PROKARYOTIC EXPRESSION AND PURIFICATION METHOD FOR CTB RECOMBINANT PROTEIN

Field of the Invention

5 **[0001]** The present application relates to the technical field of protein purification, in particular to a prokaryotic expression and purification method for CTB recombinant protein.

Background to the Invention

10 **[0002]** Cholera Toxin B subunit (CTB) is a non-toxic component of cholera toxin (CT), formed by five identical 11.6 kDa monomers linked via non-covalent bonds into a cyclic pentamer structure. CTB is known for its high-affinity binding properties to GM1 ganglioside, this property makes it a valuable immunomodulatory molecule and vaccine adjuvant. CTB is a highly effective mucosal immunomodulator that may significantly 15 enhance the immunogenicity of co-administered antigens by binding to GM1 on the surface of antigen-presenting cells (APCs) and promoting antigen internalization. CTB may also upregulate the expression of co-stimulatory molecules such as CD40 and CD86 on the surface of APCs, thereby reducing the threshold concentration for immune cell activation. CTB fusion proteins constructed by genetic engineering have been 20 successfully applied in the development of a plurality of vaccines and have shown significant immune-enhancing effects in animal models.

[0003] CTB is a new adjuvant developed for vaccines, but the existing purification processes have the problems of low yield (< 3 mg/g wet cell weight) and high impurity residue. The traditional method adopts a single affinity chromatography, which is difficult 25 to remove the host protein contamination.

[0004] In summary, how to provide a prokaryotic expression and purification method for CTB recombinant protein with high yield and less impurity residue is an urgent problem to be solved by those skilled in the art.

Statement of Invention

[0005] In view of this, the present application provides a prokaryotic expression and purification method for CTB recombinant protein

[0006] The present application belongs to the "Research and Development of Animal Vaccine Adjuvants and Protective Agents", the project of "Research on Production Process of Animal Vaccine Adjuvant Products", and the sub-project of "Research on Production Process of Compound Water Adjuvant Products for Needle-Free Injection of Animal Subunit Vaccine (2023YFD1802603-2)."

[0007] In order to achieve the above objective, the present application adopts the following technical solutions.

[0008] The prokaryotic expression and purification method for CTB recombinant protein includes the following steps:

[0009] (1) constructing and culturing a recombinant expression strain of CTB protein;

[0010] (2) bacterial fragmentation: adopting a combination of a disperser and a high-pressure homogenizer to crush the bacteria;

[0011] (3) affinity chromatography: using Ni Chromstar FF resin, equilibrating with Buffer 1, washing with Buffer 2 and eluting with Buffer 3; and

[0012] (4) cation exchange chromatography: diluting an affinity eluent with Buffer 4, using SP Beads 6FF resin, equilibrating with Buffer A and eluting with Buffer B for purification.

[0013] The beneficial effects obtained are as follows: the present application adopts a combination of "disperser + high-pressure homogenization" for bacterial fragmentation, improving lysis efficiency. The affinity chromatography process of the present application cancels NaCl to enhance the binding efficiency. The present application selects high-loading SP Beads 6FF resin in the cation exchange chromatography process. The present application optimizes the imidazole concentration gradient in Buffer 3 to 500 mM, equilibrating the elution specificity.

[0014] Further, a specific operation of step (2) includes: resuspending a CTB protein

recombinant expression strain with Buffer 1, adding nuclease, dispersing at 5,000 rpm for 30 min, homogenizing at 800 bar twice, and centrifuging to collect a supernatant; and

[0015] Buffer 1 components: 50 mM NaH_2PO_4 and 0.05% Tween-20, pH=7.0.

[0016] Further, in step (3) includes:

5 **[0017]** Buffer 1 components: 50 mM NaH_2PO_4 and 0.05% Tween-20, pH=7.0;

[0018] Buffer 2 components: 50 mM NaH_2PO_4, 0.05% Tween-20 and 20 mM imidazole pH=7.0; and

[0019] Buffer 3 components: 50 mM NaH_2PO_4, 0.05% Tween-20 and 500 mM imidazole, pH=7.0.

