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[见续页]

(54) **Title**: METHOD FOR HIGHLY EXPRESSING RECOMBINANT PROTEIN OF ENGINEERING BACTERIA AND USE THEREOF

(54) **发明名称**: 一种高效表达工程菌重组蛋白的方法及应用

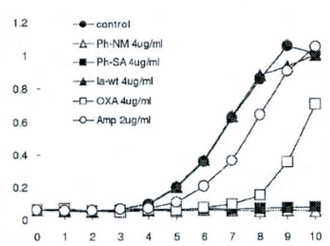


图 3 / Fig. 3

(57) **Abstract**: Provided are a method for highly expressing a recombinant protein of engineering bacteria and the use thereof. The method comprises the following steps: (1) engineering bacteria of *Escherichia coli* with pET system are transfected with recombinant mutated plasmid to obtain positive monoclonal colonies; (2) the positive monoclonal colonies are enriched to obtain a seed bacteria solution, and the seed bacteria solution is induced to enrichment and growth in a large amount; and (3) the bacteria supernatant containing the recombinant protein as the expression target is separated, and then the recombinant protein in the bacteria supernatant is extracted and purified. The method is characterized in that the engineering bacteria of *Escherichia coli* with pET system are *E.-coli*B834 (DE3). The components of the mass enrichment medium and the protein purification steps are also optimized such that a significant improvement in the yield and purity of the protein is achieved and the method is suitable for applying to the large-scale production of recombinant protein expressed by the engineering bacteria of *Escherichia coli*.

(57) **摘要**:

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— 关于申请人有权要求在先申请的优先权(细则 4.17(iii))

— 发明人资格(细则 4.17(iv))

本国际公布:

— 包括国际检索报告(条约第 21 条(3))。

提供了一种高效表达工程菌重组蛋白的方法及其应用。该方法包括如下步骤：（1）重组突变质粒转染大肠杆菌 pET 系统工程菌中得到阳性单克隆菌落，（2）对阳性单克隆菌落进行增菌得到种子菌液，诱导所述种子菌液大量增菌生长，（3）分离含有表达目的的重组蛋白的菌体上清液，提纯菌体上清液中的重组蛋白，其特征在于：所述大肠杆菌 pET 系统工程菌指 *E.coli*B834（DE3）。还对大量增菌培养基的成分及蛋白质纯化步骤进行了优化，在蛋白质产量和纯度上都得到了明显的提高，适合于应用在大肠杆菌工程菌表达重组蛋白的规模化生产中。

Specification

METHOD FOR HIGHLY EXPRESSING RECOMBINANT PROTEIN OF ENGINEERING BACTERIA AND USE THEREOF

TECHNICAL FIELD

The invention relates to biotechnology, especially to a method for highly expressing a recombinant protein of engineering bacteria and the use thereof.

BACKGROUND OF INVENTION

In earlier studies, the inventor conducted creative experiments and inventedobtained a series of new recombinant peptides with Colicin as attack point, which operationally connects a polypeptide (natural or artificial design) with identification and binding ability to target cells. For example, the new antibiotic PMC-AM1 disclosed in patent No. ZL200910092128.4, named "Novel antibiotic comprising an antibody mimetic, its preparation and uses thereof", shows a broad-spectrum antibiotic property and has stronger antibacterial activity on *Neisseria meningitidis, Multidrug-resistance Pseudomonas aeruginosa, Vancomycin-resistant Enterococcus faecalis* or *Methicillin-resistant Staphylococcus aureu* compared to the known antibiotics. The inventor's another invention in title "A novel antibiotic, its nucleotide sequence, methods of construction and uses thereof", with CN patent No.ZL200910157564.5, disclosed a series of new anti-staphylococcus antibiotics, such as PMC-SA1、PMC-SA2、PMC-SA3、PMC-SA4、

PMC-SE as well as PMC-PA. In vivo and vitro experiments, these antibiotics showed better targeting ability and stronger antibacterial activity than current antibiotics, antifungal antibiotic and chemotherapeutics drugs. Additionally compared with current antibiotics, these new antibiotics showed incomparable biological security and anti drug–resistance characteristic.

The foresaid novel antibiotics as a whole are a kind of water-soluble proteins with 600 amino acid residues, but in which there is a hydrophobic domain with 40 amino acid residues near carboxyl terminal. Compared to preparation of other water-soluble proteins with one fold structure, there is more difficult in assembling and expressing of the novel antibiotics, which inevitably affects protein yield. It is necessary to improve current expression process to achieve high yield and priority of the novel antibiotics. It will make sense for bringing the novel antibiotics into actual clinical application and practice..

Summary of invention

According to the peptide structure and characteristics of the new antibiotics disclosed in the current patent application, the present disclosure provides for a method for highly expressing recombinant protein of engineering bacteria.

In one aspect, the present disclosure provides for A method for highly expressing recombinant protein of engineering bacteria, wherein the end with hydrophilicity of the recombinant protein is colicin polypeptide and the other end with hydrophobic nature is polypeptide of target moiety which is capable of binding target, the method comprising:

(1) transfecting recombinant plasmid of expressing the recombinant protein into E. coli

engineering bacteria with pET system to obtain positive monoclonal colonies,

(2) producing seed bacteria solution of the positive monoclonal colonies, and inducing protein expression and enlargement culturing of the seed bacteria solution; the supernatant of the enlargement cultured solution contains expressed recombinant protein,

(3) extracting and purifying the recombinant protein from the supernatant, wherein the *E. coli* engineering bacteria with pET system is E.coliB834 (DE3).

In some exemplary embodiments, the enlargement culturing medium used for said inducing enriching growth of the seed bacteria solution has water as solvent and comprises following components: NaCl 6.0~6.7g/L, peptone 25.0g/L, yeast powder 7.5g/L, glucose 0.6~2.0g/L, $Na_2HPO_4 \cdot 7H_2O$ 6.8～18.3g/L, KH_2PO_4 3.0～4.3g/L, NH_4Cl 1.0~1.4g/L, $MgSO_4$ 0.2~0.4g/L, $CaCl_2$0.01g/L, methionine 0~40mg/L.

In a preferable exemplary embodiment, said enlargement culturing medium has water as solvent and comprises following components: NaCl 6.0g/L, peptone 25.0g/L, yeast powder 7.5g/L, glucose 2.0g/L, $Na_2HPO_4 \cdot 7H_2O$ 6.8g/L, KH_2PO_4 3.0g/L, NH_4Cl 1.0g/L, $MgSO_4$ 0.2g/L, $CaCl_2$0.01g/L, methionine 0~40mg/L.

In some exemplary embodiments, said enlargement culturing of the seed bacteria solution comprises following steps: the seed bacteria liquid was added into a container and started growth for 2 to 3 hours at 30℃, when the OD value reached 0.4-0.6, the seed bacteria solution was conducted heat shock at 42℃ for 30 minutes, and then cooled down to 37℃ and kept growth for 1.5 to 2 hours before being collected.

In some exemplary embodiments, wherein in conducting heat shocks the IPTG with the final density 0.5mmol/L was added into said enlargement-culturing medium.

In some exemplary embodiments, said extracting and purifying the recombinant protein from the supernatant was by CM ion exchange column, and the loading quantity of the supernatant depends on the ratio value which is 2.5mg/ml between the weight of the recombinant protein in the supernatant and the volume of Gel particles used in the CM ion exchange column.

In some exemplary embodiments, the eluent solution used for said extracting and purifying in CM ion exchange column is boric acid buffer solution with 0.2mol/L NaCl.

In most exemplary embodiments, said recombinant plasmid of expressing the recombinant protein is selected from the group consisted of pBHC-SA1、pBHC-SA2、pBHC-SA3 pBHC-SA4、pBHC-SE, pBHC-PA and pBHC-PorA1.

In an further aspect, the present disclosure provides for the applications of any foresaid methods in preparing the recombinant peptides PMC- SA1, PMC-SA2, PMC-SA3, PMC-SA4, PMC -SE, PMC-PA or PMC-AM.

In another aspect, the present disclosure provides for a medium used for *E. coli* engineering bacteria with pET system, the medium has water as solvent and comprises following components: NaCl 6.0~6.7g/L, peptone 25.0g/L, yeast powder 7.5g/L, glucose 0.6~2.0g/L, $Na_2HPO_4 \cdot 7H_2O$ 6.8～18.3g/L，KH_2PO_4 3.0～4.3g/L, NH_4Cl 1.0~1.4g/L, $MgSO_4$ 0.2~0.4g/L, $CaCl_2$0.01g/L, methionine 0~40mg/L.

In some exemplary embodiments, said *E. coli* engineering bacteria with pET system is *E.*

coli B834 (DE3), and the medium has water as solvent and comprises following components: NaCl 6.0g/L, peptone 25.0g/L, yeast powder 7.5g/L, glucose 2.0g/L, $Na_2HPO_4 \cdot 7H_2O$6.8g/L, KH_2PO_4 3.0g/L, NH_4Cl1.0g/L, $MgSO_4$0.2g/L, $CaCl_2$0.01g/L, methionine 0~40mg/L.

The pET expression system provided by Novagen Company is a common system for cloning and expressing recombinant proteins in *Escherichia* coli. In this invention, a series of BL-21 (DE3) cells are transected with recombinant mutated plasmid disclosed in former patents and produced a higher protein expression yield than the TG1 cells does in this invention. By experimental data, we found that B834（DE3）, which is parent strain of BL-21（DE3）, has a more ideal expression productivity than BL-21（DE3）. The experimental data showed that the B834 (DE3) has dozens time of protein expression productivity than TG1 system does.

Medium is used for providing required carbon source, nitrogen source and inorganic salts for bacterium growth and multiplication. In present a medium with capability of improving the expression productivity of target protein is provided in present invention, which has a optimum formula for engineering bacteria fermentation. In this invention, the medium, named FB-M9 compound medium has a increased carbon source and nitrogen source and MgSO4、CaCl2 as well as some special amino acids that are required in growth of engineering bacteria with pET system. The medium moderately improved engineering bacteria reproduction speed and protein expression rate. And material cost of the improved medium is relatively low, which provides larger research space and higher development value for enlargement production in the future.

According to guide of the product manual, the carrying rate of CM ion gel particles used in purification system in this invention could not reach the ideal standard described in the product manual, which limits the recovery rate of target protein. In present invention, the recovery rate has been significantly improved by the means of reducing loading quantity of sample while moderately increasing the gel volume, etc. The result also reflected that it is necessary to find or develop a kind of ion exchange gel with more efficient for large-scale industrial production of the target protein. In addition, the recombinant proteins has fewer impurities owing to eluent with optimized concentration used in the ion exchange steps of this invention.

In summary, this invention provides a variety of optional more optimized method of expressing E. coli engineering bacteria recombinant proteins by the means of choosing engineering strains, optimizing the composition of medium, improving the purification and recovery rate, etc. This also provides a possible research direction and technical route for finally finding optimal procedure of high-efficiently expressing fusion protein needed. Compared with the original expressing system disclosed in former patents, the expressing system developed by present invention has improved the expressing production of fusion protein dozens of times, and provided a beneficial basis of theory and practice for the subsequent large-scale industrial production.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the conductance value of eluent in the protein elution process with different volume gel column.

a. The elution process of protein with 150ml CM gel column. b. the elution process of

protein with 600ml CM gel column.

The curve signified by two arrows in the figure represent the conductance value of the eluent. The area indicated by the arrows is a conductance peak caused by the loss of the sample PMC-SA in loading process. The area of the conductance peak caused by the loss of the sample PMC-SA reduced by 70% after increasing the volume of gel.

Another curve: OD value of elutropic protein

Fig. 2 shows SDS-PAGE Gel electrophoresis of the PMC-S

From left to right in the order:

a. 1. Marker, 2. PMC-SA1 produced by TG1, 3. PMC-SA1 produced by BL-21 , 4. PMC-SA1 produced by B834;

b. 1. Marker、2. PMC-SA1 eluted by boric acid buffer solution with 0.1 M NaCl, 3. PMC-SA1 eluted by boric acid buffer solution with 0.2 M NaCl, 4. PMC-SA1 eluted by boric acid buffer solution with 0.3 M NaCl.

Fig. 3. Shows the inhibition curve of the PMC-SA against MRSA（BAA42）

Y-axis represents light absorption value; X-axis represents bacterial growth time

Control：control group; Amp：ampicillin sodium; OXA: oxacillin; Ia-wt: wild type colicin Ia; PMC-SA1: anti-staphylococcus aureus polypeptide;

PMC-AM： anti-diplococcus meningitides polypeptide.

DETAILED DESCRIPTION OF THE INVENTION

Following examples are just used for explaining the invention rather than limiting the scope of the invention.

The experimental equipment and instruments used in present invention as follows:

1. Bacterial strain

E. coli TG1 engineering bacteria (AECOM，K. Jakes).

E. coli BL-21(DE3)、B834(DE3)、Nova Blue(DE3) and 618 engineering bacteria are all purchased from Novagen company.

Staphylococcus aureus ATCC BAA-42 is purchased from ATCC (American Type Culture Collection).

