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Statistical Selection of Amino Acids Fortifying a Minimal Defined Medium for a High-level Production of the Kringle Fragments of Human Apolipoprotein(a)

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Abstract A synthetic defined medium, fortified with amino acids, was developed for the stable production of the kringle fragments of human apolipoprotein(a) (apo(a)), *rh*LK68. Using a complex rich medium containing yeast extract and a high-cell-density fed-batch culture, the expression level of *rh*LK68 reached 17% of the total cellular protein, which corresponded to 5 g l^{-1} of the culture. To replace the complex media with chemically defined media, several amino acids that positively affect cell growth and gene expression were chosen by a statistical method. The various combinations of the selected amino acids were tested for its fortifying effect on a minimal defined medium. When glutamine only was added, the overall expression level of *rh*LK68 reached 93% of the complex rich medium, increasing the specific expression level by 22.4% and decreasing the cell growth by 24%. Moreover, the addition of glutamine resulted in a 2-fold increase in the concentration of *rh*LK68 in the culture broth, compared with the minimal defined medium. The synthetic defined media developed in this study could be generally applied to high-cell-density cultures of the recombinant *Escherichia coli* BL21(DE3), especially for the production of therapeutic proteins that require a strict quality control of the culture media and fermentation processes.

Key words: *rh*LK68, defined media, amino acids, kringle protein production

Microbial cell culture media can be classified into three categories: complex, semi-defined, and defined. The complex media are generally less preferred than the defined media, since the complex media components including yeast extract and peptone can vary between batches, which makes microbial fermentation for a recombinant protein production less reproducible. With an industrial complex medium, the heterogeneities become intensified, which can finally cause the fermentation to become unstable. In addition, the use of minimal defined media might be more cost-effective than using complex rich media due to the lower medium costs with no requirement for the removal of unknown complex compounds during downstream processes [16]. However, complex media based on yeast extract and peptone have been used to boost the production of recombinant proteins during the induction phase of recombinant microbial fermentations [5, 6, 13]. The major components of yeast extract are amino acids, trace metals and vitamins. In some studies, the yeast extract and other components were replaced by individual amino acids and trace elements [7, 14, 16]. These approaches were not always successful in improving productivity and cell growth, as some amino acids might have been deficient, or inhibited gene expression and cell growth. Therefore, the effects of each amino acid on cell growth and protein production have to be evaluated in a systematic and statistical manner. For the improvement of productivity, various culture media were optimized by using a statistical experimental design [2, 18, 19].

Recently, *rh*LK68, the cryptic kringle fragments of human apolipoprotein(a) containing KIV-9, KIV-10, and KV, has been shown to have an inhibitory activity in endothelial cell proliferation and migration. Furthermore, the systematic administration of *rh*LK68 inhibited tumor growth, and this inhibition correlated with the suppression of tumor-induced angiogenesis [4].

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Table 1. Media compositions used in the high-cell-density fed-batch cultures.

Medium	Compositions (in liter culture medium)	
	Initial	Feed
Complex	Glycerol, 20.0 g; Yeast extract, 20.0 g; KH_2PO_4, 2.33 g; Na_2HPO_4, 10.22 g; $MgSO_4$, 2.0 g; EDTA, 3.0 g; NH_4Cl, 2.0 g	Glycerol, 320 g; Yeast extract, 320 g; $MgSO_4$, 10 g
Minimal defined	Glycerol, 20.0 g; KH_2PO_4, 2.33 g; Na_2HPO_4, 10.22 g; NH_4Cl, 2.0 g; EDTA, 2.0 g; $MgSO_4$, 2.0 g; Biotin, 0.06 mg; Thiamine, 0.1 mg; Pyridoxine, 1.4 mg; Nicotinic acid, 6.1 mg; Riboflavin, 0.42 mg; Pantothenic acid, 5.4 mg; Folic acid, 0.04 mg; $FeSO_4 \cdot 7H_2O$, 15 mg; $ZnSO_4 \cdot 7H_2O$, 5 mg; $Na_2Mo_4 \cdot 2H_2O$, 1 mg; $CuCl_2 \cdot 2H_2O$, 1 mg; $CoCl_2 \cdot 2H_2O$, 2.5 mg; $CaCl_2$, 15 mg; $MnSO_4$, 2.5 mg; $NiSO_4 \cdot 6H_2O$, 1 mg; HCl, 36 mg	Glycerol, 500 g; $MgSO_4$, 10.0 g; Biotin, 0.6 mg; Thiamine, 1 mg; Pyridoxine, 14 mg; Nicotinic acid, 61 mg; Riboflavin, 4.2 mg; Pantothenic acid, 54 mg; Folic acid, 0.4 mg; $FeSO_4 \cdot 7H_2O$, 150 mg; $ZnSO_4 \cdot 7H_2O$, 50 mg; $Na_2Mo_4 \cdot 2H_2O$, 10 mg; $CuCl_2 \cdot 2H_2O$, 10 mg; $CoCl_2 \cdot 2H_2O$, 25 mg; $CaCl_2$, 150 mg; $MnSO_4$, 25 mg; $NiSO_4 \cdot 6H_2O$, 10 mg; HCl, 360·mg