10 **[0020]** Further, a linear flow rate is 120 cm/h in step (3); and

[0021] an absorbance at 280 nm is measured, collection begins when UV value is higher than or equal to 300 mAU, and collection stops when the UV is less than or equal to 400 mAU.

[0022] Further, in step (4) includes:

15 **[0023]** Buffer 4 components: 50 mM NaH_2PO_4 and 0.05% Tween-20, pH=6.0;

[0024] Buffer A components: 50 mM NaH_2PO_4, 50mM NaCl and 0.05% Tween-20, pH=6.0; and

[0025] Buffer B components: 50 mM NaH_2PO_4, 500 mM NaCl and 0.05% Tween-20, pH=6.0.

20 **[0026]** Further, a linear flow rate is 120 cm/h in step (4); and

[0027] an absorbance at 280 nm is measured, collection begins when UV value is higher than or equal to 100 mAU, and collection stops when the UV is less than or equal to 100 mAU.

[0028] Use of CTB protein prepared by the method in preparation of a vaccine adjuvant
25 or an oral vaccine.

[0029] As can be seen from the above technical solutions, compared to the prior art, the

beneficial effects achieved by the present application are as follows.

[0030] The present application provides the prokaryotic expression and purification method for CTB recombinant protein. A recombinant *Escherichia coli* expression system is used, bacterial fragmentation conditions are optimized, the NaCl concentration in an affinity chromatography process is optimized to improve the purification recovery rate of recombinant protein, and cation chromatography purification is added to achieve purity. The CTB protein purified by the method of the present application has a natural pentameric conformation (verified by SDS-PAGE), achieving efficient purification of the CTB protein (recovery rate > 19 mg/g wet cell weight) with a purity of a single electrophoretic band. The CTB protein purified by the method of the present application can be used for the development of oral vaccine adjuvants or mucosal immunopotentiators, particularly suitable for preventing infections with enteric pathogens such as cholera and porcine epidemic diarrhea (PED).

Brief Description of the Drawings

[0031] In order to more clearly describe the technical solutions in the embodiments of the present application or in the prior art, the drawings required for describing the embodiments or the prior art are briefly described below. Obviously, the drawings in the following description show only some embodiments of the present application, and for those of ordinary skill in the art, other drawings may also be obtained from the provided drawings without creative labor.

[0032] FIG. 1 shows a chromatogram of an affinity chromatography process in Example 1 of the present application;

[0033] FIG. 2 shows a chromatogram of a cation exchange chromatography process in Example 1 of the present application;

[0034] FIG. 3 shows an SDS-PAGE diagram of a prokaryotic expression purification preparation process of CTB recombinant protein in Example 1 of the present application, where 1 is a supernatant after bacterial fragmentation; 2 is an affinity chromatography flow-through; 3 is an affinity chromatography washing solution; 4 is an affinity

chromatography eluent; 5 is an ion exchange chromatography flow-through; 6 is an ion exchange chromatography eluent; 7 is an ion exchange chromatography regeneration solution; M is a protein Maker; 9 is an ion exchange chromatography eluent (non-reduced, NR); 10 is an ion exchange chromatography regeneration solution (NR); Marker is a protein molecular weight standard of 10-180 kDa; NR represents in SDS-PAGE, the sample does not add a reductant, and the proteins inside also retain the original disulfide bonds;

[0035] FIG. 4 shows a product purity detection diagram (SEC) in Example 1 of the present application; and

[0036] FIG. 5 shows a comparison diagram of purification effects of different resins in the cation exchange chromatography process in Example 3 of the present application, reference products represent a purified protein, undiluted liquid represents the protein that has not been purified by chromatography, FT represents the flow-through during the sample loading, EL represents the eluate, 1 represents control group 1, 2 represents control group 2, and 3 represents the experimental group.