Plasmid: pBHC-SA1、pBHC-SA2、pBHC-SA3 pBHC-SA4、pBHC-SE，pBHC-PA，pBHC-PorA1 (These plasmids are recorded in patents ZL200910092128.4 and ZL 200910157564.5, and preserved in the applicant's laboratory. The applicant promised to offer them to the public for necessary verification tests)

2. Main reagents and medicine

Yeast powder (OXIOD LP0021), peptone (OXIOD LP0042) as well asother chemical reagent are all analytical reagent;

Dialysis bag Snake Skin Dialysis Tubing（Pierce, intercept molecular weight1×104, Lot#KD32324)；

Streptomycin Sulfate for injection (NCPC)

AMP ampicillin sodium for injection (Harbin pharmaceutical)

Anion exchange column gel（Pharmacia Biotech CM Sepharose Fast Flow LotNo.225016）.

LB liquid medium: Sodium chloride 1g, peptone 1g, and yeast 0.5g were added into a 250 ml flask with the addition of 100 ml water, dissolved and autoclaved at 120 for 8min.

LB solid medium:100mlLB solid medium containing sodium chloride 0.5-1.5g, peptone 0.5-2g, yeast 0.3-1g and agar 0.8-3g. The LB solid medium is used for plate culture of single colony after strain recovery. Reagents were added into a 250 ml flask with the addition of 100 ml water, dissolved and autoclaved at 120 for 8min.

FB-M9 complex medium: NaCl 6.0~6.7g/L，peptone 25.0g/L，yeast powder 7.5g/L，glucose 0.6~2.0g/L，$Na_2HPO_4 \cdot 7H2O$ 6.8～18.3g/L，KH_2PO_4 3.0～4.3g/L，NH_4Cl 1.0~1.4g/L，$MgSO_4$ 0.2~0.4g/L，$CaCl_2$0.01g/L，methionine 0~40mg/L.

Improved FB-M9 complex medium: NaCl 6.0g/L，peptone 25.0g/L，yeast powder 7.5g/L，glucose 2.0g/L，$Na_2HPO_4 \cdot 7H_2O$ 6.8g/L，$KH_2PO_4$3.0g/L，NH_4Cl 1.0g/L，$MgSO_4$0.2g/L，$CaCl_2$0.01g/L, methionine 0~40mg/L。The methionine is 40mg/L in the process with E.coli B834(DE3) as engineering bacteria.

3. Key Instrument

Bio-Rad Protein chromatography purification system（BioLogic Duo Flow, BioLogic Maximizer, BioLogic QuadTec UV-Vis Detector, BioLogic Econo Pump）;

Ultrasonic Cell Disruptor (Soniprep 150), protein purification ion exchange column with 5cm diameter（Pharmacia Biotech XK50）, protein purification ion exchange column with 11cm diameter (Shanghai Huamei);

Centrifuge (Beckman Coulter Avanti J-20XP, Beckman Coulter Avanti J-25);

Spectrophotometer (Bio-Rad Smart Spect Plus spectrophotometer);

Automatic fermenter (Bioengineering AG LP351-42L);

High pressure homogenizer (Italian NiroSoavi NS1001L2KSN 6564).

Statement: the biological materials adopted in this invention have been known before the application filing date and have been also preserved in this applicant's lab. The applicant promised to offer them to the public for necessary verification tests in the twenty years since application filing date.

Example1. The option experiment of engineering bacterial strains

Classic plasmid carried the colicin Ia and its immune protein gene （GenBank M13819）are from laboratory of Dr. Finkelstein. (Qiu XQ et al. An engineered multi domain bactericidal peptide as a model for targeted antibiotics against specific bacteria. Nat Biotechnol, 2003; 21(12): 1480-1485). The classic plasmid were modified into following seven kinds of restructuring mutation plasmids in former researchs: pBHC-SA1、pBHC-SA2、pBHC-SA3 pBHC-SA4、pBHC-SE, pBHC-PA, pBHC-PorA1.

Step 1. Transformation of competent cell

40μL Novagen pET system engineering bacteria BL-21(DE3)、B834(DE3)、Nova Blue(DE3)、618 were respectively transformed with 100ng recombinant mutant plasmids pBHC-SA1, and then ice-incubated for 5 minutes, heat-shocked at 42℃ for 30 seconds, kept in ice for 2minutes, added with 160μl SOC medium and shake-cultivated at 220 rpm, 37℃ for 1 hour and then coated (LB medium with 1% agar and 50ug/ml ampicillin, and cultured overnight at 37℃). Single colonies are picked out and cultivated to obtain the seed strain, which is conserved at a low temperature.

Step2: Strain Recovery

1. Preparing recovered bacteria solution

The conserved strain was thawed at 4℃; 1.5ml of the strain is transferred into 10ml LB medium (containing 50μg/ml of AMP) and cultivated at 220 rpm, 37℃ for 5-8 hours.

2. Inoculation of single colony

The recovered bacteria solution was diluted 10^4 or 10^5 times; and 10ul of the diluted bacteria solution was transferred on to LB solid medium plate (containing 50μg/ml of AMP) and coated on the plate. The plate was placed in a humid box and cultivated in incubator at 37℃ for 10-12 hours till round single colonies have grown out on the surface of the medium.

Step 3. Enlargement culturing the single colonies (1) Single colonies with regular round shape and smooth edge were picked up from the plate and respectively added into 1.5ml LB medium, and cultivated at 220rpm, 37℃ for 5-8hours.

(2) Each 1.5ml LB bacteria solution was transferred into a 100ml LB medium, and cultivated at 220rpm, 37℃ for 5-8hours.

(3) Primary stage of enlargement culturing: the 100ml of bacteria solution from the last step was added into 700ml of improved FB-M9 complex medium and cultivated at 220 rpm, 37℃ for 5-8 hours.

(4) Secondary stage of enlargement culturing: 700ml of bacteria solution from the primary stage is added into 6×700ml of the improved FB-M9 complex medium and cultivated at 220rpm, 37℃ for 5-8hours.

(5) Third stage of enlargement culturing: 6×700ml of bacteria solution from the secondary stage was added into 20L of the improved FB-M9 complex medium and cultivated in a

fermenter with stirring rate of 220rpm and maximum oxygen flow volume, 37℃ for 3-5 hours.

(6) Fermentation of engineered bacteria and induced expression of protein: 20L of bacteria solution from the third stage of enlargement culturing was added into 200L of improved FB-M9 complex medium and cultivated in a fermenter for induced expression of protein with stirring rate of 220rpm and maximum oxygen flow volume, at 30℃ for 2~4hours; 42 ℃ for 0.5 hours; and 37℃for 1~2hours, note that IPTG is added at 42℃with a final concentration of 0.5mM.

Step 4, Collecting bacteria by centrifugation

6000 g fermentation liquor obtained from step 3 was centrifuged at 4℃ for 20min. The precipitate was collected and added into 50mM boric acid buffer (pH9.0) for resuspend of the bacteria. Note: the boric acid buffer has 2mM PMSF (Phenylmethylsulfonyl fluoride serine protease inhibitor). All consequent steps after bacteria resuspend was conducted at 4 ℃.

Step5.Cells fragmentation

After suspension in pH9.0 boric acid buffer completely, the bacteria cells was fragmented by a High Pressure Homogenizer at 500~600 bar for 7 times, with intervals of 3~5 minutes.

Step6. Precipitation of the bacteria DNA

The fragmented bacteria solution was centrifuged at 55000 g, 4℃ for 40 min. The supernatant was added with streptomycin sulfate (16 bottles of 1 million unit streptomycin sulfate were added into every 200ml liquid supernatant), and stirred for 1 h with a

magnetic stirrer.

Step7. Dialysis

The bacteria solution from the step 6 was centrifuged at 55000g, 4 for 20min. The supernatant was placed into a dialysis bag and dialyzed for 8~12 hours in boric acid buffer, which was changed once every 4 hours.

Step8. Purifying the protein medicine and obtaining antibacterial-engineered polypeptide

The dialyzed bacteria solution was centrifuged at 55000g, 4℃for 20min. The supernatant was measured the protein concentration in unit volume and placed into a Bunsen beaker for conducting protein purification by ion exchange method. The supernatant with known protein concentration was uploaded onto a CM ion exchange column. The sample loading and its ratio with the CM iron gel particular are according to the Product Manuals of CM ion exchange column. After being washed completely the CM ion exchange column was eluted with 50mM boric acid buffer containing 0.3M NaCl to obtain the novel antibacterial-engineered polypeptide.

The results are shown as table 1, the expressing efficiency of PMC-SA by ***E.coli*** B834 (DE3) is the highest.

Table 1. Expressing efficiency of different bacterial strain

(Average unit production=Gross production of extracted PMC-SA1/ volume of bacterial liquid)

Engineering strain	TG1	BL-21	618	NavaBlue	B834
Average unit production（mg/L）	0.8	10	5.8	8.1	24.4

The same operation was conducted on the other six restructuring mutation plasmids, the

results appeared similar trend as the result listed in Table 1, namely, in contrast to other engineering bacteria, *E. coli* B834 (DE3) showed the highest expressing efficiency on all seven restructuring mutation plasmids.

The operation of heat shock as following adopted to inducing expression of protein in this embodiment was different from that in prior arts: After transferring the seed bacteria liquid into the tank, cultured the bacteria at an initial temperature 30℃for 2 hours, when OD value had reached 0.4-0.6, conducted the heat shock at 42℃ for 30 minutes, then when the temperature low down to 37℃, cultured the bacteria again for 1.5 to 2 hours again. At this stage the OD value of bacteria liquid can reach to 1-3 or even more, and can be conducted collection. During this process, 0.5mM IPTG was added to induce expression of pET engineering bacteria.

Before proposing present method, the usual process for preparing the recombinant peptides was as following:

100ng of the mutant plasmids was ice-incubated with 40ul competent cell of BL-21 engineered bacteria for 5 minutes, heat-shocked at 42℃ for 30 seconds, ice-incubated for 2 minutes, added with 160ul of SOC medium, shake-cultivated at 220 rpm, 37℃ for 1hour and then coated plate (LB medium with 1% agar and 50ug/ml ampicillin, and cultured overnight at 37℃). Single colonies were picked out for enlargement culturing.

Enlargement culturing: 8-10L FB medium, 250rpm, at 37℃ for 3-4 hours; was added with IPTG, 250rpm, at 28°C grew for 4 hours again; conducted centrifugation to precipitate bacteria at 4°C, 6000g, 20 minutes. The precipitated bacteria was added with 80-100ml 50mM boric acid buffer (pH9.0, 2mM EDTA) kept at 4℃ to suspend, then added with

50ug PMSF and broken by ultra sonication (4℃, 400 w, 1 minutes, repeat 4 to 5 times with intermittent 2-3 minutes for keeping the temperature of the liquid). Then the broken bacteria was conducted high-speed centrifugation (4 ℃, 75000g, 90minutes), the supernatant was added with 5 million units streptomycin sulfate to precipitate DNA (4℃ stirred for 1 hour), and 10000g, 4℃, for 10minutes centrifugation. The supernatant was put into dialysis bag with the molecular weight 15000 on 4℃, and dialyzed by 10L 50 mM boric acid buffer overnight, then conducted centrifugation at 4℃, 10000g, for10minutes once again. The supernatant was loaded on CM ion exchange column, after being flushed completely, eluted by 0.3M NaCl + 50mM boric acid buffer, the new antibiotics can be obtained.

Example 2 Improving medium

The classic FB medium for colicin Ia preparation (Qiu XQ et al. An engineered multi domain bactericidal peptide as a model for targeted antibiotics against specific bacteria. Nat Biotechnol, 2003; 21(12): 1480-1485 /Karen Jakes, Charles Abrams, Alan Finkelstein, et al. Alteration of the pH-dependent Ion Selectivity of the Colicin E1 Channel by Site-directed Mutagenesis.JBC, 1990; 265(12): 6984-6991) has components as follows: peptone 25.0g/L, yeast powder 7.5g/L, NaCl 6.0g/L and glucose 1.0g/L.

In this invention, we adopted FB medium without glucose, the components of which as follows: peptone 25.0g/L, yeast powder 7.5g/L and NaCl 6.0g/L. And the FB medium without glucose was configured with M9 medium at a special volume proportion to obtain the FB-M9 compound medium.

The mother liquor of M9 medium is 5×M9 and has components as follows: :

$Na_2HPO_4 \cdot 7H_2O$ 64.0g/L, KH_2PO_4 15.0g/L, NH_4Cl 5.0g/L, NaCl 2.5g/L, $MgSO_4$ 1.5g/L, $CaCl_2$ 0.05g/L, and 2% glucose.

A preliminary attempt of the compound medium:

FB-M9: volume ratio between FB:M9 was 7:10, the components as follows: NaCl 6.7g/L, peptone 25.0g/L, yeast powder 7.5g/L, $Na_2HPO4 \cdot 7H2O$18.3g/L, $KH_2PO_4$4.3g/L, NH_4Cl 1.4g/L, $MgSO_4$0.4g/L, $CaCl_2$0.01g/L and glucose 0.6g/L.