In this study, *E. coli* was used as an expression system for *rh*LK68 production. It has been proved that the degree of glycosylation of *rh*LK68 does not affect its anti-angiogenic activity [3]. The effective dosage of anti-angiogenic proteins, including *rh*LK68, was so high that culture medium optimization for the stable and economical production of *rh*LK68 might be the first step toward successful commercialization. For this reason, amino acids that assist cell growth and the expression of *rh*LK68 were selected using a statistical method, and the amino acid combinations were applied to fortify a synthetic defined medium for the stable and efficient cultivation of the recombinant *E. coli* BL21(DE3) for the production of *rh*LK68.

MATERIALS AND METHODS

Strain and Plasmid

The cloning and expression of *rh*LK68 containing the KIV-9, KIV-10, and KV kringle domain of human apo(a) had been described by Kim *et al.* [4]. A DNA fragment of apo(a), spanning the nucleotides from 12,052 to 12,975 [11], was amplified by PCR from human liver cDNA. The amplified 924-bp fragment was ligated into the *E. coli* expression vector, pET11a (Novagen, U.S.A.). The final construct for *rh*LK68 expression, pET11a/LK68, was introduced into *E. coli* BL21 (DE3).

Culture

A seed culture, containing 100 ml of fortified Luria-Bertani (FLB) medium (Bacto-tryptone, 10 g/l; yeast extract, 20 g/l; NaCl, 5 g/l) was prepared in a 500-ml flask and incubated in a shaking incubator at 37°C for 12 h. The main cultures were carried out in a 2.5-l fermentor, BiofloIII (New Brunswick Sci. Co., U.S.A.), with an initial volume of 1.0 l, or in a 75-l fermentor, BiostatD DCU50 (B. Braun Biotech., Germany), with an initial volume of 25.0 l. The compositions of the culture media used in this study are shown in Table 1. For *rh*LK68 production, a dissolved-oxygen (DO)-stat fed-batch culture was adopted as described previously [9]. In all the fed-batch cultures, the DO level was controlled above 20% saturation by controlling the agitation speed and by introducing pure oxygen into the inlet air flow using the computational command of AFS (Advanced Fermentation Software, New Brunswick Sci. Co., U.S.A.). The *rh*LK68 expression was induced by the addition of 20 g/l of lactose 13 h after initiation of the fermentation.

Analytical Methods

The cell concentration was determined by measuring the absorbance of the culture broth at 600 nm (OD_{600}) with a spectrophotometer (LKB Biochrom, Ultrospec II, U.K.), or by measuring the dry cell weight (DCW) in an electronic moisture analyzer (Sartorius MA 40, Sartorius, Germany).

The protein concentration was determined using a BCA (bicinchoninic acid) method, as modified by Smith *et al.* [17].

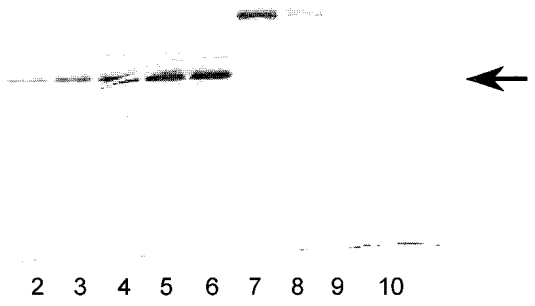


Fig. 1. SDS-PAGE of the cell lysates sampled from the 5-l scale fed-batch culture for *rh*LK68 production.
Lanes 1-6: 0, 1, 2, 3, 4, and 5 h elapsed, respectively, following the induction. Lanes 7-10: bovine serum albumin (BSA) standards for protein quantification. Arrow indicates *rh*LK68.

