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Optimization and One-Step Purification of Recombinant V Antigen Production from *Yersinia pestis*

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Abstract

The purpose of this study was to develop an efficient and inexpensive method for the useful production of recombinant protein V antigen, an important virulence factor for *Yersinia pestis*. To this end, the synthetic gene encoding the V antigen was subcloned into the downstream of the intein (INT) and chitin-binding domain (CBD) from the pTXB1 vector using specific primers. In the following, the produced new plasmid, pTX-V, was transformed into *E. coli* ER$_{2566}$ strain, and the expression accuracy was confirmed using electrophoresis and Western blotting. In addition, the effects of medium, inducer, and temperature on the enhancement of protein production were studied using the Taguchi method. Finally, the V antigen was purified by a chitin affinity column using INT and CBD tag. The expression was induced by 0.05 mM IPTG at 25 °C under optimal conditions including TB medium. It was observed that the expression of the V-INT–CBD fusion protein was successfully increased to more than 40% of the total protein. The purity of V antigen was as high as 90%. This result indicates that V antigen can be produced at low cost and subjected to one-step purification using a self-cleaving INT tag.

Keywords V antigen · *Yersinia pestis* · Intein · Expression · Purification · Optimization

Introduction

Yersinia pestis has evolved from gastrointestinal pathogens and its antibiotic resistance can cause a dangerous disease, i.e., plague. Therefore, plague is still considered as a major threat to human health [1-3]. The human plague vaccine (USP) is a killed whole-cell plague bacilli that prevents plague infections through subcutaneous injection [4]. However, some reports have recently demonstrated the cytotoxic effects of whole-cell vaccines and their poor protection against virulent strains without capsules [5, 6]. The low calcium response (Lcr) of V antigen (LcrV) and the component 1 (F1) capsular antigen are the two important virulence factors which have been considered as vaccine candidates tested for their efficacy on humans and primates. LcrV is known as the virulence and multifunctional protein. This crucial protein has been shown to act at the level of secretion control by binding to other proteins in order to modulate the host immune response by altering cytokine production [7, 8]. Genetic engineering can be used to produce recombinant vaccines using different parts of *Yersinia*, such as V antigen and F1 [9, 10]. To this purpose, the selection of effective methods to recombinant protein purification is a key factor of production. Highly purified recombinant proteins with the lowest and shortest time are the major problems for biotechnology researchers. Intein (INT) is the internal part of the protein that is derived from immature proteins during protein splicing process [11-13]. Since the discovery of the INT feature in 1990, this fusion tag has been used in many biotechnology applications. One of its wonderful applications is in the INT-tagged self-cleaving step to purify recombinant proteins [14, 15]. The INT tag is separated from the target protein by thiol induction, as well as by temperature and pH changes for one-step protein purification. The advantage of this technique over other protein purification procedures is that the protease phase is eliminated, making the method economically affordable [16-18]. Thus, in the commercial pTXB1 and pTWIN vectors, intein is fused to the chitin-binding domain (CBD). The expressed fusion protein can be ligated to the chitin beads and then, pH,

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temperature changes, or thiol induction leads to cleavage and separation of the target protein from INT tag and CBD. Finally, the target protein can be purified from the column without any additional amino acid [19, 20]. On the other hand, the traditional reaction optimization method involves the influence of one variable factor at a time. This method requires costly, time-consuming, and laborious experiments. Statistical methods including Response surface and Taguchi methodologies are usually used to optimize the effective factors in increasing the production of recombinant proteins. In Response Surface method, the relationship between the independent factors and the effective response can be evaluated in designed experiments to predict, with more tests than Taguchi approach [21, 22]. In turn, in Taguchi methodology, more variables and qualitative factors can be investigated [23, 24]. Generally, when the cost and time limitations make it difficult to perform more experiments in the optimization process and also when discrete or qualitative factors such as media culture types are investigated, Taguchi design is preferred in bench-scale fermenter [25].

In this work, the INT tag was used to purify V antigen from *Y. pestis* in order to develop a new purification strategy for this important protein. In addition, detailed studies were conducted to find optimal conditions of temperature, medium, inducer concentrations, and overexpressed V-INT–CBD fusion protein using the Taguchi method.