This invention adopted this formula for bacteria fermentation. The process was as step3 in Example 1. The result shows in table2, wet bacteria weight got from per liter culture solution is significantly higher than that done through FB medium. The collected protein production is significantly improved with average production up to 30 mg/L.

Table 2 contrast of target protein production from test medium (PMC - SA1 / BL - 21 engineering bacteria)

	Fermenting in BF medium		Fermenting in FB-M9（7：10）medium	
	Bacterial weight (g).	Protein contents(mg)	Bacterial weight (g)	Protein contents (mg)
1	255.07	280.8	349.82	847.82
2	246.3	519.94	343.47	643.71
3	302.28	461.965	366	779.3
4	276.67	465.179	388.44	946.34
AVG	270.8	431.971	361.9325	804.2925

The final improved FB-M9 medium was obtained by further researchs and repeated

comparison in this invention. The production rate of the target protein can reach 34 mg/L as Table 3 shows, in the same fermentation conditions as example 1.

The components of the improved FB-M9 medium as follows: NaCl 6.0g/L, peptone 25.0g/L, yeast powder 7.5g/L, glucose 2.0g/L, $Na_2HPO_4 \cdot 7H_2O$6.8g/L, $KH_2PO_4$3.0g/L, NH_4Cl 1.0g/L, $MgSO_4$0.2g/L and $CaCl_2$0.01g/L. As the methionine was required in the growth of B834 engineering bacterium, in the process of B834 as engineering bacteria the methionine (40 mg/L) is added into the final improved FB-M9 medium.

Table 3 comparison of improved FB - M9 medium with other medium on productivity

Engineering strains and the medium	BL-21			B834		
	FB	FB-M9（7：10）	Improved FB-M9	FB	FB-M9（7：10）	Improved FB-M9
Average unit production（mg/L）	10	24	25	22.3	30	34

Example 3. Optimizing conditions for purifying protein

The basic structure of recombinant polypeptide (PMC- SA1、PMC-SA2、PMC -SA3，PMC -SA4、PMC -SE，PMC -PA，PMC-AM) prepared in this invention is Colicin Ia. The isoelectric point of colicin Ia is about 9.15, therefore the classic purification adopted is Ion Exchange Chromatography (Qiu XQ et al. An engineered multidomain bactericidal peptide as a model for targeted antibiotics against specific bacteria. Nat Biotechnol, 2003；21(12)：1480-1485).

The principle is: In PH9.0 boric acid buffer system, the majority of PMC-SA molecules exist as positive charge ions. When the CM gel particles with negative charge go through the chromatographic column, the recombinant protein molecules with positive charge was hung on the CM gel particles due to the electric charges attraction, while the other miscellaneous protein was rushed out of the gel column.

In this example, the other steps were as that in example 1, but after the miscellaneous protein was rushed out completely, using boric acid buffer of 0.1 to 0.3M NaCl gradiently to elute the gel column.

Owning to Na^+ ions having stronger positive property than the recombinant protein molecules, the recombinant protein was replaced from CM gel particles by Na^+ ion. There are two variables to be manipulated in the process of ion exchange and purification for a better protein yield: ①NaCl with different concentration within 0.05-1M can be chosen respectively to elute protein molecules with different positive charge mounted on CM gel particles ②The amount of CM gel particles adopted can be optimized: In the environment with certain ionic strength, the amount of protein carried by every CM gel particles is relatively constant. The volume of gel column is indispensable to be enlarged in order to increase the amount of protein carried by gel column.

CM Sepharose Fast Flow is anion exchange column gel produced by GE company. According to the manual, every 100 ml gel can combine with 9mM cation. The actual usable combination capacity varies with the nature of sample in the process of dynamic combination, and molecular weight is inversely proportional with combined capacity. Its standard sample that has equivalent molecular weight with the recombinant peptides

manufactured in this invention is Bovine COHb-(Mr69kD), which has theoretically dynamic combined capacity 30mg/ml. Namely, with 100 ml CM Sepharose Fast Flow glue to retrieve recombinant protein molecules, the theoretically highest recovery rate is about 300 mg (0.004mM). But according to its manual operation, the actual dynamic combination capacity of recombinant protein molecules to CM gel particles reached only 3 mg/ml, just reached 10% of theoretical combined capacity.

In the experiment, we found that in the latter half process of washing out the miscellaneous protein, conductance curve will raise a small peak (as shown in figure 1 a). According to this phenomenon, we speculate that when there is a large amount of recombinant protein in the sample, due to the limited capacity of CM gel particles with target protein, only a little part of the recombinant protein molecules can be recovered. The recombinant protein without being mounted on the CM gel particles has to be flushed out gel column together with miscellaneous protein. As the recombinant protein is positively charged, a short rising peak appears in the conductance curve.

In an optimized example of this invention: in order to reduce the loss of recombinant proteins, we reduced loading amount of sample to 1/3 of the manual regulation, and increased the volume of gel from 150 ml to 600 ml, namely the protein amount in the supernatant fluid: gel particle volume=2.5mg/ml. The loss of the recombinant protein decreased in the process of elution. The experimental data showed that the recovery rate of recombinant proteins was increased 3.5 times; the results shown in figure 1b.

In addition, we set the gradient concentration of NaCl as 0.1M-0.2M-0.3 M in the boric acid buffer used in elution, and 0.2M showed the highest eluting efficiency and protein

purity, as shown in figure 2b.

Example 4. Detecting protein purity and activity

Step 1. SDS-PAGE electrophoresis

The fusion protein samples obtained by optimized conditions of example 4 were conducted SDS-PAGE electrophoresis and silver nitrate dyeing. As shown in figure 2, there is a clear protein-imprinting stripe at the point of about 70kD relative molecular weight, namely PMC - SA1 manufactured in this invention in the electrophoresis map a the map b shows the protein has eliminated mixed zone through the improved gradient elution in example 4 and the purity is improved. The rest six kinds of recombinant proteins manufactured through the optimized method of this invention have also showed similar improved purification.

Step 2. Detecting the antibacterial activity

With the recombinant protein PMC - SA1 and PMC-AM that produced by the improved manufacturing method in the example1, 2, 3, we conduct the following antibacterial activity test.

The *Methicillin-resistant staphylococcus aureus* (MRSA, ATCC BAA-42) bacteria liquid 10ul (10^5cfu/ml) was inoculate into t10 ml BM medium and added with antimicrobial agents. According to the antimicrobial agents we set six parallel groups: ampicillin sodium 2ug/ml, oxazocilline 4ug/ml, wild type colicin Ia, PMC-SA and Ph-NM (4ug/ml), and blank control group. Culturing at 37℃, 210rpm, and testing optical density value per hour

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(595 nm), drawing the bacteriostasis curve, as shown in figure 3.

The bacteriostatic curve shows that the recombinant proteins produced by improved methods of this invention have good antibacterial activity.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

The reference in this specification to any prior publication (or information derived from it), or to any matter which is known, is not, and should not be taken as an acknowledgment or admission or any form of suggestion that that prior publication (or information derived from it) or known matter forms part of the common general knowledge in the field of endeavour to which this specification relates.

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CLAIMS

1. A method for highly expressing recombinant protein of an engineered bacteria, wherein the hydrophilic end of the recombinant protein is colicin polypeptide and the hydrophobic end of the recombinant protein is a polypeptide target moiety which is capable of binding target, the method comprising:

(1) transfecting the recombinant plasmid which expresses the recombinant protein into E.coli engineering bacteria with pET system to obtain positive monoclonal colonies,

(2) producing seed bacteria solution of the positive monoclonal colonies, and inducing protein expression and enlargement culturing of the seed bacteria solution; wherein the supernatant of the enlargement cultured solution contains expressed recombinant protein,

(3) extracting and purifying the recombinant protein from the supernatant, wherein the *E.coli* engineering bacteria with pET system is E.coliB834 (DE3); wherein the enlargement culturing medium used for said enlargement culturing of the seed bacteria solution has water as solvent and comprises the following components: NaCl 6.0~6.7g/L, peptone 25.0g/L, yeast powder 7.5g/L, glucose 0.6~2.0g/L, $Na_2HPO_4 \cdot 7H_2O$ 6.8 ~ 18.3g/L, KH_2PO_4 3.0 ~ 4.3g/L, NH_4Cl 1.0~1.4g/L, $MgSO_4$ 0.2~0.4g/L, $CaCl_2$0.01g/L and methionine 0~40mg/L.

2. The method according to claim 1, wherein the enlargement culturing medium has water as solvent and comprises following components: NaCl 6.0g/L, peptone 25.0g/L, yeast powder 7.5g/L, glucose 2.0g/L, $Na_2HPO_4 \cdot 7H_2O$ 6.8g/L, KH_2PO_4 3.0g/L, NH_4Cl 1.0g/L, $MgSO_4$ 0.2g/L, $CaCl_2$0.01g/L and methionine 0~40mg/L.

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3. The method according to claim 1 or claim 2, wherein said enlargement culturing of the seed bacteria solution comprises the following steps: (i) the seed bacteria liquid is added into a container and grown for 2 to 3 hours at 30℃, (ii) when the OD value reaches 0.4-0.6, the seed bacteria solution is heat shocked at 42℃ for 30 minutes, and then cooled down to 37℃ and grown for 1.5 to 2 hours before being collected.

4. The method according to claim 3, wherein in conducting heat shock, IPTG with final concentration 0.5mmol/L is added into said enlargement culturing medium.

5. The method according to any one of claims 1 to 4, wherein said extracting and purifying the recombinant protein from the supernatant is by CM ion exchange column, and the loading quantity of the supernatant samples depends on the ratio value which is 2.5mg/ml between the weight of the recombinant protein in the supernatant and the volume of Gel particles used in the CM ion exchange column.

6. The method according to claim 5, wherein the eluent solution used for said extracting and purifying in CM ion exchange column is boric acid buffer solution with 0.2mol/L NaCl.

7. The method according to any one of claims 1 to 6, wherein the recombinant plasmid expressing the recombinant protein is selected from the group consisting of pBHC-SA1、pBHC-SA2、pBHC-SA3 pBHC-SA4、pBHC-SE, pBHC-PA and pBHC-PorA1.

8. Use of the method according to any one claims 1 to 7 in preparing the recombinant peptides PMC-SA1, PMC-SA2, PMC -SA3, PMC-SA4, PMC-SE,

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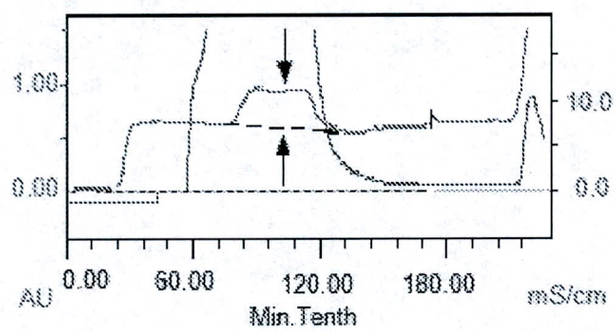
PMC-PA or PMC-AM.

9. An enlargement culturing medium used for *E.coli* engineering bacteria with pET system, wherein the medium has water as solvent and comprises following components: NaCl 6.0~6.7g/L, peptone 25.0g/L, yeast powder 7.5g/L, glucose 0.6~2.0g/L, $Na_2HPO_4 \cdot 7H_2O$ 6.8 ~ 18.3g/L, KH_2PO_4 3.0 ~ 4.3g/L, NH_4Cl 1.0~1.4g/L, $MgSO_4$ 0.2~0.4g/L, $CaCl_2$0.01g/L and methionine 0~40mg/L.

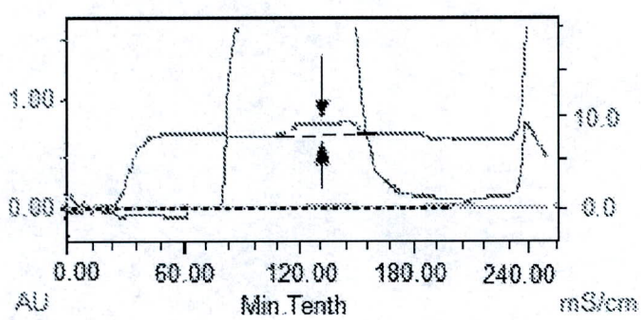
10. The medium according to claim 9, wherein said *E.coli* engineering bacteria with pET system is *E.coli* B834 (DE3), and the medium has water as solvent and comprises the following components: NaCl 6.0g/L, peptone 25.0g/L, yeast powder 7.5g/L, glucose 2.0g/L, $Na_2HPO_4 \cdot 7H_2O$ 6.8g/L, KH_2PO_4 3.0g/L, NH_4Cl 1.0g/L, $MgSO_4$ 0.2g/L and$CaCl_2$ 0.01g/L, methionine 0~40mg/L.

