 0.07	15.7 ± 0.09
P			494 ± 2.0	515 ± 1.4	579 ± 2.2	485 ± 1.9	469 ± 2.1
P/X			32.5 ± 0.83	32.6 ± 0.21	34.3 ± 0.09	31.5 ± 0.03	29.7 ± 0.12
Methionine							
X			7.7 ± 0.09	7.9 ± 0.02	7.7 ± 0.03	7.7 ± 0.03	7.2 ± 0.08
P			398 ± 2.91	432 ± 1.9	455 ± 2.3	427 ± 1.3	406 ± 3.0
P/X			51.2 ± 1.18	54.7 ± 0.09	58.7 ± 0.89	54.9 ± 1.01	52.3 ± 1.25
Phenylalanine							
X			15.3 ± 0.04	15.9 ± 0.05	18.1 ± 0.02	15.5 ± 0.03	15.7 ± 0.06
P			583 ± 2.1	630 ± 1.6	732 ± 2.1	569 ± 1.7	522 ± 1.6
P/X			38.1 ± 1.02	39.6 ± 0.07	40.4 ± 0.16	36.7 ± 0.10	33.6 ± 0.09
Threonine							
X		15.2 ± 0.20	15.7 ± 0.05	15.7 ± 0.12	15.4 ± 0.04	15.3 ± 0.07	15.0 ± 0.02
P		767 ± 1.5	860 ± 2.2	722 ± 1.9	712 ± 2.5	629 ± 1. 7	626 ± 3.1
P/X		48.7 ± 1.02	54.6 ± 0.71	52.0 ± 1.01	45.2 ± 0.86	39.9 ± 0.82	39.7 ± 0.92
Valine							
X		11.0 ± 0.01	10.8 ± 0.09	10.2 ± 0.03	10.4 ± 0.02	10.1 ± 0.02	10.1 ± 0.08
P		277 ± 1.5	391 ± 0.3	303 ± 1.2	299 ± 0.2	272 ± 0.4	266 ± 1.8
P/X		25.2 ± 0.06	37.8 ± 0.08	29.7 ± 0.10	28.8 ± 0.03	29.6 ± 0.09	25.7 ± 0.12

Recombinant *E. coli* DH5α was cultured at 30 °C, initial pH 7, and 250 rpm for 40 h. Control culture contained no amino acids. Data represent the means of three experiments and error bars represent standard deviation

X cell mass (g/l), *P* β-carotene production (mg/l), *P/X* specific β-carotene content (mg/g cells)

Glycerol is the best carbon source for carotenoid production (Kim et al. 2011). The initial concentration of glycerol in a 7 l fermentor was varied from 5 to 25 g/l to evaluate the effect of the initial glycerol concentration on β-carotene production by recombinant *E. coli* DH5α (Fig. 3). In batch cultures, β-carotene production was inhibited above 20 g glycerol/l. The specific content of β-carotene decreased with increasing glycerol concentration, and it decreased substantially above 10 g glycerol/l. Therefore, fed-batch cultures were

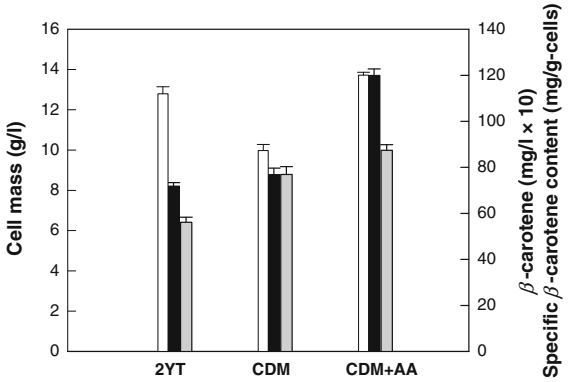

Fig. 2 Cell growth and β-carotene production in a 7 l fermentor using a complex medium, chemically defined medium, and a chemically defined medium supplemented with amino acids. Cell mass (*white bar*), β-carotene (*black bar*), and specific β-carotene content (*gray bar*). Recombinant *E. coli* DH5α was cultured at 30 °C, pH 7, and 300–1,200 rpm for 36 h. Data represent the means of three experiments and *error bars* represent standard deviation