Materials and Methods

Primer Designing and Amplification of V Antigen

To amplify the V antigen encoding sequence, specific primers were designed based on the V antigen gene sequence retrieved from Gene bank (Accession No. AF167310.1). The *NdeI* enzyme restriction site sequence was added to the forward primer (5′-GGTGGT CATATGATT AGAG CCT AC GAAC-3′) and *SapI* enzyme restriction site sequence was also added to the reverse primer (5′-GGTGGTT GCTCT TCCGC ATTTACCAGACGTGTCATC-3′). The pET-V (pET28a containing the V antigen encoding sequence) was amplified using the polymerase chain reaction (PCR). The PCR mixture (25 µl) contained 1 × PCR buffer, 4 mM magnesium sulfate, 300 mM of each dNTP, 40 pmol per primer, 5 µl (1 ng) V antigen in pET28 vector, and 0.2 unit Pfu DNA polymerase (Fermentase). The amplification was performed using the Techne thermocycler, with initial denaturation at 94 °C for 4 min, 35 cycles at 94 °C for 60 s, 30 s at 53 °C, and 90 s at 72 °C, and the final extension was performed at 72 °C for 10 min. The PCR product was analyzed using 1% agarose gel electrophoresis.

Cloning of V Antigen in *pTXB1* Vector

The PCR product was purified using a gel purification kit (bioneer). The PCR product and the *pTXB1* vector (NEB #N6707, Biolab) were double-digested with *SapI* and *NdeI* enzymes and then ligated together with *T4* DNA ligase. The cloning of V antigen in the pTXB1 vector was verified by restriction enzyme mapping.

The Expression and Purification of the Fusion Protein

After confirming the pTX-V construct (Fig. 1c), the plasmid was transformed into the competent ER_{2566} strain of *E. coli*, prepared by the calcium chloride method [24]. The transformed bacteria were grown in a 1-l Lysogeny broth (LB) containing 100 µg/ml ampicillin at 37 °C in an air shaker (250 rpm) until the optical density (OD600) at 600 nm reached 0.5–0.6. Then, the T7 promoter was induced by 0.3 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 16 °C for 16 h (to increase the soluble protein). The expression of V antigen was assessed by SDS-PAGE and Western blotting at different times using specific anti-chitin-binding domain serum antibodies (NEB #S6654, New England Biolabs Inc.). The cells were harvested and resuspended in 100 ml of column buffer (20 mM Tris–HCl, pH 8.0, 0.5 M NaCl) and the crude cell extracts were prepared by sonication for 10 cycles of 30 s (Hielscher Ultrasonic, Germany). The supernatant was separated from the cell debris by centrifugation at 12,000×*g* for 30 min at 4 °C and passed through a 1 × 10 cm column (Bio-Rad, Hercules, CA) containing 10 ml of chitin beads (NEB #S6651). The flow rate was 0.5 ml/min. After loading the supernatant on the column, the flow rate was increased to 2 ml/min, and the column was thoroughly washed with the column buffer until the eluted non-specific protein content reached a minimum. Thereafter, a column buffer containing 50 mM dithiothreitol (DTT) was slowly passed through the column, the flow was stopped, and the column was incubated at room temperature for 16 to 40 h. Each fraction (1 ml) containing V antigen was obtained by eluting the column with the column buffer. All samples were analyzed by SDS-PAGE using 12% Tris–glycine gel. The protein concentration was estimated using the Bradford method. After purification, DTT was removed from the buffer and the protein was concentrated using a Millipore centricon tube.

Optimization of V Antigen Expression

The enhancement of recombinant protein production and subsequently, the purification of the target protein, the effects
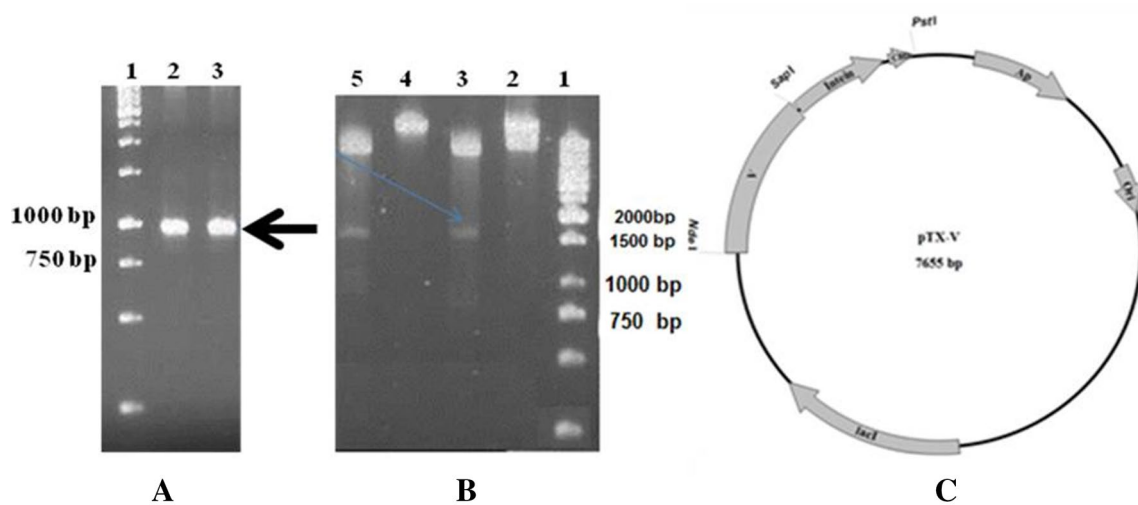
Fig. 1 a Analysis of the PCR product with specific primers, pfu enzyme and *pET-V* plasmid as a template. 1—DNA Ladder 1 kb, 2 and 3—PCR products at 60 °C and 57 °C. **b** Double digestion of *pTX-* *V* using *PstI/NdeI*. 1—DNA Ladder 1 kb, 2—undigested *pTXB1*, 4—undigested *pTX-V*, 3,5—double-digested *pTX-V* in the first and second clones. **c** pTX-V constructs

