

Production of recombinant CAMP – Sialidase protein and preparation of chitosan nanoparticles carrying this protein to be used as a candidate for vaccines targeting *Propionibacterium acnes*

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ABSTRACT

BACKGROUND AND OBJECTIVE: Acne vulgaris is one of the most common skin diseases that imposes too much mental pressure and high costs on patients. The existing treatments have low efficacy and include antibiotics and anti-inflammatory drugs, which have many complications due to the chronic nature of the disease, especially at young age, including antibiotic resistance and allergic reactions. For this reason, one of the new therapeutic approaches is the use of a vaccine with the help of the bacterium or its components. The aim of this study is to produce nanoparticles carrying the recombinant CAMP – Sialidase protein as a new chimeric antigen to be used in acne vaccine.

METHODS: To express the recombinant CAMP – Sialidase protein, *E. coli* BL21 DE3 was used as the host. Purification of protein was done through combined urea/imidazole method and using a nickel-nitroacetic acid column. The recombinant protein was confirmed using Western Blotting by Anti – Histidine Antibody. Then, the loaded nanoparticles were prepared by recombinant protein using ionic gelation technique and tripolyphosphate. Finally, the size and zeta potential of the nanoparticles were determined by the DLS device.

FINDINGS: The recombinant CAMP – Sialidase protein was confirmed after expression and purification. The size and zeta potential of nanoparticles containing recombinant CAMP – Sialidase protein at a concentration of 0.6 mg/ml were determined to be 80 nm and +27 mV, respectively, using the DLS device. The loading rate of the protein in the nanoparticles was found to be 88%.

CONCLUSION: The results show that the recombinant protein is expressed completely and is successfully encapsulated in the chitosan nanoparticles.

KEY WORDS: *Acne Vulgaris, Camp – Sialidase Protein, Chitosan Nanoparticle, Vaccine.*

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Introduction

Acne vulgaris is one of the most common skin diseases and almost 85% of people in the community experience acne is a period of their life (1). This disease causes both physical and mental complications in patients, including scarring in the skin, depression, anxiety and low self-esteem. Acne is a multifactorial disease, and one of its main causes is the proliferation of *Propionibacterium acnes*.

The main treatment of this disease is mainly done through the administration of retinoic acid, as well as antibiotic therapy. Acne, especially in severe forms such as acne fulminans, causes a lot of problems for patients who are often young, causing very high costs for the treatment. These treatments are usually temporary and include hormonal and anti-inflammatory drugs as well as antibiotic therapy. Unfortunately, in general, existing therapies have low efficacy due to the chronic nature and long duration of treatment, several complications such as the occurrence of antibiotic resistance and the development of strains resistant to normal flora of the intestine and skin, as well as allergic reactions in the patient. Nowadays, the attention of researchers has been drawn to the use of other therapies due to the side effects of these drugs.

One of these methods is the vaccine treatment method, using either inactivated vaccines (in the old times) or the bacterial components at present. Since old times, inactivated *P. acnes* was obtained from the infection site of the acne on patient's skin, and was used as autovaccine for the treatment of acne in that patient (2, 3). However, due to the complications of the old vaccine, new vaccines containing bacterial antigens began to be used. In this regard, two important antigens, including Sialidase Factor and CAMP Factor, have been tested in separate studies as vaccine against acne (3, 4).

Sialidase is a cell wall – bound protein, which, as a pathogen, causes tissue destruction and infections in the host. Cell wall – bound sialidase is one of the five bacterial sialidases and plays an important role in adhesion and infection (4).

The CAMP factor is a secreted protein that, along with host sphingomyelinase, causes hemolysis of red blood cells, which can be toxic to keratinocytes and macrophages (3, 5). In the previous studies, a chimeric protein containing two parts of the sialidase antigen and CAMP antigen of *P. acnes* was designed bioinformatically for the first time. After determining

the second and third structure and analyzing its various properties, it was cloned with the aim of being used in acne vaccine (6). The aim of this study is to express the recombinant CAMP–Sialidase protein, purification by affinity chromatography, confirmation by specific antibodies using western blotting technique, and the production and evaluation of chitosan nanoparticles containing this recombinant protein for oral administration in the acne vaccine.

Methods

E. coli BL21 DE3 was used in this experimental study, LB medium was used for its growth. Chemicals, kits and molecular markers were obtained from Merck, CinnaGen, Qiagen and Fermentas. Ni-NTA chromatography column was purchased from Qiagen Company. The secondary antibody (conjugata) attached to HRP against mouse immunoglobulin G and Mouse anti Histidine tag antibody was purchased from Sigma Company. Chitosan with average molecular weight and sodium triphosphate were purchased from Sigma Company. In order to investigate the size and zeta potential of nanoparticles, the DLS device from the Baqiyatallah University of Medical Sciences was used.