Table 2. Effects of addition of yeast extract on expression of *rh*LK68 and cell growth in *E. coli**.

	Yeast extract (%)		Dry cell mass (g/l)	Expression level (% of total protein)	Relative volumetric expression level** (%)	Plasmid stability *** (%)
	Initial	Feed				
Complex	2	32	56	17.0	100	>90
Minimal defined	-	-	30.0	14.1	44.4	>90
Semi-defined 1	0.5	-	34.4	12.8	46.3	>90
Semi-defined 2	0.5	10	50.4	17.2	91.1	>90

*All cultures were performed in 5-l fermentor with conditions of pH 7.0, 970 rpm, and 37°C. The mode of feed medium was DO-stat method as explained in Materials and Methods.

**The volumetric expression level is defined as the sum of dry cell weight and expression.

***Plasmid stability was measured at the time of maximal expression level.

The percentage expression level of the *rh*LK68, in the total cell lysate of *E. coli*, was determined by scanning a gradient gel stained with Coomassie brilliant blue, as previously described [8]. The total cell lysates were electrophoresed on 4–20% Tris-Glycine gradient gels (NOVEX pre-cast gels, U.S.A.). The stained gels were scanned by using ScanJet IIcx (Hewlett Packard, U.S.A.) with scanning software (Hewlett Packard, DeskScan II v2.1, U.S.A.). The scanned images were analyzed using an image analysis program (Biomed Instruments, Zeineh Programs, Universal Software, U.S.A.).

RESULTS AND DISCUSSION

Control Experiments using Complex Media

A complex rich medium, originated from Terrific Broth, was designed as the standard preparation of high-cell-density culture media for the production of the heterologous proteins in *E. coli*. The ratio of the carbon to nitrogen source (glycerol/yeast extract) was optimized using yeast extract (YE) from Difco (U.S.A.), and that was substituted for an industrial reagent grade [10]. As shown in Fig. 1, the expression level of *rh*LK68 and the cell density reached

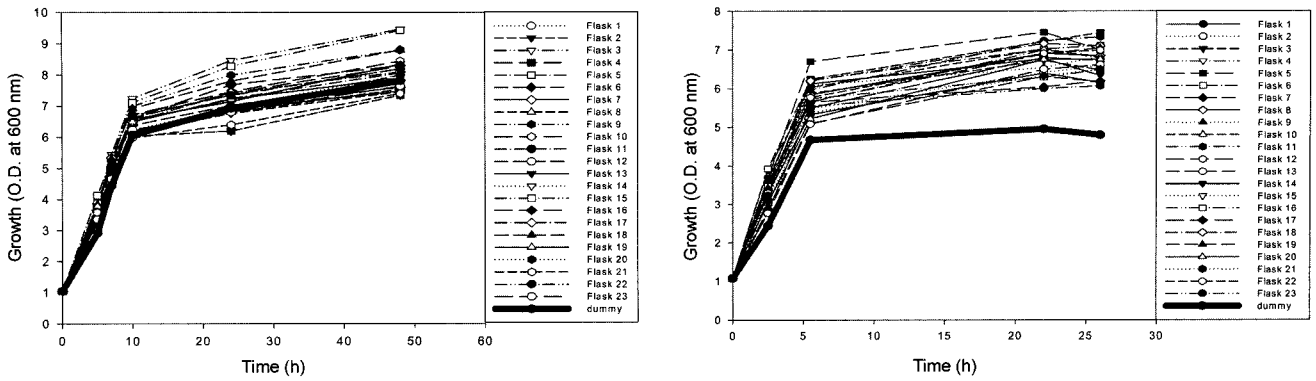
Table 3. Plackett-Burman design for flask experiments with 20 amino acids*.