Detailed Description

[0037] The following technical solutions in the embodiments of the present application are described clearly and completely with reference to the drawings in the embodiments of the present application. It is obvious that the described embodiments are only some of the embodiments of the present application, rather than all embodiments of the present application. Based on the embodiments in the present application, all other embodiments obtained by those of ordinary skill in the art without making creative efforts are included in the scope of protection of the present application.

[0038] The medication required by the present application is conventional experimental medication, purchased from commercially available channels. Experimental methods not mentioned are conventional experimental methods and will not be described in detail here.

[0039] **Example 1**

[0040] A prokaryotic expression and purification method for CTB recombinant protein included the following steps:

[0041] 1) constructing a recombinant expression strain of CTB protein

[0042] where based on an amino acid sequence of CTB protein (Unirpo: Q57193)
5 published by Unipro (www.uniprot.org), combined with a codon preference of *Escherichia coli*, a gene sequence of CTB protein was designed, gene sequence of CTB protein was synthesized by chemical synthesis; the CTB gene sequence was inserted into a pET-22b expression vector using NcoI/BlpI digestion to obtain a recombinant expression vector expressing CTB protein; and the recombinant expression plasmid was transformed into
10 *Escherichia coli* BL21 (DE3) expression strain to obtain the recombinant expression strain for CTB protein;

[0043] 2) culturing a recombinant expression strain of CTB protein

[0044] where initial parameters (37°C, pH = 6.90, 300 rpm, DO = 100%) were set after sterilization of a fermentation tank, bacterial solution (inoculation amount of 6%) was
15 inoculated, cultured to OD_{600} = 10-15, cooled to 20°C, and IPTG was added to induce expression; after induction, a fed-batch culture medium was added to maintain DO = 40-50%, fermentation was completed after 6 h of induction, and bacteria were collected by centrifugation and stored at -20°C after subpackaging;

[0045] 3) bacteria fragmentation

20 [0046] where the recombinant expression strain of CTB protein was resuspended according to the bacteria: Buffer 1 = 1 g : 10 mL, added 5 μg/L nuclease, dispersed at 5,000 rpm for 30 min, homogenized at 800 bar twice, and centrifuged (8,500 rpm, 30 min) to collect the supernatant; and

[0047] Buffer 1 components: 50 mM NaH_2PO_4 and 0.05% Tween-20, pH=7.0;

25 [0048] 4) affinity chromatography

[0049] where Ni Chromstar FF resin (loading 10-15 mg Protein/mL) was used, equilibrated with Buffer 1, washed with Buffer 2, and isocratically eluted with Buffer 3;

[0050] Buffer 1 components: 50 mM NaH_2PO_4 and 0.05% Tween-20, pH=7.0;

[0051] Buffer 2 components: 50 mM NaH_2PO_4, 0.05% Tween-20 and 20 mM imidazole pH=7.0;

[0052] Buffer 3 components: 50 mM NaH_2PO_4, 0.05% Tween-20 and 500 mM imidazole, pH=7.0;

[0053] elution conditions were as follows:

[0054] flow rate: a linear flow rate was 120 cm/h;

[0055] elution volume: 3 CV (column volume);

[0056] collection part: the absorbance at 280 nm was measured, and collection began when the ultraviolet absorbance (UV) was higher than or equal to 300 mAU, and the collection stopped when the UV was less than or equal to 400 mAU;

[0057] target product: CTB protein in an elution peak; and

[0058] a chromatogram of affinity chromatography process is shown in FIG. 1; and

[0059] 5) cation exchange chromatography (SP Beads 6FF resin)

[0060] where the affinity eluent of step 4) was diluted according to the eluent: Buffer 4 = 1 mL: 5 mL, adjusted to pH = 6.0, SP Beads 6FF resin (loading 50-80 mg/mL) was used, equilibrated with Buffer A, and purification was performed by isocratic elution with Buffer B (containing 500 mM NaCl);

[0061] Buffer 4 components: 50 mM NaH_2PO_4 and 0.05% Tween-20, pH=6.0;

[0062] Buffer A components: 50 mM NaH_2PO_4, 50 mM NaCl and 0.05% Tween-20, pH=6.0;