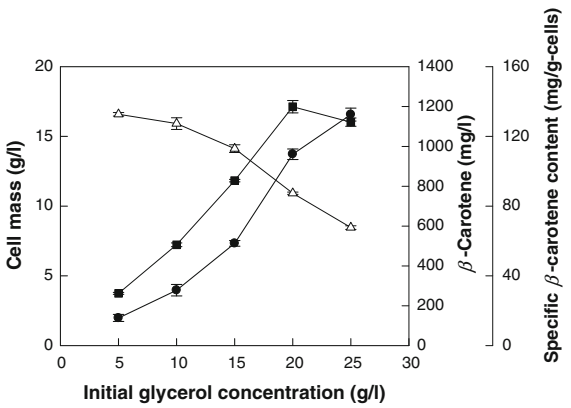

Fig. 3 Effect of the initial glycerol concentration on cell growth and β-carotene production by recombinant *E. coli* in a 7 l fermentor. Cell mass (*filled circles*), β-carotene (*filled squares*), and specific β-carotene content (*open triangles*). Recombinant *E. coli* DH5α was cultured at 30 °C, pH 7, and 300–1,200 rpm for 36 h. Data represent the means of three experiments and error bars represent standard deviation

performed at an initial glycerol concentration of 20 g/l and the glycerol concentration was maintained near to 5 g/l after 20 h (Fig. 4). In the fed-batch culture of glycerol, the cell mass and the concentration, specific content, and productivity of β-carotene in the chemically defined medium supplemented amino acids were 34 g/l, 2,470 mg/l, 72 mg/g cells, and 77 mg/l h after 32 h, respectively. These values were, respectively, 6, 43, 33, and 25 % higher than those after 28 h obtained

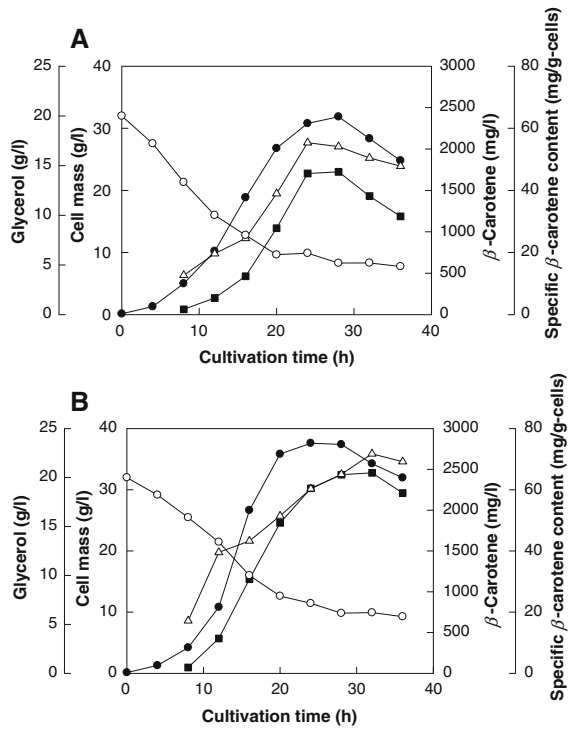

Fig. 4 Fed-batch cultures of glycerol by recombinant *E. coli* DH5α for β-carotene production in a 7 l fermentor. **a** complex medium, **b** chemically defined supplemented medium with amino acids. Cell mass (*filled circles*), β-carotene (*filled squares*), specific β-carotene content (*open triangles*), and glycerol (*open circles*). Recombinant *E. coli* DH5α was cultured at 30 °C, pH 7, and 300–1,200 rpm for 36 h

in the complex medium in the fed-batch culture. The narrower peak for β-carotene production around 1,700 mg/l occurred in the complex medium between 24 and 28 h, whereas the broader peak around 2,400 mg/l occurred in the chemically defined medium supplemented amino acids between 24 and 34 h. These results indicate that the chemically defined medium has advantage in the industrial production and can be used for the efficient production of β-carotene instead of the complex medium.