Expression and purification of recombinant protein: The pET28a vector containing the recombinant CAMP – Sialidase gene sequence from the previous study was used in this study. In order to express the recombinant CAMP – Sialidase protein, *E. coli* BL21 DE3 expression host was used. Overnight culturing of bacteria in the presence of kanamycin (20 µg/ml) was used for induction. When optical absorption reached around 0.7 at a wavelength of 600 nm, IPTG (1 mM) was used for induction. After that, the cells were incubated at 37 °C for 16 hours and at 150 rpm. After centrifugation, cell precipitate was solved in PBS and was broken up by sonication of the cells and was centrifuged.

Then, the precipitate was dissolved in urea buffer and centrifuged at ambient temperature for one hour. Then, the supernatant and non-induced solutions were examined on 12% SDS-PAGE gel for the presence of the desired band. Combined urea/imidazole preparation method and nickel nitrilotriacetic acid were used to purify the protein. For this purpose, after collecting the bacterial precipitate, the lysis buffer was added and, after centrifugation and sonication, the supernatant was passed through an affinity column. Then, the buffer

containing 20 mM imidazole was used for washing and 500 mM buffer was used as a recombinant protein removing buffer. The dialysis process against PBS was then performed to remove urea.

Recombinant protein confirmation using western blotting technique: Western blotting technique was used to confirm the recombinant protein using Anti-His Antibody. The cell extract of induced and non-induced sample, and the Protein Marker PS-107 was electrophoresed on 12% SDS-PAGE gel. Blotting was performed on nitrocellulose paper and transfer buffer containing 192 mM glycine, 25 mM Tris, 1% SDS and methanol 20%. Nitrocellulose paper was blocked overnight in a PBST containing 5% dry milk. After the washing process, the nitrocellulose paper was exposed to a mouse polyclonal anti-6-His Tag Antibody with a dilution of 1:1000. After washing, the color reaction of the protein was performed by adding diaminobenzidine in 50 mM Tris buffer and H₂O₂, and the reaction stopped after the appearance of chemical band.

Preparation of chitosan nanoparticles: Chitosan nanoparticles were prepared by ionic gelation. The 2 mg/ml chitosan solution was prepared in 2% acetic acid and 1 mg/ml triphenyl phosphate (TPP) solution. Then, the target antigen was dropped and added to 7.5 ml of chitosan solution for five minutes, so that the final concentration of the antigen was 1 mg/ml. The pH of the solution was then adjusted to 5.5 using a saturated NaOH solution and the solution was allowed to stay on the stirrer for 30 minutes at ambient temperature so that the pH conditions would be similar in all solutions. 5 ml of sodium tripolyphosphate solution was added drop by drop to the protein-containing chitosan solution. For this purpose, each milliliter of TPP was added in 5 minutes. It should be noted that after adding one milliliter of TPP, sonication was performed in five 20-second cycles. This was done to prevent the accumulation of nanoparticles. Finally, the final solution was centrifuged for 40 minutes at a rate of 13000 rpm and the supernatant was removed. Determination of protein loading in the supernatant was calculated based on the following formula from the difference in the amount of initial protein added to the chitosan solution and the final amount of supernatant.

$$LC\% = [(A-B)/A] \times 100$$

LC=Loading Capacity

A: Initial amount of protein

B: The amount of protein remaining in the supernatant after centrifugation

The size of the nanoparticles was measured using the Malvern DLS instrument.

Results

Expression and purification of recombinant CAMP

– **Sialidase protein:** After inducing the target gene, collecting cells and breaking the cells, the total protein content was analyzed on 12% SDS-PAGE gel. High expression of protein was observed in the induced samples, which could not be observed in non-induced samples (Fig 1). The results of purification of the protein using a nickel-nitroacetic acid column indicate that a recombinant protein with a weight of 65 kDa was in the output of imidazole 500 mM with high purity (Fig 2).

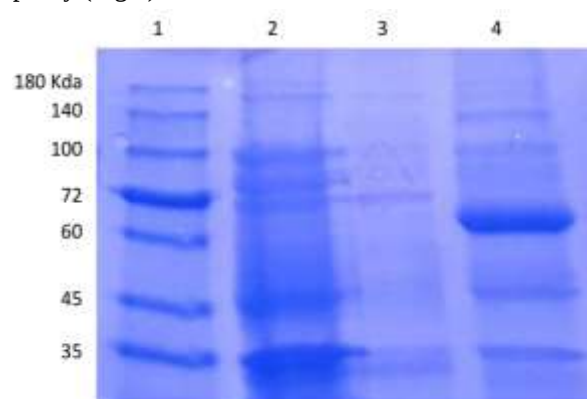


Figure 1. Electrophoresis gel before and after protein induction. Column 1: Protein Molecular Marker (kilodalton); Column 2: Induced Sample; Column 3: The Supernatant of the Sample Dissolved in PBS; Column 4: The Supernatant of the Sample Dissolved in Urea Buffer.