[0063] Buffer B components: 50 mM NaH_2PO_4, 500 mM NaCl and 0.05% Tween-20, pH=6.0;

[0064] elution conditions were as follows:

[0065] flow rate: a linear flow rate was 120 cm/h;

[0066] elution volume: 3 CV (column volume);

[0067] collection part: the absorbance at 280 nm was measured, and collection began when the ultraviolet absorbance (UV) was higher than or equal to 100 mAU, and the collection stopped when the UV was less than or equal to 100 mAU; and

5 [0068] target product: CTB protein in the elution peak (excluding non-specific impurities).

[0069] The chromatogram of the cation exchange chromatography process is shown in FIG. 2.

[0070] The SDS-PAGE diagram of the prokaryotic expression and purification of CTB

10 recombinant protein is shown in FIG. 3. In the affinity chromatography process, FT and washing loss were less, and the elution effect was better. The FT loss in the cation exchange chromatography process was less, the elution purity was high, and the impurity protein removal effect was good.

[0071] The purity of the product is shown in FIG. 4, with the final product purity higher

15 than 95% (purity = target protein peak area/total peak area).

[0072] **Example 2**

[0073] Optimization of Affinity Chromatography Buffer System

[0074] Control group 1: traditional buffer (300 mM NaCl was added to Buffer 1);

[0075] control group 2: traditional buffer (150 mM NaCl was added to Buffer 1); and

20 [0076] experimental group: buffer (Buffer 1) of the present application.

[0077] The remaining operations were the same as in Example 1.

[0078] The recovery rate was calculated, and the calculation formula is as follows:

[0079] Recovery rate = protein quality / bacteria used

[0080] The recovery results of affinity chromatography using different buffers are as

25 follows.

[0081] Table 1: Results of Different Buffer Recovery Rates

Group	Protein concentration A280 (mg/mL)	Volume (mL)	Quality (mg)	Bacteria used (g)	Recovery rate (mg/g bacteria)
Experiment group	4.15	76	315.40	50	6.31
Control group 1	3.66	75	274.50	50	5.49
Control group 2	3.91	74	289.34	50	5.79

[0082] It can be seen from the above table that the recovery rate of the target protein in the experimental group was 14.94% higher than that in the control group 1. The recovery rate of target protein in the experimental group was 8.98% higher than that in the control group 2.

5 **[0083]** **Example 3**

[0084] Comparison of Resin for Cation Exchange Chromatography

[0085] Control group 1: SP Sepharose High Performance;

[0086] control group 2: SP Chromstar HP; and

[0087] experimental group: SP Beads 6FF used in the present application.

10 **[0088]** The remaining operations were the same as in Example 1.

[0089] The comparison diagram of purification effect is shown in FIG. 5. With the same purification method, only a chromatographic resin was changed. The purity of the target protein was the same by comparison of the three resins. The FT of SP Beads 6FF used in the present application had the least, so it was finally selected.

15 **[0090]** **Example 4**

[0091] CTB protein was prepared by the method of Example 1, and the recovery rate of the protein was detected. The results are shown in Table 2.

[0092] Table 2: Recovery Rates Between Different Batches

Inventory data	Batch number	Volume (L)	Concentration (mg/mL)	Quality (g)	Total single batch inventory (g)	Bacteria used (kg)	Recovery rate (g/kg)
2025.03.26	YF/CTB/250326-1	0.45	53.99	24.30	24.30	1.25	19.44

[0093] Each embodiment in this specification is described in a progressive way, and each embodiment focuses on the differences from other embodiments. The same or similar parts between the various embodiments can be referenced to each other.

[0094] The above description of the disclosed embodiments enables those skilled in the art to realize or use the present application. Various modifications to these embodiments are obvious to those skilled in the art, and the general principles defined herein can be realized in other embodiments without departing from the spirit or scope of the present application. Therefore, the present application is not to be limited to the embodiments shown herein, but is to be conformed to the widest scope consistent with the principles and novel features disclosed herein.