Flask	Ala	Arg	Asn	Asp	Cys	Glu	Gln	Gly	His	Ile	Leu	Lys	Met	Phe	Pro	Ser	Thr	Trp	Tyr	Val	A.s**	A.cl***	Dummy
1	1	-1	-1	-1	-1	1	-1	1	-1	-1	1	1	-1	-1	1	1	-1	1	-1	1	1	1	1
2	1	1	-1	-1	-1	-1	1	-1	1	-1	-1	1	1	-1	-1	1	1	-1	1	-1	1	1	1
3	1	1	1	-1	-1	-1	-1	1	-1	1	-1	-1	1	1	-1	-1	1	1	-1	1	-1	1	1
4	1	1	1	1	-1	-1	-1	-1	1	-1	1	-1	-1	1	1	-1	-1	1	1	-1	1	-1	1
5	1	1	1	1	1	-1	-1	-1	-1	1	-1	1	-1	-1	1	1	-1	-1	1	1	-1	1	-1
6	-1	1	1	1	1	1	-1	-1	-1	-1	1	-1	1	-1	-1	1	1	-1	-1	1	1	-1	1
7	1	-1	1	1	1	1	1	-1	-1	-1	-1	1	-1	1	-1	-1	1	1	-1	-1	1	1	-1
8	-1	1	-1	1	1	1	1	1	-1	-1	-1	-1	1	-1	1	-1	-1	1	1	-1	-1	1	1
9	1	-1	1	-1	1	1	1	1	1	-1	-1	-1	-1	1	-1	1	-1	-1	1	1	-1	-1	1
10	1	1	-1	1	-1	1	1	1	1	1	-1	-1	-1	-1	1	-1	1	-1	-1	1	1	-1	-1
11	-1	1	1	-1	1	-1	1	1	1	1	1	-1	-1	-1	-1	1	-1	1	-1	-1	1	1	-1
12	-1	-1	1	1	-1	1	-1	1	1	1	1	1	-1	-1	-1	-1	1	-1	1	-1	-1	1	1
13	1	-1	-1	1	1	-1	1	-1	1	1	1	1	1	-1	-1	-1	-1	1	-1	1	-1	-1	1
14	1	1	-1	-1	1	1	-1	1	-1	1	1	1	1	1	-1	-1	-1	-1	1	-1	1	-1	-1
15	-1	1	1	-1	-1	1	1	-1	1	-1	1	1	1	1	1	-1	-1	-1	-1	1	-1	1	-1
16	-1	-1	1	1	-1	-1	1	1	-1	1	-1	1	1	1	1	1	-1	-1	-1	-1	1	-1	1
17	1	-1	-1	1	1	-1	-1	1	1	-1	1	-1	1	1	1	1	1	-1	-1	-1	-1	1	-1
18	-1	1	-1	-1	1	1	-1	-1	1	1	-1	1	-1	1	1	1	1	1	-1	-1	-1	-1	1
19	1	-1	1	-1	-1	1	1	-1	-1	1	1	-1	1	-1	1	1	1	1	1	-1	-1	-1	-1
20	-1	1	-1	1	-1	-1	1	1	-1	-1	1	1	-1	1	-1	1	1	1	1	1	-1	-1	-1
21	-1	-1	1	-1	1	-1	-1	1	1	-1	-1	1	1	-1	1	-1	1	1	1	1	1	-1	-1
22	-1	-1	-1	1	-1	1	-1	-1	1	1	-1	-1	1	1	-1	1	-1	1	1	1	1	1	-1
23	-1	-1	-1	-1	1	-1	1	-1	-1	1	1	-1	-1	1	1	-1	1	-1	1	1	1	1	1
Dummy	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1

*'1' means adding and '-1' means omitting. The concentrations of each amino acid used in this experiment are as follows (mg/l); alanine 43.5, arginine 59.2, asparagine 30.3, aspartate 30.5, cysteine 10.5, glutamate 36.8, glutamine 36.5, glycine 43.7, histidine 13.9, isoleucine 36.2, leucine 56.2, lysine 59.5, methionine 20.6, phenylalanine 29.1, proline 24.2, serine 21.5, threonine 28.7, tryptophan 11.0, tyrosine 23.7, valine 47.1.