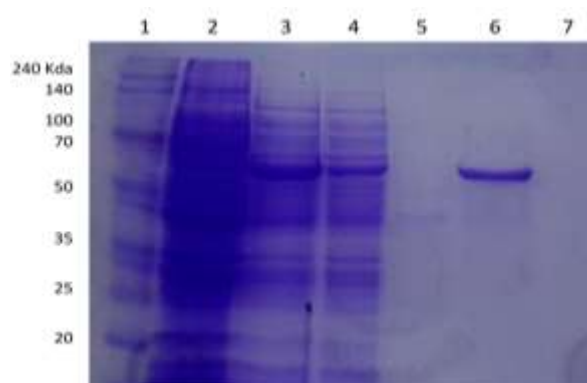


Figure 2. Electrophoresis gel after purification of the protein using nickel column (Ni-NTA). 1: Molecular protein marker; 2: Control or non-induced sample; 3: Sample induced before the column; 4: Primary output specimen before sample; 5: 20 mM imidazole output sample; 6: 500 mM imidazole output sample; 7: MES output buffer sample.

Western Blotting Analysis by His-tag Antibody:

After the removal of urea by dialysis, the expression of the protein was confirmed by the Western Blotting technique because of having His-tag sequence and the use of anti-His-tag antibody and the target band was placed in the correct weight position and no band was observed in the control column (Fig. 3).

Preparation of chitosan nanoparticles and evaluation of protein loading: After making chitosan nanoparticles containing recombinant proteins with ion gelation and measuring the size, the results of the DLS device indicated the production of chitosan nanoparticles with an average size of 80 nm (Fig. 4). The zeta potential of nanoparticles containing recombinant proteins was +27 mV. The results also showed that the loading rate of the protein in the nanoparticles was 88%.

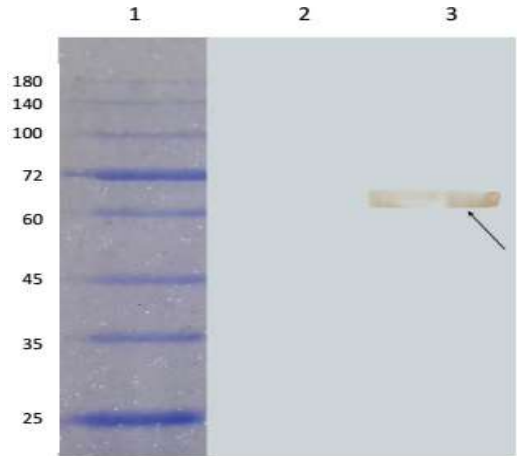


Figure 3. Confirmation of recombinant protein by Western blotting. Column 1: Protein molecular marker; Column 2: The control sample (no IPTG); Column 3: Target protein

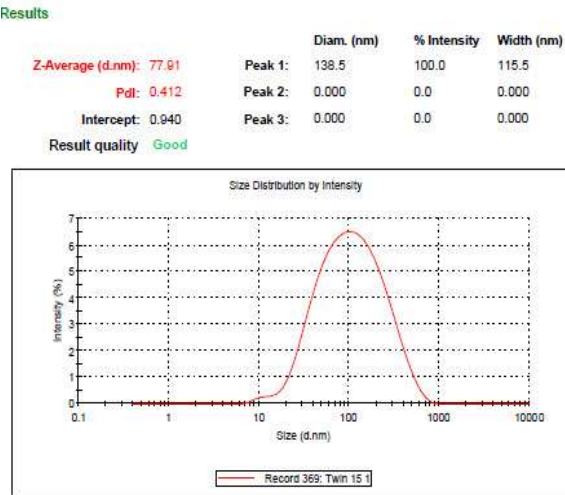


Figure 4. DLS device results regarding chitosan nanoparticles

Discussion

In the present study, the CAMP-Sialidase chimeric structure, which was designed and cloned from two proteins of CAMP and Sialidase of *Propionibacterium acnes* from our previous study (6), was first expressed and used as a candidate for vaccine. In the first step of the present study, after inducing the gene, the recombinant protein was significantly expressed, using a combination of urea – imidazole and nickel column to purify it. Then, SDS-PAGE and Western blotting techniques were used to confirm the presence of protein, and the concentration of recombinant CAMP-Sialidase protein was measured by 0.6 mg/ml Bradford Reagent. The present study is a continuation of studies by Nakatsuji et al., which used two different acne bacteria antigens separately and in independent studies. In several studies, they expressed the deactivated bacteria in several and Sialidase and CAMP antigens individually and evaluated them in terms of immunogenicity (2,4,5).