Drawings

FIG.1

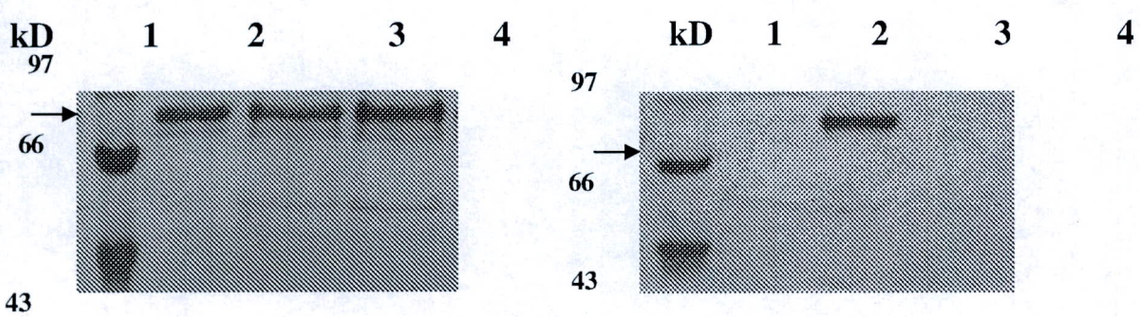


a



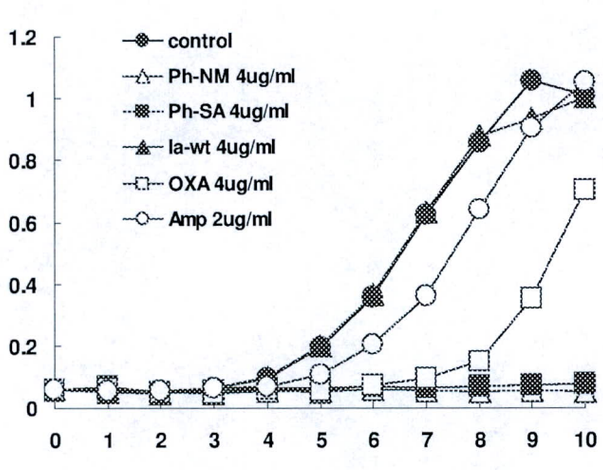
b

FIG.2


kD
1
2
3
4
97
66
43
kD
1
2
3
4
97
66
43

a b

FIG.3


control
Ph-NM 4ug/ml
Ph-SA 4ug/ml
Ia-wt 4ug/ml
OXA 4ug/ml
Amp 2ug/ml
1.2
1
0.8
0.6
0.4
0.2
0
0 1 2 3 4 5 6 7 8 9 10

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审查员 蔡玉品

权利要求书1页 说明书8页 附图2页

(54)发明名称

一种高效表达工程菌重组蛋白的方法及应用

(57)摘要

本发明“一种高效表达工程菌重组蛋白的方法及应用”,属于生物制药工程。该方法包括如下步骤:(1)重组突变质粒转染大肠杆菌pET系统工程菌中得到阳性单克隆菌落,(2)对阳性单克隆菌落进行增菌得到种子菌液,诱导所述种子菌液大量增菌生长,(3)分离含有表达的目的重组蛋白的菌体上清液,提纯菌体上清液中的重组蛋白,其特征在于:所述大肠杆菌pET系统工程菌指E.coli B834(DE3)。本发明还进一步对大量增菌培养基的成分及蛋白质纯化步骤进行了优化,在蛋白质产量和纯度上都得到了明显的提高。使本发明的方法适合于应用在大肠杆菌工程菌表达重组蛋白的规模化生产中。

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1. 一种高效表达工程菌重组蛋白的方法，包括如下步骤：

(1) 重组突变质粒转染大肠杆菌 pET 系统工程菌中得到阳性单克隆菌落，

(2) 对阳性单克隆菌落进行增菌得到种子菌液，诱导所述种子菌液大量增菌生长，

(3) 分离含有表达的目的重组蛋白的菌体上清液，提纯菌体上清液中的重组蛋白，

其特征在于：所述大肠杆菌 pET 系统工程菌指 E. coli B834(DE3)，步骤 (2) 中所述大量增菌生长的培养基为：NaCl 6.0 ～ 6.7g/L，蛋白胨 25.0g/L，酵母粉 7.5g/L，葡萄糖 0.6 ～ 2.0g/L，$Na_2HPO_4 \cdot 7H_2O$ 6.8 ～ 18.3g/L，KH_2PO_4 3.0 ～ 4.3g/L，NH_4Cl 1.0 ～ 1.4g/L，$MgSO_4$ 0.2 ～ 0.4g/L，$CaCl_2$ 0.01g/L，甲硫氨酸 40mg/L；

所述重组突变质粒指 pBHC-SA1、pBHC-SA2、pBHC-SA3 pBHC-SA4、pBHC-SE，pBHC-PA 或 pBHC-PorA1；

所述诱导采用热冲击诱导，操作方式如下：种子菌液入罐后，30℃起始生长 2 ～ 3 小时，测 OD 值达 0.4-0.6 时，于 42℃热冲击 30 分钟，然后将温度下调至 37℃，再生长 1.5-2 小时后收菌；

所述热冲击诱导步骤中，在所述培养基中加入终浓度为 0.5mM 的 IPTG。

2. 根据权利要求 1 所述的方法，所述大量增菌生长的培养基为 NaCl 6.0g/L，蛋白胨 25.0g/L，酵母粉 7.5g/L，葡萄糖 2.0g/L，$Na_2HPO_4 \cdot 7H_2O$ 6.8g/L，KH_2PO_4 3.0g/L，NH_4Cl 1.0g/L，$MgSO_4$ 0.2g/L，$CaCl_2$ 0.01g/L，甲硫氨酸 40mg/L。

3. 根据权利要求 1 所述的方法，所述提纯菌体上清液中的重组蛋白，采用 CM 离子交换柱，其中上样量为上清液蛋白质量 / 凝胶颗粒体积为 2.5mg/ml。

4. 根据权利要求 1 所述的方法，所述提纯菌体上清液中的重组蛋白步骤中，采用 CM 离子交换柱，洗脱所用的 NaCl 浓度 0.2M。

5. 权利要求 1 ～ 4 任一所述方法在制备重组多肽 PMC-SA1、PMC-SA2、PMC-SA3，PMC-SA4、PMC-SE，PMC - PA 或 PMC-AM 中的应用。

一种高效表达工程菌重组蛋白的方法及应用

技术领域

[0001] 本发明涉及生物技术领域，特别是一种高效表达工程菌重组蛋白的方法及其应用。

背景技术

[0002] 本发明的发明人在前期的研究中，经过创造性的尝试和实验验证，得到了以大肠菌素为攻击端，可操作地连接其它识别靶细胞的识别多肽（天然或人工设计的）得到一系列新型重组多肽，如申请号为200910092128.4，发明名称为“一种含抗体模拟物的新型抗生素及其制备方法与应用”中公开的新型抗生素PMC-AM1具有广谱抗生性能，对脑膜炎双球菌，多重耐药绿脓杆菌、耐万古霉素肠球菌、耐甲氧西林金葡菌都具有较现有抗生素效果明显的抗菌效果；申请号为200910157564.5，名称为“一种新型抗生素及其核苷酸序列、制备方法与应用”中公开了一系列抗葡萄球菌新型抗生素，如PMC-SA1、PMC-SA2、PMC-SA3、PMC-SA4、PMC-SE以及PMC-PA六种新型抗生素。在体外和体内试验中，表现出比现有抗生素、抗真菌抗生素、化疗药物更为优越的靶向性和杀伤效率，这些新型抗生素不仅抗菌效果明显，且具有其它现有抗生素不能比拟的生物安全性和抗耐药性。

[0003] 由于上述系列重组蛋白是一类以水溶性蛋白，一般由600余个氨基酸残基组成，但在该类蛋白的肽链中靠近羧基端有一段40个氨基酸残基组成的疏水区段。相较于其他结构单一的水溶性蛋白而言，该蛋白的装配和表达更为困难，不可避免地影响其生产率，改善上述重组蛋白的表达工艺流程，提高蛋白的表达率，解决了大规模生产以将上述系列重组蛋白推向实际临床应用和实践中存在的技术难题。

发明内容

[0004] 本发明针对发明人在现有专利申请中公开的重组多肽的结构和性质特点，提供一种高效率表达工程菌蛋白的方法。

[0005] 一种高效表达工程菌重组蛋白的方法，包括如下步骤：

[0006] (1) 重组突变质粒转染大肠杆菌pET系统工程菌中得到阳性单克隆菌落，

[0007] (2) 对阳性单克隆菌落进行增菌得到种子菌液，诱导种子菌液大量增菌生长，

[0008] (3) 分离含有表达的目的重组蛋白的菌体上清液，提纯菌体上清液中的重组蛋白，

[0009] 其特征在于：所述大肠杆菌pET系统工程菌指E.coli B834(DE3)。

[0010] 所述大量增菌采用的培养基为NaCl 6.0～6.7g/L，蛋白胨25.0g/L，酵母粉7.5g/L，葡萄糖0.6～2.0g/L，$Na_2HPO_4 \cdot 7H_2O$ 6.8～18.3g/L，KH_2PO_4 3.0～4.3g/L，NH_4Cl 1.0～1.4g/L，$MgSO_4$ 0.2～0.4g/L，$CaCl_2$ 0.01g/L，甲硫氨酸0～40mg/L。

[0011] 所述大量增菌采用的培养基为NaCl 6.0g/L，蛋白胨25.0g/L，酵母粉7.5g/L，葡萄糖2.0g/L，$Na_2HPO_4 \cdot 7H_2O$ 6.8g/L，KH_2PO_4 3.0g/L，NH_4Cl 1.0g/L，$MgSO_4$ 0.2g/L，$CaCl_2$ 0.01g/L，甲硫氨酸0～40mg/L。在以E.coli B834(DE3)为工程菌的工艺中，甲硫氨酸为40mg/L。

[0012] 所述诱导采用热冲击诱导，操作方式如下：种子菌液入罐后，30℃起始生长2～3

小时，测 OD 值达 0.4-0.6 时，于 42℃热冲击 30 分钟，然后将温度下调至 37℃，再生长 1.5-2 小时后收菌。

[0013] 所述热冲击诱导步骤中，在所述培养基中加入终浓度为 0.5mM 的 PTG。

[0014] 所述提纯菌体上清液中的重组蛋白，采用 CM 离子交换柱，其中上样量为上清液蛋白质量 / 凝胶颗粒体积为 2.5mg/ml。

[0015] 所述提纯菌体上清液中的重组蛋白，采用 CM 离子交换柱，洗脱所用的硼酸缓冲液中 NaCl 浓度为 0.2M。

[0016] 所述重组突变质粒指 pBHC-SA1、pBHC-SA2、pBHC-SA3 pBHC-SA4、pBHC-SE，pBHC-PA 或 pBHC-PorA1。

[0017] 上述任一方法在制备重组多肽 PMC-SA1、PMC-SA2、PMC-SA3，PMC-SA4、PMC-SE，PMC-PA 或 PMC-AM 中的应用。

[0018] Novagen 公司的 pET 系统是目前在大肠杆菌中克隆和表达重组蛋白的常用系统。本发明中首先将质粒转染入一系列 BL-21(DE3) 细胞，得到了比原 TG1 细胞更好的蛋白表达产率。通过实验数据对比，我们发现作为 BL-21(DE3) 的母本菌株 B834(DE3) 具有比 BL-21(DE3) 更理想的表达产率。试验数据证实质粒 B834(DE3) 可以达到超过原质粒 TG1 体系数十倍的蛋白表达产率。

[0019] 培养基为细菌生长繁殖提供所需的碳源、氮源和无机盐，本发明通过改进培养基的组分提高了工程菌的生长速率，从而提高目的蛋白的表达产率。通过逐步改进、调整培养基中各个成分的含量，找到工程菌发酵生产的最佳培养基配方。本发明中我们改进的 FB-M9 复合培养基适度提高了碳源和氮源的含量，加入了 MgSO4、CaC12、以及 pET 系统菌生长所需的氨基酸，适度提高了细菌的繁殖速率和蛋白表达效率。该改进培养基配方所需的成本较为低廉，这就为今后的大规模生产提供了较大的研究空间和开发价值。

[0020] 本发明中所采用纯化体系中的 CM 离子凝胶颗粒携载率并未达到产品手册中描述的理想水准，这限制了目的蛋白的回收率，本发明通过减少上样量、适度加大凝胶体积等手段，使回收率得到了明显的的改善。该结果提示仍需寻找效率更高的离子交换凝胶，以便进行更高效的大规模工业化生产。此外，本发明通过优化离子交换步骤中的洗脱液浓度，使得到的重组蛋白杂质更少。

[0021] 综合而言，本发明通过选择理想的工程菌株、优化培养基组成、改进纯化、回收效率等手段，提供了多种可选择的更优化的大肠杆菌工程菌表达重组蛋白的工艺，这同时也为最终找到高效表达所需融合蛋白的最优组合提供了一个可能的发展方向和技术路线。与原表达体系相比较，本研究所发展的表达体系将融合蛋白的表达产量提高了数十倍，为继后的大规模工业化生产提供了有益的理论及实践依据。