**Ammonium sulfate.

***Ammonium chloride.



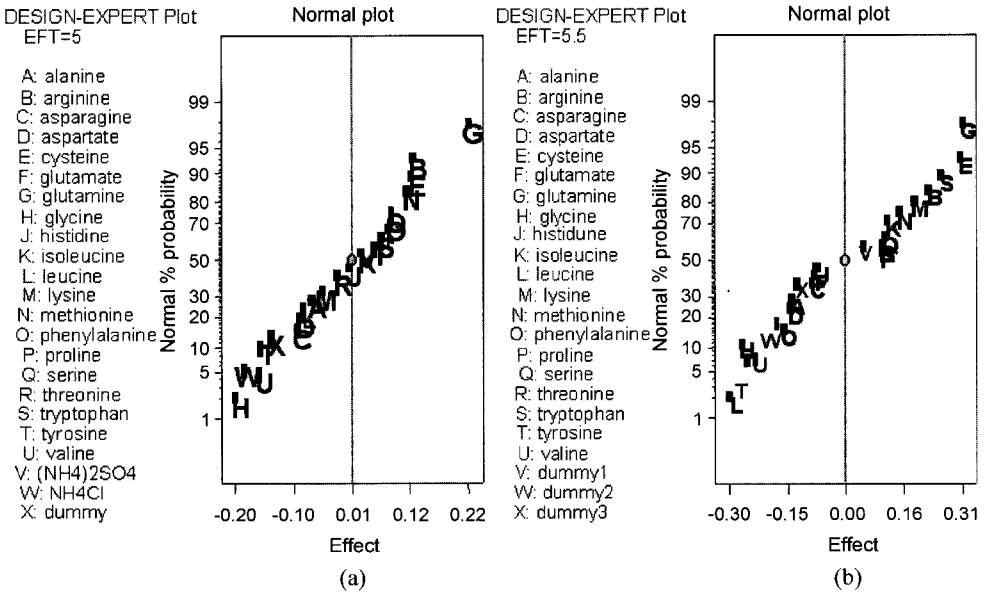
(a) (b)

Fig. 2. (a) Experimental series #1. Cell growth profiles of each flask containing amino acids, as designed following the Blackett-Burman method, with the following basal components (g/l): Glycerol, 20.0; EDTA, 3.0; NH_4Cl, 2.0; $MgSO_4$, 2.0; Na_2HPO_4, 10.22; KH_2PO_4, 2.33; metals and vitamins listed in Table 1. (b) Experimental series #2. Cell growth profiles of each flask containing amino acids, as designed following the Blackett-Burman method, with the following basal components (g/l): Glycerol, 10.0; EDTA, 1.5; NH_4Cl, 1.0; $MgSO_4$, 1.0; Na_2HPO_4, 5.11; KH_2PO_4, 1.17; metals and vitamins listed in Table 1.
The bold lines in (a) and (b) indicate the growth profiles of the control flasks containing only the basal components.

about 17% of the total cell protein and a cell concentration of 56 g/l, respectively, in the 5-l fermentation. This expression level and cell concentration corresponded to about 5–6 g of *rh*LK68 per liter of culture broth.

Development of Semi-Defined Medium

The major reasons for the design of culture media for the production of therapeutics for human uses include: the development of cost-effective, pyrogen-free, unknown factor-free, and consistency guaranteed media. To fulfill these conditions, the development of defined media is inevitable. Using the minimal defined medium, shown in Table 1, the expression level decreased by 44.4% of that obtained with the complex medium. Fortification, with a small quantity of YE in the initial or feed medium, was attempted so as to compensate for the decreased *rh*LK68 production level. As summarized in Table 2, the addition of 0.5% (w/v) and 10% (w/v) YE to the initial and feed media, respectively, was the most effective concentration, with cell concentration and expression level reaching about 90 and 91%, respectively, of the corresponding results obtained with the complex medium.



(a) (b)

Fig. 3. Normal probability plot for the analysis of the effects of the amino acid on the cell growth.
(a) Experimental series #1. (b) Experimental series #2.

Table 4. Amino acid affecting cell growth positively or negatively, by analysis of normal probability of effects estimated.

Priority	Positive		Negative	
	Series 1	Series 2	Series 1	Series 2
1	Glutamine	Glutamine	Glycine	Leucine
2	Arginine	Cysteine	Valine	Glycine
3	Cysteine	Tryptophan	Tyrosine	Tyrosine
4	Methionine	Arginine	Asparagine	Phenylalanine
5	Serine	Lysine	Aspartate	Aspartate

Development of Completely Defined Medium

As another approach, a minimal defined medium fortified with specific amino acids was designed to develop a defined medium without any additional YE. To assess the effect of each amino acid on cell growth, a statistical method, the Plackett-Burman design, was used [12]. Each flask contained a certain amino acid predetermined by experimental design (Table 3) with a given amount of glycerol, EDTA, NH_4Cl, $MgSO_4$, Na_2HPO_4, KH_2PO_4, other metals, and a vitamin solution (*Korea patent no.*0230580). The concentration of the specific amino acids added to each flask was determined by estimating the average amount of amino acid in 10 g dry weight of *E. coli* [1]. The growth profiles, at the late exponential phase, were compared among the cultures (Fig. 2), and the normal probability of the estimated effects plotted using a computer program (Design-Expert ver. 6.0.3, Stat-Ease Inc., U.S.A.) to analyze the amino acids for their effects on cell growth (Fig. 3). The flask culture with each formulation was performed twice. The second experimental series of flask cultures were performed with low glycerol and salt concentrations to show a distinct effect of the amino acids on cell growth. As a result, the amino acids including glutamine, arginine, and cysteine were found to exert positive effects on cell growth, whereas the amino acids including glycine, tyrosine, and aspartate exhibited a negative effect on two experimental series, as shown in Table 4.