But in the present study, we synthesized these two antigens as a single-chimeric protein and transformed into nanoparticle for the first time in order to investigate immunogenicity in the future study. In order to design oral vaccines, attention to factors such as confronting gastrointestinal proteases, stability in the gastrointestinal tract, permeability of the intestinal wall and basement membrane, and entry into the bloodstream and solubility at near – neutral pH is necessary (7). As a result, many studies are today inclined toward the use of nanoparticles as efficient system for drug delivery.

The advantage of using polymer nanoparticles, such as chitosan, is to protect bioactive molecules through encapsulation against hydrolytic and enzymatic degradation. The physicochemical properties of nanoparticles play an important role in their interaction with antigens, biological systems and immune system cells and also affect the efficacy of the vaccine (8, 9).

Among the most widely used nanoparticles, chitosan, PLGA and TMC can be mentioned. In this study, for the first time, chitosan nanoparticle was used to remove the pain and discomfort associated with injection, non-invasive oral route for drug delivery, and also to preserve the target vaccine from being degraded by digestive enzymes. This polysaccharide is highly biocompatible, available and non-toxic, and is easily removed from the living creature. It also has antimicrobial properties and, in addition, it has good

adhesion strength, degree of deacetylation and controllable molecular weight and ability of controlled release, and can therefore be used to transfer peptide medications (10, 11). In this research, ionic gelation technique was used to prepare the chitosan nanospheres.

The advantages of this method are the absence of harmful organic solvents for proteins, high efficiency, high percentage of encapsulation and controlled release (12). In the present study, chitosan solution with pH = 5.5 was used to make chitosan nanospheres, because at this pH, the molecular field of chitosan is more open and there are more active sites for the formation of hydrogen bonds with protein molecules (13). Moreover, due to the acidic nature of PI in the synthesized protein, the process of nanoparticulation with chitosan was well done.

The results of this part of the study indicate that the size of the nanoparticle contains an antigen of about 80 nm and a zeta potential of +27 mV and a protein loading rate of 88%. In addition, according to the results of this study, the best size of nanoparticles in the concentration of 1 mg/ml of chitosan is obtained in relation to the chitosan nanoparticle. According to previous studies, the size of the nanoparticle produced in this study is appropriate and its loading rate is approximately equal to these studies. The use of polymer nanoparticles in recent years has been of great interest to researchers, and several studies have been carried out to produce various vaccines.

In separate studies, Soleymani et al., and Bagheripour et al. used TMC and chitosan nanoparticles as carriers of neutrophil-activating protein from *helicobacter pylori* and also as carriers of the recombinant protein binding site of BONT / E (7, 14). In these studies, the size of the nanoparticles, the zeta potential and the loading rate of the protein were

measured to be 210 nm, +11 mV and 285 nm, 91%, respectively. Vila et al. also used chitosan nanoparticles as carrier proteins for tetanus vaccine and estimated the size of nanoparticles to be 350 nm, zeta potential of +40 mV and protein loading rates of 50 – 60%, and yielded acceptable results in providing a nasal vaccine (15). In a study by Jesus et al., PCI chitosan nanoparticles were used as carriers of the recombinant protein of the hepatitis B antigen. The size of the nanoparticles was 201 nm, the zeta potential was +18.6 mV and the loading rate of the protein was 96% (16). In a study by Nesalin et al., zidovudine was loaded in different concentrations of chitosan nanoparticles. The dimensions of the produced nanoparticles were approximately in the range of 342 – 486 nm and the zeta potential was 20.4 to 37.08 mV (17). Zolfagharinia et al. used TMPC nanoparticles for insulin delivery.

In this study, the size of the produced nanoparticles was 268 nm, the zeta potential was 28.3 mV, and the loading efficiency was reported to be $91 \pm 2.6\%$ (18). Overall, studies have shown that polymer nanoparticles can be used to produce oral vaccines. Our results indicate that the expressed recombinant protein is completely healthy and is encapsulated in the chitosan nanoparticles. In the next study, the immunogenesis of this chimeric structure will be examined in oral mice model and its efficacy as an adjuvant treatment method for acne disease will be investigated.

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