附图说明

[0022] 图 1 不同体积凝胶柱的蛋白洗脱过程洗脱液电导值

[0023] a. 150ml CM 凝胶柱的蛋白洗脱过程。b. 600ml CM 凝胶柱的蛋白洗脱过程。

[0024] 图中箭头所指曲线代表洗脱液的电导值，箭头所示部分即上样过程中流失的 PMC-SA 所造成的电导小峰。加大凝胶体积后，流失 PMC-SA 所造成的电导小峰面积减少了 70%。

[0025] 另外一条曲线：洗脱蛋白 OD 值，

[0026] 图 2. PMC-SA 的 SDS-PAGE 凝胶电泳图谱

[0027] 从左到右依次为：

[0028] a. 1. Marker、2. TG1 产 PMC-SA1、3. BL-21 产 PMC-SA1、4. B834 产 PMC-SA1；

[0029] b. 1. Marker、2. 0. 1M NaCl 硼酸缓冲液洗脱的 PMC-SA1、3. 0. 2M NaCl 硼酸缓冲液洗脱的 PMC-SA1、4. 0. 3 M NaCl 硼酸缓冲液洗脱的 PMC-SA1。

[0030] 图 3. PMC-SA 对 MRSA(BAA42) 的抑菌曲线

[0031] 纵坐标为吸光值，横坐标为菌生长时间

[0032] Control：对照组；Amp：氨苄西林钠；OXA：苯唑西林；Ia-wt：野生型大肠菌素；PMC-SA1：抗金黄色葡萄球菌多肽；PMC-AM：抗脑膜炎双球菌多肽。

具体实施方式

[0033] 通过以下实施例说明本发明。本发明采用的实验材和仪器如下：

[0034] 1. 菌株：

[0035] E. coli TG1 工程菌 (AECOM, K. Jakes 赠与)；

[0036] E. coli BL-21(DE3)、B834(DE3)、NovaBlue(DE3) 和 618 工程菌均购自 Novagen 公司；

[0037] 金黄色葡萄球菌 ATCC BAA-42 购自 ATCC(American Type Culture Collection)。

[0038] 质 粒：pBHC-SA1、pBHC-SA2、pBHC-SA3 pBHC-SA4、pBHC-SE，pBHC-PA，pBHC-PorA1(记载于申请号为 200910092128. 4，及 200910157564. 5 的两个发明申请的说明书中)。

[0039] 2. 主要试剂与药品：

[0040] 酵母粉 (OXIOD LP0021)，蛋白胨 (OXIOD LP0042)，及其它化学试剂均为国产分析纯；

[0041] 透析袋 Snake Skin Dialysis Tubing(Pierce，截留相对分子质量 1×104，Lot#KD32324)；

[0042] 注射用硫酸链霉素 (华北制药)；

[0043] 注射用氨苄西林钠 AMP(哈尔滨制药)；

[0044] 阴离子交换柱凝胶 (Pharmacia Biotech CM Sepharose Fast Flow LotNo. 225016)。

[0045] LB 液体培养基：100ml 氯化钠 1g，蛋白胨 1g，酵母 0. 5g，将各种试剂称好后放入 250ml 烧瓶中，加入 100ml 自来水，使其溶解，在高压灭菌锅内 120℃ 8min 灭菌

[0046] LB 固体培养基：100ml 氯化钠 0. 5 ～ 1. 5g，蛋白胨 0. 5 ～ 2g，酵母 0. 3 ～ 1g，琼脂 0. 8 ～ 3g，LB 固体培养基用于菌种复苏后划平板培养单克隆菌落用。将各种试剂称好后放入 250ml 烧瓶中，加入 100ml 自来水，使其溶解，在高压灭菌锅内 120℃ 8min 灭菌。

[0047] FB-M9 复合培养基：NaCl 6. 0 ～ 6. 7g/L，蛋白胨 25. 0g/L，酵母粉 7. 5g/L，葡萄糖 0. 6 ～ 2. 0g/L，$Na_2HPO_4 \cdot 7H_2O$ 6. 8 ～ 18. 3g/L，KH_2PO_4 3. 0 ～ 4. 3g/L，NH_4Cl 1. 0 ～ 1. 4g/L，$MgSO_4$ 0. 2 ～ 0. 4g/L，$CaCl_2$ 0. 01g/L，甲硫氨酸 0 ～ 40mg/L。

[0048] 改良的 FB-M9 复合培养基：NaCl 6. 0g/L，蛋白胨 25. 0g/L，酵母粉 7. 5g/L，葡萄糖

2.0g/L，$Na_2HPO_4 \cdot 7H_2O$ 6.8g/L，$KH_2PO_4$3.0g/L，NH_4Cl 1.0g/L，$MgSO_4$0.2g/L，$CaCl_2$0.01g/L，甲硫氨酸 0 ～ 40mg/L。在以 E.coli B834(DE3) 为工程菌的工艺中，甲硫氨酸为 40mg/L

[0049] 3. 主要仪器：

[0050] Bio-Rad 蛋白质层析纯化系统 (BioLogic Duo Flow，BioLogic Maximizer，BioLogic QuadTecUV-Vis Detector，BioLogic Econo Pump)；

[0051] 超声破碎仪 (Soniprep 150)，直径 5cm 蛋白纯化离子交换柱 (Pharmacia Biotech XK50)，直径 11cm 蛋白纯化离子交换柱（上海华美）；

[0052] 离心机 (Beckman Coulter Avanti J-20XP，Beckman Coulter Avanti J-25)；

[0053] 分光光度计 (Bio-Rad Smart Spect Plus spectrophotometer)；

[0054] 全自动发酵罐（瑞士比欧 LP351-42L)；

[0055] 细菌高压均质机（意大利 Niro Soavi NS1001L2KSN 6564)。

[0056] 声明：本发明所采用的生物材料都为申请日前已知的，且本单位已有保存，保证自申请日起二十年内向公众发放用于验证试验。

[0057] 实施例 1. 工程菌菌种的优化选择

[0058] 载有大肠菌素 Ia 及其免疫蛋白基因 (GenBank M13819) 的经典质粒来自于 Dr.Finkelstein 实验室 (Qiu XQ et al.An engineered multidomain bactericidal peptide as a model for targetedantibiotics against specific bacteria.Nat Biotechnol，2003；21(12)：1480-1485)，经本实验室改造后得到 7 种重组突变质粒：pBHC-SA1、pBHC-SA2、pBHC-SA3 pBHC-SA4、pBHC-SE，pBHC-PA，pBHC-PorA1。

[0059] 步骤 1. 转化感受态细胞

[0060] 将重组突变质粒 pBHC-SA1 100ng 分别转染入 4040ul Novagen 数种 pET 系统工程菌 BL-21(DE3)、B834(DE3)、Nova Blue(DE3)、618 中，冰孵 5 分钟，42℃热冲击 30 秒，再置入冰中 2 分钟，加入 SOC 培养基 160ul，220rpm，37oC 摇菌 1 小时后铺板 (LB 培养基加 1％琼脂，加 50ug/ml 氨苄青霉素，37℃过夜)，挑取单克隆菌落增菌，获得菌种，低温保存菌种；

[0061] 步骤 2. 菌种复苏

[0062] 1. 菌种复苏

[0063] 取出保存菌种 4℃解冻，取 1.5ml 倒入 10ml LB 培养基中（含 AMP 50μg/ml)，220rpm、37℃培养 5-8 小时。

[0064] 2. 接种单克隆菌

[0065] 复苏后的菌液稀释 10^4 或 10^5 倍，取 10ul 稀释后的菌液加入到制备好的 LB 固体培养基 (AMP 50μg/ml) 平板上，涂平板。放湿盒中，37℃恒温培养箱培养 10-12 小时，培养基表面长出圆形单菌落。

[0066] 步骤 3. 挑菌和扩菌

[0067] (1) 用已灭菌牙签或接菌环从长好的平板上挑选规则圆形，边缘光滑的单菌落，放入 1.5mlLB 培养基中，振荡培养：220rpm、37℃培养 5-8 小时。

[0068] (2)1.5mlLB 菌液翻至 100ml LB 培养基中，振荡培养：220rpm、37℃培养 5-8 小时。

[0069] (3) 一级扩大培养：将上步的 100ml 菌液加入到 700ml 改良的 FB-M9 复合培养基中培养，振荡培养：220rpm、37℃培养 5-8 小时。

[0070] (4) 二级扩大培养：将上步 700ml 菌液分别加入到 6×700ml 改良的 FB-M9 复合培

养基中，振荡培养：220rpm、37℃培养 5-8 小时。

[0071] (5) 三级扩大培养：将上步 6×700ml 菌液加入到 20L 改良的 FB-M9 复合培养基中，在发酵罐中培养：搅拌速度 220rpm、最大通氧量，37℃培养 3-5 小时。

[0072] (6) 工程菌发酵和诱导表达蛋白：将上步 20L 菌液加入到 200L 改良的 FB-M9 复合培养基中，在发酵罐中培养和诱导表达蛋白：搅拌速度 220rpm，最大通氧量，30℃，2 ～ 4 小时；42℃，0.5 小时；37℃，1 ～ 2 小时，注：达到 42℃时加入终浓度为 0.5mM 的 IPTG。

[0073] 步骤 4、离心收菌

[0074] 培养液 6000g，4℃离心 20min。收取离心后的沉淀，放入 50mM 的硼酸缓冲液 (pH9.0) 中，使菌体重悬浮于硼酸缓冲液中，注：硼酸缓冲液中加入 2mM 的 PMSF（中文名：苯甲基磺酰氟丝氨酸蛋白酶抑制剂，自菌体重悬浮之后的操作须在 4℃进行。

[0075] 步骤 5、破碎菌体

[0076] 待菌体完全悬浮于 pH9.0 硼酸缓冲液后，用高压匀质机 500 ～ 600bar 高压破碎菌体，反复破碎 7 次，每次破菌间隔 3 ～ 5 分钟。

[0077] 步骤 6、沉淀菌体 DNA

[0078] 将破碎后的菌液，55000g，4℃离心 40min。取上清液，加入硫酸链霉素（每 200ml 液体加入 16 瓶 100 万单位的硫酸链霉素），在磁力搅拌器上搅拌 1h。

[0079] 步骤 7、透析

[0080] 将上步的菌液 55000g，4℃离心 20min，取上清液，装入透析袋中，置硼酸缓冲液中透析 8h ～ 12 小时，每 4h 换透析液一次。

[0081] 步骤 8、蛋白药物纯化得到抗菌工程多肽药物

[0082] 将透析后的菌液 55000g，4℃离心 20min，取上清液放入烧杯中，采用离子交换法纯化蛋白。取上清上样于 CM 离子交换柱，测蛋白浓度计算单位体积中蛋白质含量，上样量与 CM 离子凝胶颗粒的比例按操作手册，充分冲洗后，用含 0.2M NaCl 的 50mM 硼酸缓冲液洗脱得到新型抗菌工程多。

[0083] 结果如表 1 所示，E.coli B834(DE3) 对于 PMC-SA 的表达效率是最高的。

[0084] 表 1 菌株表达效率比较（平均单位产量＝提取的 PMC-SA1 的总产量 / 菌液体积）

[0085]

工程菌种	TG1	BL-21	618	NavaBlue	B834
平均单位产量（mg/L）	0.8	10	5.8	8.1	24.4

[0086] 对于其余 6 种重组突变质粒进行相同操作和比较，结果均与表 1 表示的结果出现相同的趋势，即相较于其余的工程菌，E.coli B834(DE3) 对于这 7 种重组突变质粒的表达效率都是最高的。

[0087] 本实施例中，采用的与过去不同的生长诱导方法为热冲击诱导：即种子菌液入罐后，大规模增菌的起始温度为 30℃，待菌液生长 2 小时左右，OD 值达到 0.4-0.6 时，42℃热冲击 30 分钟，热冲击结束后，将温度下调至 37℃，再生长 1.5-2 小时后即可收菌，此时增殖菌液 OD 值可达 1-3 甚至更高。时还加入了 IPTG 来诱导 pET 系列工程菌，加入量为 0.5mM。

[0088] 在本申请之前，常用的生产制备上述重组多肽的方法如下：

[0089] 将突变质粒 100ng 与制备的 BL-21 工程菌感受态细胞 40ul 冰孵 5 分钟，热冲击 42℃，30 秒，再置入冰中 2 分钟，加入 SOC 培基 160ul，220rpm，37℃摇菌 1 小时后铺板 (LB