The production of β-carotene and lycopene by several microorganisms is summarized in Table 2. In this study, the cell mass and the concentration, productivity, and content of β-carotene were 136, 373, 313, and 273 % higher than the previously highest reported values using recombinant *E. coli* (Kim et al. 2009a); and they were 136, 373, 313, and 273 % higher than the previously highest reported values for lycopene production. The previously highest reported concentration and specific

Table 2 β-Carotene or lycopene production by microorganisms

Microorganism	Product	Medium	Cell mass (g/l)	Product (mg/l)	Productivity (mg/l h)	Content (mg/g)	Reference
Blakeslea trispora	β-Carotene	Chemically defined	NR	2,870	14.9	NR	(Mantzouridou et al. 2002)
Blakeslea trispora	β-Carotene	Chemically defined	20	1,800	9.4	90.0	(Nanou et al. 2007)
Rhodotorula glutinis	β-Carotene	Complex	68	159	1.8	2.3	(Bhosale and Gadre 2001)
Sphingomonas sp.	β-Carotene	Chemically defined	10	45	0.6	4.5	(Silva et al. 2004)
Escherichia coli[a]	β-Carotene	Complex	39	390	7.8	10.0	(Kim et al. 2006)
Escherichia coli[a]	β-Carotene	Complex	10	503	3.5	50.3	(Yoon et al. 2007)
Escherichia coli[a]	β-Carotene	Complex	NR	464	3.8	NR	(Yoon et al. 2009)
Escherichia coli[a]	β-Carotene	Complex	25	663	24.6	26.5	(Kim et al. 2009a)
Escherichia coli[a]	β-Carotene	Chemically defined	34	2,470	77.1	72.6	This study
Blakeslea trispora	Lycopene	Complex	12	779	8.1	64.9	(Choudhari et al. 2008)
Phchia pastoris[a]	Lycopene	Complex	16	74	1.8	4.6	(Bhataya et al. 2009)
Escherichia coli[a]	Lycopene	Complex	48	260	4.3	5.4	(Kim et al. 2009b)
Escherichia coli[a]	Lycopene	Chemically defined	42	1,350	39.7	32.1	(Kim et al. 2011)

NR not reported

[a] Metabolically engineered cells

content of β-carotene were 90 mg/g (Nanou et al. 2007) and 2,870 mg/l (Mantzouridou et al. 2002), respectively, in the carotenogenic fungus, *B. trispora*, which were 116 and 124 % higher than those in recombinant *E. coli* in this study, respectively. However, β-carotene productivity observed with recombinant *E. coli* in this study was 517 % higher than that using *B. trispora*. This productivity is the highest among the production of β-carotene and lycopene.

A short bioprocess time for industrial production of metabolites has several advantages, such as high amount production per day and labor and production cost savings. The reagent-grade cost of the complex medium ($13 per 1 l of medium) for β-carotene production was estimated to be 44 % higher than that of the chemically defined medium supplemented amino acids ($9 per 1 l of medium). Moreover, β-carotene production in the chemically defined medium supplemented amino acids was 43 % higher than that in the complex medium. Therefore, the chemically defined medium supplemented amino acids is more cost-benefit than the complex medium for β-carotene production.

In summary, this study demonstrates that β-carotene in recombinant *E. coli* can be produced effectively in a chemically defined medium supplemented with amino acids instead of a complex medium. To the best of our knowledge, this is the first report where a chemically defined medium was used for β-carotene production. β-Carotene production using the medium in the fed-batch culture of glycerol has shown the highest productivity of β-carotene ever reported. The present study has considerable importance for the possible commercial manufacture of β-carotene using a microbiological process.

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