With a combination of positive amino acids selected from the flask cultures, high-cell-density fed-batch cultures were performed in a 75-l fermentor, and their expression levels and cell growths were compared with each other. All media shown in Table 5 had the same composition as the minimal defined medium, as shown in Table 1, except amino acids added to fortify each experiment set. The medium in the experiment set 1 contained no amino acids. Set 2 contained cysteine at a concentration of 1.0 g/l in the initial medium and 10.0 g/l in the feed medium. Set 3 contained glutamine at a concentration of 0.6 g/l in the initial medium and 6.0 g/l in the feed medium. Set 4 contained glutamine, arginine and cysteine at a concentration of 0.6, 1.2, and 0.5 g/l in the initial medium and 1.8, 3.0, and 1.2 g/l in the feed medium, respectively. Set 5 contained glutamine, arginine, cysteine, methionine, serine, and tryptophane at a concentration of 0.2, 0.3, 0.05, 0.10, 0.10, and 0.15 g/l in the initial medium, and 3.7, 6.0, 1.0, 2.0, 2.0, and 3.0 g/l in the feed medium, respectively. As a result, the sole addition of glutamine was more effective than all the combinations. The expression level and cell growth increased by 48% and 34% compared to those of the control experiment in an amino acid-free medium (Table 5 and Fig. 4). Compared to the complex rich medium, as the specific level of expression increased by 22.4%, and the cell growth decreased by 24%, the overall level of *rh*LK68 expression reached 93% when the medium was fortified with glutamine.

The host strain used in this study, *E. coli* BL21 (DE3), is not auxotrophic to any amino acids. However, glutamine, which can be considered as a high-energy nitrogen donor, provides the nitrogen for amino sugars, NAD, and *p*-aminobenzoate, and provides the remaining nitrogen for purines, pyrimidines, histidine, tryptophan, and sometimes asparagine [15]. Not surprisingly, glutamine, as a nitrogen source, supported the rapid growth of the host cells and the high-expression of *rh*LK68 in the minimal defined medium (Table 1) with the aid of NH_4Cl as an inorganic N-source. Contrary to expectations, the sole addition of cysteine drastically decreased the cell growth and expression level. Cysteine is quite toxic to enteric bacteria, and therefore it has to be utilized with care [10]. It has also been reported as an inhibitory amino acid in the growth of *Bacillus brevis* [14]. Therefore, the positive cell growth effect of cysteine

Table 5. Effect of amino acid selected by statistical method on cell growth and expression level in a 75-l fermentor.

Experiment set	1	2	3	4	5
Amino acid added	No amino acid	Cysteine	Glutamine	Glutamine, Arginine, Cysteine	Glutamine, Arginine, Cysteine, Methionine, Serine, Tryptophan
Cell mass (g/l)	31.5	30.4	42.6	32.8	30.4
Expression level (% of *rh*LK68 in total cell protein)	14.1	18.2	20.8	20.5	19.0
Inclusion body* (g/l)	3.6	-	5.6	-	4.4

*Inclusion bodies were isolated from 30 ml of culture broth by sonication with 5 mM EDTA and 20 mM TrisHCl buffer, pH 7.2. Isolated inclusion bodies were solubilized with 7 M urea and then protein concentration was measured with the BCA method (described in Materials and Methods).