培基加 1%琼脂，加 50ug/ml 氨苄青霉素，37℃过夜），挑取单克隆菌落大量增菌；

[0090] 大量增菌：8-10 升 FB 培养基，250rpm，37℃，3-4 小时；加入 IPTG，250rpm，28℃再生长 4 小时；4℃，6000g，20 分钟离心沉淀菌体，取 4℃，50mM 硼酸缓冲液（pH 9.0，2mM EDTA）80-100ml 悬浮菌体，加入 PMSF 50ug 后超声破碎菌体（4℃，400W，1 分钟，重复 4-5 次，间歇 2-3 分钟确保菌液温度），高速离心沉淀破碎的菌体（4℃，75,000g，90 分钟），取上清加入硫酸链霉素 500 万单位沉淀 DNA（4℃搅拌 1 小时），10,000g，4℃，10 分钟离心沉淀后，取上清装入分子量 15,000 透析袋于 4℃，50mM 硼酸缓冲液 10 升透析过夜后，再次 10,000g，4℃，10 分钟离心沉淀，取上清上样于 CM 离子交换柱，充分冲洗后，0.3M NaCl+50mM 硼酸缓冲液洗脱即可得到所制备的新型抗生素。

[0091] 实施例 2. 改进培养基

[0092] 经典的大肠菌素 Ia 生长培养基为 FB 培养基（Qiu XQ et al. An engineered multidomainbactericidal peptide as a model for targeted antibiotics against specific bacteria. Nat Biotechnol, 2003；21(12)：1480-1485/Karen Jakes, Charles Abrams, Alan Finkelstein, et al. Alteration of thepH-dependent Ion Selectivity of the Colicin E1 Channel by Site-directed Mutagenesis. JBC, 1990；265(12)：6984-6991）。其配方如下：25g/L 蛋白胨，7.5g/L 酵母粉，6g/L NaCl，1g/L 葡萄糖。

[0093] 在本发明中，我们采了不含葡萄糖的 FB 培养基，配方为：蛋白胨 25.0g/L，酵母粉 7.5g/L，NaCl 6.0g/L，并将上述无糖配方的 FB 培养液与 M9 培养液以一定体积例复合配置，从而得到了本发明的 FB-M9 复合培养基。

[0094] M9 培养基母液为 5×M9，其配方为：$Na_2HPO_4 \cdot 7H_2O$ 64.0g/L，KH_2PO_4 15.0g/L，NH_4Cl 5.0g/L，NaCl 2.5g/L，$MgSO_4$ 1.5g/L，$CaCl_2$ 0.05g/L，2%葡萄糖。

[0095] 初步尝试两种培养基的复合：

[0096] FB-M9：FB:M9 的体积比＝7：10，配方为：NaCl 6.7g/L，蛋白胨 25.0g/L，酵母粉 7.5g/L，Na2HPO4 • 7H2O 18.3g/L，KH2PO4 4.3g/L，NH4Cl 1.4g/L，MgSO4 0.4g/L，CaCl2 0.01g/L，葡萄糖 0.6g/L。

[0097] 由于 B834 工程菌生长中需要甲硫氨酸，因此在以 B834 为工程菌的工艺中，FB-M9 培养基中还加入了甲硫氨酸（40mg/L）。

[0098] 本发明中用此配方进行发酵生产，发酵步骤同实施例 1，结果显示如表 2，每升培养液所得到的湿菌重量显著高于仅用 FB 培养基所得到的湿菌重量，所回收的蛋白产量明显提高，平均产量可达到 30mg/L。

[0099] 表 2 所试培养基目的蛋白产量对比（PMC-SA1/BL-21 工程菌）

[0100]

	用FB培养基发酵		用FB-M9（7：10）培养基发酵	
	菌重g	蛋白含量mg	菌重g	蛋白含量mg
1	255.07	280.8	349.82	847.82
2	246.3	519.94	343.47	643.71
3	302.28	461.965	366	779.3
4	276.67	465.179	388.44	946.34
平均值	270.8	431.971	361.9325	804.2925

[0101] 本发明进一步通过反复对比，得到最终改良的FB-M9培养基，在如实施例1的发酵条件下，可达到目的蛋白产率34mg/L，如表3。

[0102] 该改良的FB-M9配方为：NaCl 6.0g/L，蛋白胨25.0g/L，酵母粉7.5g/L，葡萄糖2.0g/L，$Na_2HPO_4 \cdot 7H_2O$6.8g/L，$KH_2PO_4$3.0g/L，NH_4Cl 1.0g/L，$MgSO_4$0.2g/L，$CaCl_2$0.01g/L。在以B834为工程菌的培养基中加入甲硫氨酸40mg/L。

[0103] 表3 改良FB-M9培养基与其他培养基产率比较

[0104]

工程菌种及采用的培养基	BL-21			B834		
	FB	FB-M9（7：10）	改良FB-M9	FB	FB-M9（7：10）	改良FB-M9
平均单位产量（mg/L）	10	24	25	22.3	30	34

[0105] 实施例3 优化蛋白纯化条件

[0106] 本发明中制备的重组多肽（PMC-SA1、PMC-SA2、PMC-SA3，PMC-SA4、PMC-SE，PMC-PA，PMC-AM）之基本结构为大肠菌素Ia，而大肠菌素Ia的等电点约为9.15，因此其经典纯化方式采用了离子亲和层析交换法（Qiu XQ et al. An engineered multidomainbactericidal peptide as a model for targeted antibiotics against specific bacteria. Nat Biotechnol，2003；21(12)：1480-1485）。

[0107] 其工作原理为：在pH9.0的硼酸缓冲液体系中，绝大部分的PMC-SA分子都以正电荷离子的形式存在，在层析柱中流经带负电荷的CM凝胶颗粒时，带正电荷的上述重组蛋白分子即因电荷间的吸引力而挂在了带负电荷的CM凝胶颗粒上。其他杂蛋白则被次第冲出凝胶柱。

[0108] 本实施例中，其它步骤同实施例1，待杂蛋白冲洗干净后，分别以带有0.1-0.3M NaCl的硼酸缓冲液对凝胶柱进行梯度洗脱。

[0109] 由于Na^+离子较重组蛋白分子离子的正电荷性更强，于是重组蛋白离子被Na^+离子自CM凝胶颗粒上置换下来。在离子交换纯化中有两个变量可以操纵，从而得到更好的目的蛋白回收率：①. 可以在0.05-1M范围内选择不同浓度的NaCl，分别洗脱CM凝胶颗粒上挂载的、带有不同正电荷强度的蛋白分子。②所用CM凝胶颗粒的量可以进一步优化：在一定的离子强度环境中，每个CM凝胶颗粒携载的蛋白数量是相对恒定的，要想加大凝胶柱对蛋

白的携载量，则必需加大凝胶柱的体积。

[0110] CM Sepharose Fast Flow 是 GE 公司生产的阴离子交换柱凝胶，根据其使用手册可知每 100ml 凝胶最多可结合 9mM 阳离子。动态结合过程中实际可用的结合容量随样品的性质变化，分子量与结合容量呈反比关系。其标准样品中与本发明中制备的重组多肽的分子量较接近的是 Bovine COHb-(Mr69kD)。其理论动态结合容量为 30mg/ml。即用 100ml 容量的 CMSepharose Fast Flow 胶回收重组蛋白分子样品时，理论上其最高回收率大约 300mg(0.004mM)，按其使用手册操作，本发明中 CM 凝胶颗粒与重组蛋白分子之间的实际动态结合容量是 3mg/ml，只达到理论值的 10%。

[0111] 在试验中，我们发现在杂蛋白冲洗过程的后半期中，电导曲线会出现一过性的小上升峰（如图 1a 所示）。根据此现象我们推测，当样品中重组蛋白量较大时，由于 CM 凝胶颗粒与目的蛋白结合容量有限，即只能回收样品中重组蛋白分子的一部分。未能挂载在 CM 凝胶颗粒上的重组蛋白随杂蛋白一并被冲洗出凝胶柱。由于重组蛋白带正电荷，所以出现了电导曲线的短暂上升峰。

[0112] 本发明的优化方案中：为减少目的重组蛋白流失，我们将每批上样量减为使用手册规定的 1/3，同时加大所用凝胶的体积，胶体积由 150ml 增加到 600ml，即上清液中蛋白质量：凝胶颗粒体积＝ 2.5mg/ml，洗脱过程中流失的重组蛋白减少，实验数据显示重组蛋白的回收率提高了 3.5 倍，结果由图 1b 所示。

[0113] 此外，我们将洗脱所用硼酸缓冲液中的 NaCl 梯度设为 0.1M-0.2M-0.3M，0.2M 的洗脱效率和蛋白纯度最高，由图 2b 所示。

[0114] 实施例 4. 蛋白纯度及活性检测

[0115] 步骤 1. SDS-PAGE 电泳

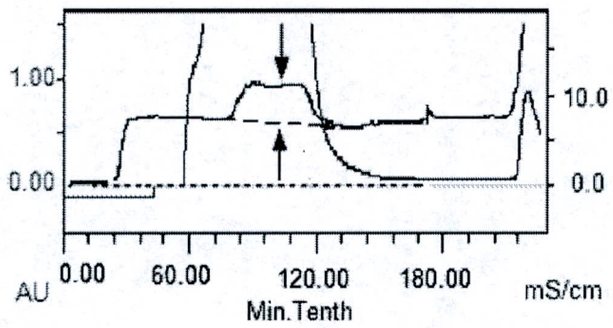
[0116] 对实施例 4 优化方法提取的融合蛋白样品进行 SDS-PAGE 电泳，硝酸银染染色。如图 2 所示，电泳图谱 a 中，在相对分子质量约为 70kD 处有一清晰的蛋白印记条带，即为本发明制备的 PMC-SA1。图谱 b 显示通过实施例 4 中改进的梯度洗脱方法收到的蛋白消除了杂带，纯度提高。本发明的优化方法制备得到其余六种重组蛋白在纯度上显示出同样的提高。

[0117] 步骤 2. 抑菌活性检测

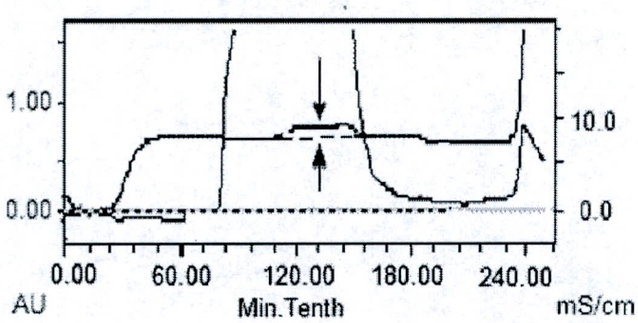
[0118] 经实施例 1、2、3 改进制备方法后制备得到的重组蛋白 PMC-SA1 和 PMC-AM，我们进行了以下抗菌活性测试。

[0119] 在 10ml BM 培养基中接种耐甲氧西林金黄色葡萄球菌 (MRSA，ATCC BAA-42) 菌液 10ul(10^5CFU/ml)，加入抗菌药物，根据抗菌药物分为 6 个平行组：氨苄西林钠 2ug/ml，苯唑西林 4ug/ml，野生型大肠菌素 Ia，PMC-SA 和 Ph-NM(4ug/ml)，空白对照组。37℃，210rpm 振荡培养，每小时测定菌液光密度值 (595nm)，绘制生长曲线图，如图 3。

[0120] 抑菌试验曲线显示经本发明的改进方法所制备的重组多肽具有良好的抗菌活性。

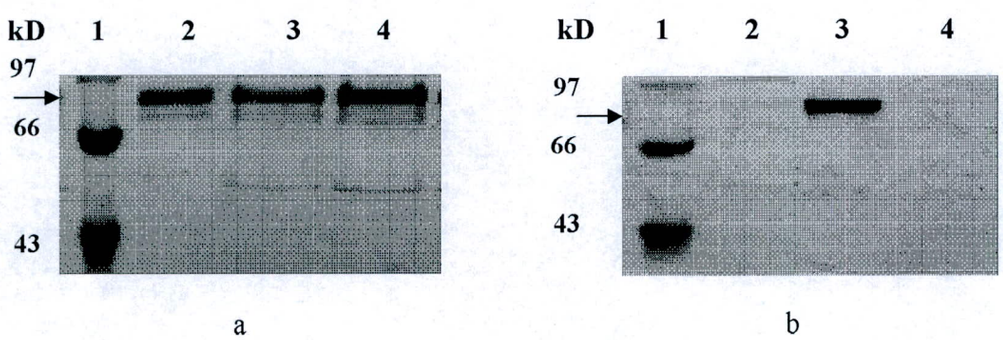

1.00
0.00
10.0
0.0
AU
0.00
60.00
120.00
180.00
mS/cm
Min.Tenth

a


1.00
0.00
10.0
0.0
0.00
60.00
120.00
180.00
240.00
AU
Min.Tenth
mS/cm

b

图 1


kD
1
2
3
4
97
66
43
kD
1
2
3
4
97
66
43

a b

图 2

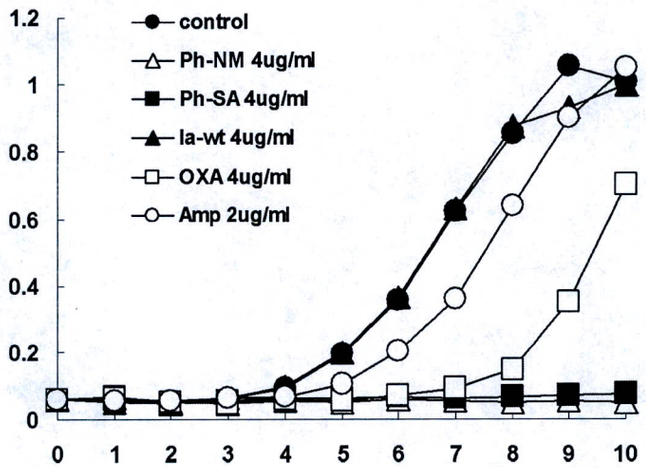

1.2
1
0.8
0.6
0.4
0.2
0
0 1 2 3 4 5 6 7 8 9 10
control
Ph-NM 4ug/ml
Ph-SA 4ug/ml
la-wt 4ug/ml
OXA 4ug/ml
Amp 2ug/ml

图 3

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权利要求书 1 页 说明书 16 页 附图 4 页

(54)发明名称

一种含抗体模拟物的新型抗生素及其制备方法与应用

(57)摘要

本发明属于生物医药领域，特别是涉及“一种含抗体模拟物的新型抗生素及其制备方法与应用”，一种含抗体模拟物的新型抗生素，由大肠菌素 E1、Ia、Ib、A、B、N 或其水性孔道结构域及抗体模拟物构成，所述抗体模拟物是由免疫球蛋白的 V_HCDR1 的羧基端连接 V_HFR2 的氨基端，V_HFR2 的羧基端再连接 V_LCDR 的氨基端构成；所述免疫球蛋白特异性识别细菌膜孔蛋白。该抗生素的杀菌能力是常用抗生素千倍以上，由于其作用机理特殊，因此致病菌很难产生耐药性，杀菌过程中不会伤害人体正常细胞；可用于制备抗抗脑膜炎双球菌、抗耐万古霉素肠球菌、抗耐甲氧西林金葡菌或抗多重耐药绿脓杆菌的药物中。

CN 101633699 B

1. 一种含抗体模拟物的新型抗生素，由大肠菌素 E1、Ia、Ib、A、B、N 或其水性孔道结构域及共价连接在所述大肠菌素或其水性孔道结构域的肽链羧基端的抗体模拟物构成，所述抗体模拟物的氨基酸序列如 Seq ID No. 2 所示。

2. 根据权利要求 1 所述的新型抗生素，所述大肠菌素为 Ia。

3. 一种肽链分子，其特征在于由大肠菌素 E1、Ia、Ib、A、B、N 或其水性孔道结构域的肽链及共价结合在其羧基端的抗体模拟物肽链连接而成，所述抗体模拟物肽链的氨基酸序列如 Seq ID No. 2 所示。

4. 根据权利要求 3 所述肽链分子，其氨基酸序列如 Seq ID No. 6 所示。

5. 编码权利要求 3 或 4 所述肽链分子的核苷酸序列。

6. 根据权利要求 5 所述核苷酸序列，如 Seq ID No. 5 所示。

7. 包含权利要求 5 所述核苷酸序列且能表达权利要求 1 所述的新型抗生素的重组表达载体。

8. 权利要求 1 或 2 所述的新型抗生素的制备方法，指将权利要求 7 所述的重组表达载体导入到表达系统中进行表达；分离纯化表达的多肽获得抗生素。

9. 权利要求 1 或 2 所述的新型抗生素在制备抗细菌药物中的应用。

10. 根据权利要求 9 所述的应用，所述抗细菌药物指抗脑膜炎双球菌、抗耐万古霉素肠球菌、抗耐甲氧西林金葡菌或抗多重耐药绿脓杆菌的药物。

一种含抗体模拟物的新型抗生素及其制备方法与应用

技术领域

[0001] 本发明涉及生物医药领域，特别涉及一种含抗体模拟物的新型抗生素及其制备方法与应用。

背景技术

[0002] 自 1944 年青霉素等抗生素投入使用以来，不仅仅是脑膜炎双球菌，其它很多威胁生命的致病菌，如金葡球菌、肺炎链球菌、绿脓杆菌、结核杆菌都已对其产生了耐药性。据美国疾病控制中心 (CDC) 历年发表的有关报告预测，再过 10 年至 20 年，这些抗生素将可能完全失效。

[0003] 目前常用的抗生素主要通过抑制细胞壁合成、抑制或干扰细菌的核酸和蛋白质代谢与合成途径来达到抗菌目的。然而这些抗菌方式容易诱导细菌发生突变而产生耐药性。因此人们一直在致力于开发新型的抗生素。模仿同种异株细菌之间互相杀伤的工作方式来开发新型抗生素是比较有前途的方向之一。自然界中有不少细菌毒素直接在细菌胞膜上形成离子通道来杀死细菌。其模式标本就是大肠杆菌分泌的一种细菌毒素 - 大肠菌素。其中大肠菌素 Ia 自 1952 年被 Jacob 发现之后，经过数代人的努力，1996 年终于揭示了大肠菌素 Ia 在人工脂质双分子膜上所形成离子通道开放和关闭时的跨膜立体结构 (Qiu et al, Major transmemebranemovement soociated with colicin Ia channel gating. J. Gen. Physiology, 107 :313-328(1996))，为在分子水平上设计和制备新型的抗菌素奠定了理论基础。

[0004] 如上所述，大肠菌素是一种理想的离子通道抗生素原型，但野生型大肠菌素只能作用于同种异株的大肠杆菌，因此必需改变它的靶向性，才能使大肠菌素转而攻击其它致病菌 ；膜孔蛋白 Porin 是存在于细菌质膜的外膜、线粒体和叶绿体的外膜上的通道蛋白，它们允许较大的分子通过，，具有较强的免疫原性，能在宿主体内诱导出特异性的高水平的单抗，如能利用对抗细菌表面 Porin 蛋白的抗体作为原型，设计出具有更理想识别性能的抗体模拟物，用这个模拟物来改变大肠菌素的靶向性，应该是一种理想的抗生素开发方向。

发明内容

[0005] 本发明针对上述领域的空白，提供一种的新型抗生素，该抗生素的杀菌能力是常用抗生素千倍以上，由于其作用机理特殊，因此致病菌很难产生耐药性，杀菌过程中不会伤害人体正常细胞。

[0006] 一种含抗体模拟物的新型抗生素，由大肠菌素 E1、Ia、Ib、A、B、N 或其水性孔道结构域及共价连接在所述大肠菌素或其水性孔道结构域的肽链羧基端的抗体模拟物构成，所述抗体模拟物是由免疫球蛋白的 V_HCDR1 的羧基端连接 V_HFR2 的氨基端，V_HFR2 的羧基端再连接 V_LCDR 的氨基端构成 ；所述免疫球蛋白特异性识别细菌膜孔蛋白。

[0007] 所述细菌指脑膜炎双球菌，所述膜孔蛋白指脑膜炎双球菌表面的膜孔蛋白 PorA。

[0008] 所述免疫球蛋白指在具有 PUBMED 登录号为 2MPA_H 所记载的氨基酸序列的肽链与

具有登录号为 2MPA_L 所记载的氨基酸序列的肽链共价结合而得的抗体蛋白。

[0009] 所述大肠菌素为 Ia。

[0010] 一种肽链分子，其特征在于由大肠菌素 E1、Ia、Ib、A、B、N 或其水性孔道结构域的肽链及共价结合在其羧基端的抗体模拟物肽链连接而成，所述抗体模拟物肽链由免疫球蛋白的三个区域 V_HCDR1、V_HFR2、V_LCDR3 的肽链顺次以羧基端连接下一区域肽链的氨基端构成；所述免疫球蛋白特异性识别细菌膜孔蛋白。

[0011] 所述肽链分子，具有 Seq ID No.6 所示氨基酸序列。

[0012] 编码上述肽链分子的核苷酸序列。

[0013] 所述核苷酸序列，如 Seq ID No.5 所示。

[0014] 包含上述核苷酸序列的重组表达载体。

[0015] 上述的新型抗生素的制备方法，指将上述的重组表达载体导入到表达系统中进行表达；分离纯化表达的多肽获得抗生素。

[0016] 所述的新型抗生素在制备抗细菌药物中的应用。

[0017] 所述抗细菌药物指抗脑膜炎双球菌、抗耐万古霉素肠球菌、抗耐甲氧西林金葡菌或抗多重耐药绿脓杆菌的药物。

[0018] 本发明利用大肠菌素能在靶细菌膜上形成离子通道，使靶细胞内胞质泄漏而死，而本发明中起靶向性作用的结构是对抗细菌表面膜孔蛋白即 Porin 蛋白的免疫球蛋白的部分区域被重构后获得的模拟抗体物，包含该免疫球蛋白的重链可变区的第一互补决定区 V_HCDR1、重链可变区的第二骨架区 V_HFR2、轻链可变区的第三互补决定区 V_LCDR3，三个区域顺次以羧基端链接下一区域的氨基端构成 V_HCDR1-V_HFR2-V_LCDR3 的线性分子；众所周知，免疫球蛋白起识别作用的活性区域称为互补决定区，互补决定区仅有几个到十几个氨基酸组成，与完整的免疫球蛋白或者目前有报道的单链抗体 seFv、Fab 等人工改造抗体相比，本发明的分子量极小，组织透过性好，同时由于其结构简单，去除了完整免疫球蛋白分子的大部分框架结构及 Fc 段，使其对患者的免疫原性大大降低，非常利于带领大肠菌素到达致病部位识别致病菌。在治疗中，大肠菌素被引向致病菌细胞膜表面，大肠菌素在靶细菌膜上形成离子通道，使靶细胞内胞质泄漏而死，对于目前已经产生耐药性的菌株也具有相同的杀菌能力。由于其识别位点为细菌表面特有的抗原物质，人体组织细胞膜上不存在这样的识别位点，因此其对于人体是安全的。相对于其他易于产生耐药性的抗生素，本发明的抗生素，由于其起靶向作用的抗体模拟物仅仅需将大肠菌素引向致病菌就完成了任务，我们并不需要通过膜孔蛋白位点作用致死细菌，而是通过大肠菌素作用于细菌的生物膜，使生物膜产生离子通道导致细胞泄漏而死，传统的细菌耐药性一般通过改变膜孔蛋白的结构对抗生素进入细菌细胞内造成障碍而产生耐药性，而本发明仅仅需要膜孔蛋白的抗体识别位点的活性即可到达杀菌的目的，可以说，是一种声东击西的策略，即识别膜孔蛋白位点，但大肠菌素结合在细胞膜的另外一个位点形离子通道致死细菌，真正的作用位点不在 Porin 蛋白，因此细菌很难通过变异、进化而丢掉或改变膜孔蛋白这种生存必须的结构，因此细菌很难对本发明的抗生素产生耐药性。由于本发明根据不同细菌表面膜孔蛋白的多样性及与其识别的免疫球蛋白的多样性，根据本发明这一设计构思获得的新型抗生素也是多样的。

[0019] 由于脑膜炎双球菌引起的脑膜炎对国内外的婴儿以及青少年的健康造成了极大的威胁，且其对目前常用药的抗药性表现非常明显，为了有效抑制该菌而使药剂量越来越

高，这严重危害患者健康，因此，发明人采用本发明的构思，针对脑膜炎双球菌的膜孔蛋白 Porin A 的抗体，其重链肽链的 PUBMED 登录号为 2MPA_H，轻链肽链的 PUBMED 登录号为 2MPA_L，进行重构获得抗体模拟物，即如 Seq ID No.2 所示的氨基酸序列；并可操作地连接在大肠菌素 Ia 的肽链的羧基端，获得具有如 Seq ID No.6 所示的氨基酸序列的新型抗生素 PMC-AM1。如图 7 所示，在脑膜炎双球菌致死量感染小鼠的存活实验中，注射本发明抗生素 PMC-AM1 的小鼠的 8 天存活率为 90%，说明本发明的抗生素比现用抗生素青霉素、庆大霉素等具有无法比拟的抗菌活性和体内保护效果；同时，通过杀菌效果比较，如图 6A 所示，比较本发明的抗生素 PMC-AM1 与头孢它啶、氨苄青霉素对脑膜炎双球菌的最低抑菌浓度 (MIC 值)，实验结果显示 PMC-AM1 的 MIC 0.11nMol，头孢它啶的 MIC 3.02nMol，氨苄青霉素的 MIC1.35nMol；说明其杀菌能力明显高于目前常用于抑制脑膜炎双球菌的抗生素。

[0020] 发明人用本发明的新型抗生素 PMC-AM1 对其他目前产生耐药性严重的致病菌进行试探性实验，发现 PMC-AM1 还对多重耐药绿脓杆菌、耐万古霉素肠球菌、耐甲氧西林金葡菌有极强的抗菌效力，如图 5 所示，PMC-AM1 对多重耐药绿脓杆菌的杀菌效果比头孢它啶、左氧氟沙星、庆大霉素等现用抗菌素强 127 ～ 3800 倍以上；PMC-AM1 对耐万古霉素肠球菌、耐甲氧西林金葡菌有明显的抑制效果，如图 6B、C 所示。