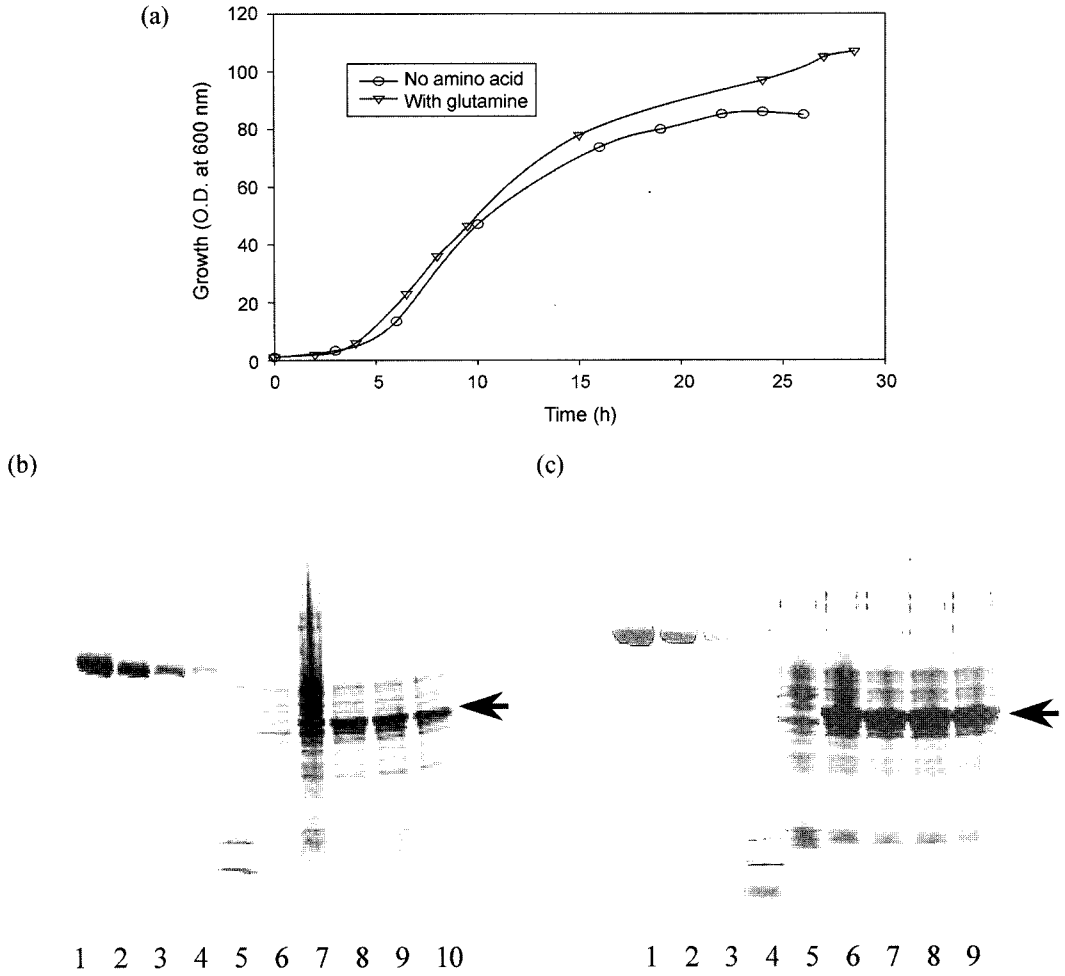


Fig. 4. (a) Time profiles of the cell growth in the cultures with glutamine and with no amino acid. (b) SDS-PAGE of the total cell lysate sampled from the cultures with no additional amino acids. Lanes 1-4: protein standards (BSA). Lane 5: size marker. Lanes 6-10: 0, 1, 2, 3, and 4 h elapsed, respectively, following induction. (c) SDS-PAGE of the total cell lysate sampled from the culture with glutamine. Lanes 1-3: protein standards (BSA). Lane 4: size marker. Lanes 5-9: 0, 1, 2, 3 and 4 h elapsed, respectively, following induction. Arrows indicate *rh*LK68.

on the *rh*LK68 producing strain might only be exerted in the presence of other amino acids. Instead of adding cysteine directly, the addition of cysteine building blocks such as sulfate and serine to the culture medium was tested in fed-batch cultures, but no effect on the expression level of *rh*LK68 was observed (data not shown).

These results suggest that certain amino acids may inhibit or support protein production, and hence the balance of each amino acid may be important in the development of a synthetic defined media that can avoid feedback inhibition in the amino acid biosynthetic pathways. In addition, the defined medium developed in this study costs less than 10% of the complex rich media containing yeast extract, which was negligible compared to the total expense of recombinant protein production. The medium formulation developed in this study could be applied to other recombinant *E. coli* BL21(DE3) cultivations for the production of industrial recombinant proteins.

Acknowledgments

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