[0021] 本发明的抗生素可用于制备抗菌药物，特别是在制备抗脑膜炎双球菌、抗绿脓杆菌、抗耐万古霉素肠球菌及抗耐甲氧西林金葡菌的药物中的应用。

[0022] 可将编码本发明抗生素的核苷酸克隆到表达载体中构建重组表达载体，这些载体可在宿主中表达融合蛋白，分离融合蛋白获得本发明的抗生素蛋白。

[0023] 根据密码子的兼并性，编码本发明的抗生素或者抗体模拟物的核苷酸序列是可调整的，可根据宿主细胞对密码子的偏好性调整核苷酸序列，只要编码的氨基酸不变，都属于本发明要求保护的范围。

附图说明

[0024] 图 1 含抗体模拟物和大肠菌素 Ia 的重组质粒 pBHC-PorA1 的结构

[0025] 其中抗体模拟物的肽链连接在 Ia 的肽链羧基端，抗体模拟物的氨基酸序列如 Seq IDNo.2 所示。

[0026] 图 2 含抗体模拟物肽链和大肠菌素 Ia 的重组质粒 pBHC-PorA2 的结构

[0027] 其中抗体模拟物肽链连接在 Ia 的羧基端，抗体模拟物肽链是：重链可变区的第一互补决定区的羧基端链接重链第二骨架区，轻链可变区的第三互补决定区的肽链羧基端氨基酸链接在重链第二骨架区的羧基端氨基酸上，如 Seq ID No.4 所示。

[0028] 图 3 本发明新型抗生素的结构

[0029] 其中 T 和 R 是大肠菌素 Ia 位于氨基端的两个信号识别结构域；channel-forming 是大肠菌素 Ia 位于羧基端的形成离子通道结构域；AM 是抗体模拟物。

[0030] 图 4 为本发明新型抗生素 PMC-AM1 对脑膜炎双球菌的抑制实验

[0031] 图中曲线从左到右依次为对照，5μg/ml 氨苄青霉素，5μg/ml PMC-AM2，5μg/mlPMC-AM1，10μg/ml PMC-AM1。

[0032] 本图横座标为细菌生长时间，单位为小时；纵座标为培养液 600nm 光密度，显示细菌生长数量。

[0033] 图 5. 琼脂二倍稀释法测定新型抗生素的最低抑菌浓度 (MIC)

[0034] 平皿显示 :(Con),空白对照,(A),头孢它啶为 16μg/ml、(B),左氧氟沙星为 8μg/ml、(C),庆大霉素大于 512μg/ml、(D),新型抗生素 (PMC-AM1) 对多重耐药绿脓杆菌的最低抑菌浓度为 8μg/ml。

[0035] 图 6. 本发明新型抗生素与常用抗菌素对耐甲氧西林金葡菌 (ATCC BAA-42)、耐万古霉素肠球菌 (ATCC 700802)、多重耐药绿脓杆菌 (华西医院临床分离株 13578) 和脑膜炎双球菌 (中国菌种保存中心 29332) 最小抑菌浓度的比较实验

[0036] 纵座标为最小抑菌浓度 (nMol) ;

[0037] 其中 A 图为脑膜炎双球菌, (1)PMC-AM1, MIC = 0.11nMol, (2) 头孢它啶, MIC = 3.02nMol, (3) 氨苄青霉素, MIC = 1.35nMol ;

[0038] B 图为耐万古霉素肠球菌, (1)PMC-AM1, MIC = 0.23nMol, (2) 万古霉素, MIC = 21.54nMol, (3) 氨苄青霉素, MIC = 10.78nMol ;

[0039] C 图为耐甲氧西林金葡菌, (1)PMC-AM1, MIC = 0.06nMol, (2) 氨苄青霉素, MIC = 21.55nMol, (3) 苯唑西林, MIC = 14.1nMol ;

[0040] D 图为多重耐药绿脓杆菌, (1)PMC-AM1, MIC = 0.91nMol, (2) 左氧氟沙星, MIC = 43.2nMol, (3) 头孢它啶, MIC = 29.3nMol, (4) 庆大霉素, MIC > 889.4nMol。

[0041] 图 7. 本发明新型抗生素与野生型大肠菌素、抗金葡菌多肽 (ZL 01128836.1) 对耐甲氧西林金葡菌 (ATCC BAA-42)、耐万古霉素肠球菌 (ATCC 700802)、多重耐药绿脓杆菌 (华西医院临床分离株 13578) 抑制作用的比较之生存曲线

[0042] 纵座标为最小抑菌浓度 (nMol) ;

[0043] 其中 A 图为耐万古霉素肠球菌, (1) 抗金葡菌多肽, MIC = 0.91nMol, (2) 野生型大肠菌素 Ia, MIC = 0.91nMol, (3)PMC-AM1, MIC = 0.23nMol ;

[0044] B 图为耐甲氧西林金葡菌, (1) 抗金葡菌多肽, MIC = 0.06nMol, (2) 野生型大肠菌素 Ia, MIC = 0.23nMol, (3)PMC-AM1, MIC = 0.06nMol ;

[0045] C 图为多重耐药绿脓杆菌, (1) 抗金葡菌多肽, MIC = 0.91nMol, (2) 野生型大肠菌素 Ia, MIC = 0.91nMol, (3)PMC-AM1, MIC = 0.23nMol。

[0046] 图 8 本发明新型抗生素对脑膜炎双球菌感染动物的体内保护试验之生存曲线横座标为鼠存活时间, 单位为天 ; 纵座标为动物存活数, 单位为每只鼠 1)PMC-AM1, 本发明新型抗生素 ;2)Gen, 庆大霉素 ;3)PEN, 青霉素 ;4)Con., 对照 ;

[0047] 所有药物注射浓度均为 1.5mg/kg。

具体实施方式

[0048] 结合附图, 通过本发明较佳实施例的描述具体说明本发明。

[0049] 【实施例 1】表达新型抗生素的质粒的构建和新型抗生素制备

[0050] 原始质粒为装载了大肠菌素和 immunity 蛋白基因的 pSELECT™-1 质粒 (8.3kb)。经双链寡聚核苷酸点突变技术 (QuickChange™ Kit, Strategene 公司) 将编码抗体模拟物基因, 如 Seq ID No.1 和 Seq ID No.3 所述的核苷酸序列分别插入到大肠菌素变构多肽基因的 626 位点上, 制备了新型抗生素的突变质粒 pBHC-PorA1、pBHC-PorA2(如图 1- 图 2 所示)。突变质粒转染入 E.coli BL-21 工程菌里制备新型抗生素。

[0051] 突变程序按 Strategene QuickChange SiteDirected Mutagenesis Kit(catalog#200518) 药箱手册进行 :即

[0052] 1. 准备点突变反应物 :

[0053] 5ul 10X buffer

[0054] 2ul(10ng) 装载了大肠菌素变构多肽和 immunity 蛋白基因的原始质粒

[0055] 1.25ul(125ng) 设计的 5’-3’ 寡聚核苷酸引物 (见所列引物序列)

[0056] 1.25ul(125ng) 设计的 3’-5’ 寡聚核苷酸引物 (见所列引物序列)

[0057] 1ul dNTP

[0058] 双蒸水 50ul

[0059] 1ul pfu

[0060] (除质粒、引物和双蒸水外,均为药箱所备试剂)

[0061] 2. 进行 PCR 扩增,扩增条件 :变性 95℃,35 秒,退火 53℃,70 秒,延伸 68℃,17 分,共 20 个循环 ;

[0062] 3. 加入 Dpn 1 内切酶 1ul 消化母体 DNA 链后 (37℃,1 小时),取 1ul 反应物与 XL1-Blue 感受态细胞 50ul 冰孵 30 分钟,热冲击 42℃,45 秒,再置入冰中 2 分钟 ;

[0063] 4. 加入 NZY 培基 0.5ml,220rpm,37℃摇菌 1 小时,取 50-100ul 反应物铺板 (LB 培基加 1%琼脂,加 50ug/ml 氨苄青霉素,37℃过夜) ;

[0064] 5.18 小时后挑菌,提取质粒后测序确定突变成功 ;

[0065] 6. 将突变质粒 100ng 与制备的 BL-21 工程菌感受态细胞 40ul 冰孵 5 分钟,热冲击 42℃,30 秒,再置入冰中 2 分钟,加入 SOC 培基 160ul,220rpm,37℃摇菌 1 小时后铺板 (LB 培基加 1%琼脂,加 50ug/ml 氨苄青霉素,37℃过夜),挑取单克隆菌落大量增菌 ;

[0066] 7. 大量增菌,8-10 升 FB 培基,250rpm,30℃,3-4 小时 ;升温至 42℃,250rpm 生长 0.5 小时 ;降温至 37℃,250rpm 生长 1.5 小时 ;4℃,6000g,20 分钟离心沉淀菌体,取 4℃,50mM 硼酸缓冲液 (pH 9.0,2mMEDTA)80-100ml 悬浮菌体,加入 PMSF 50ug 后超声破碎菌体 (4℃,400W,1 分钟,重复 4-5 次,间歇 2-3 分钟确保菌液温度),高速离心沉淀破碎的菌体 (4℃,75,000g,90 分钟),取上清加入硫酸链霉素 500 万单位沉淀 DNA(4℃搅拌 1 小时),10,000g,4℃,10 分钟离心沉淀后,取上清装入分子量 15,000 透析袋于 4℃,50mM 硼酸缓冲液 10 升透析过夜后,再次 10,000g,4℃,10 分钟离心沉淀,取上清上样于 CM 离子交换柱,充分冲洗后,0.3M NaCl+50mM 硼酸缓冲液洗脱即可得到所制备的新型抗生素。对应于以上 2 种质粒,可分别获得 PMC-AM1 和 PMC-AM2 两种抗生素,其氨基酸序列分别如 Seq ID No.6、Seq ID No.8 所示。

[0067] 其中 AM1 是重链可变区的第一互补决定区、重链第二骨架区、轻链可变区第三互补决定区,三个区域顺次以羧基端链接下一区域的氨基端,氨基酸序列如 Seq ID No.2 所示 ;AM2 是重链可变区的第一互补决定区的羧基端链接重链第二骨架区,轻链可变区的第三互补决定区的肽链羧基端氨基酸链接在重链第二骨架区的羧基端氨基酸上,氨基酸序列如 SeqID No.4 所示。

[0068] 将 PMC-AM2 作为 PMC-AM1 的对照,验证本发明设计的抗体模拟物的氨基酸区段之间在不同连接方式下产生的抗生素的功能。

[0069] 上述制备质粒中所设计的寡聚核苷酸引物序列如下 :

[0070] 5’-3’(SEQ ID NO.9)

[0071] gcg aat aag ttc tgg ggt att TCT TAT TGG CTG CAT TGG ATTAAA CAG taa ata aaa tat aag aca

[0072] ggc

[0073] 3’-5’(SEQ ID NO.10)

[0074] gcc tgt ctt ata ttt tat tta CTG TTTAAT CCA ATG CAG CCA ATA AGA aat acc cca gaa ctt att cgc

[0075] 5’-3’(SEQ ID NO.11)

[0076] tgg ctg cat tgg att aaa cag AGA CCT GGT CAG GGA CTG TGG ATC GGA taa ata aaa tat aag aca

[0077] ggc

[0078] 3’-5’(SEQ ID NO.12)

[0079] gcc tgt ctt ata ttt tat tta TCC GAT CCA CAG TCC CTG ACC AGG TCT ctg ttt aat cca atg cag cca

[0080] 5’-3’(SEQ IDNO.13)

[0081] ggt cag gga ctg tgg atc gga TCT CAG TCC ACG CAT GTG CCG AGA ACC taa ata aaa tat aag aca

[0082] ggc

[0083] 3’-5’(SEQ ID NO.14)

[0084] gcc tgt ctt ata ttt tat tta GGT TCT CGG CAC ATG CGT GGA CTG AGA tcc gat cca cag tcc ctg

[0085] acc

[0086] pBHC-PorA 2

[0087] 5’-3’(SEQ IDNO.15)

[0088] gcg aat aag ttc tgg ggt att TCT TAT TGG CTG CAT TGG ATT AAA CAG taa ata aaa tat aag aca

[0089] ggc

[0090] 3’-5’(SEQ ID NO.16)

[0091] gcc tgt ctt ata ttt tat tta CTG TTT AAT CCA ATG CAG CCA ATA AGA aat acc cca gaa ctt att cgc

[0092] 5’-3’(SEQ ID NO.17)

[0093] tgg ctg cat tgg att aaa cag AGA CCT GGT CAG GGA CTG TGG ATC GGA taa ata aaa tat aag aca

[0094] ggc

[0095] 3’-5’(SEQ ID NO.18)

[0096] gcc tgt ctt ata ttt tat tta TCC GAT CCA CAG TCC CTGACCAGG TCT ctg ttt aat cca atg cag cca

[0097] 5’-3’(SEQ ID NO.19)

[0098] ggt cag gga ctg tgg atc gga ACC AGA CCG GTG CAT ACG TCC CAG TCT taa ata