Table 1 Factors and levels of study in Taguchi experiment

Culture medium	IPTG concentration (mM)	Induction temperature (°C)	Surface factor
TB	0.05	15	1
SB	0.1	20	2
32Y	0.15	25	3

TB terrific broth (tryptone 1.2%, yeast extract 2.4%, glycerol 0.5%), *SB* Super Broth (tryptone 3%, yeast extract 2%, MOPS 1%, Glucose 2%), 32Y (tryptone 0.8%, yeast extract 3.2%, NaCl 0.58% in Tris–HCl 10 mM, pH 7.6)

Table 2 Suggested experiments based on the Taguchi method

Factor	Type of culture medium	Inducer concentration (mM)	Induction temperature (°C)
1	TB	0.1	15
2	SB	0.05	15
3	32Y	0.15	15
4	TB	0.15	20
5	SB	0.1	20
6	32Y	0.05	20
7	TB	0.05	25
8	SB	0.15	25
9	32Y	0.1	25

of temperature on the induction (15, 20, and 25 °C), media (Terrific Broth or TB, contains tryptone 1.2%, yeast extract 2.4%, and glycerol 0.5%; Supper Broth or SB which contains tryptone 3%, yeast extract 2%, MOPS 1%, and Glucose 2%; and 32Y which contains tryptone 0.8%, yeast extract 3.2%, and NaCl 0.58% in 10 mM Tris–HCl, pH 7.6), and inducer concentration (0.05, 0.1 and 0.15 mM) were investigated using the Taguchi statistical method [20] (Table 1). The Taguchi method is used to evaluate the factors considered. This method allows the simultaneous study of different effective factors and their interactions. For this purpose, the experiments were designed on the L9 orthogonal array (Table 2) based on which each proposed test condition was repeated twice. Then the results (final concentration of the recombinant protein) were analyzed using Minitab-16 software. Taguchi uses analysis techniques that do not depend on a model that relates the response to the factor effects. Rather, it uses a calculated response average to identify factors and their levels that yield maximum quality or quantity

of the considered response (in this research the amount of recombinant protein). To compute the average performance or mean effect of any factor, amount of recombinant protein of experiments related to any level of each parameter are added and divided by the number of such trials.

Results

Genetic Construct of *pTXB1- V*

After the PCR reaction, the product was confirmed by the emergence of a 981 bp band on 1% agarose gel electrophoresis at about 981 bp (Fig. 1a). To clone the V antigen in pTXB1, the PCR product of the previous step and pTXB1 were digested by *SapI* and *NdeI* restriction enzymes, and

Table 3 The optimization results of cultivating conditions and induction in the production of V antigen based on orthogonal array
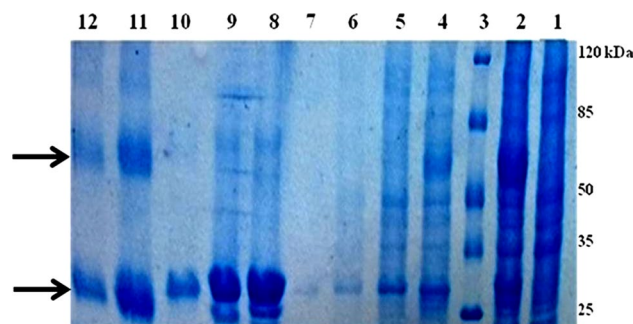
Factor	Induction temp. (°C)	Medium	IPTG (mM)	OD$_{600}$	Dry weight (g/l)	Total protein (g/l)	Producing time (h)	Expression (%)	Productivity (g/l/h)	Recombinant protein (g/L)
1	15	TB	0.1	12	5.25	3.26	54	42.5	0.026	1.38
2	15	SB	0.05	6	2.76	1.77	54	47.8	0.015	0.83
3	15	32Y	0.15	6	2.76	1.8	54	48	0.015	0.86
4	20	TB	0.15	8	3.68	2.02	30	26.7	0.015	0.52
5	20	SB	0.1	5	2.3	1.33	30	37.8	0.017	0.49
6	20	32Y	0.05	5	2.3	1.36	30	39.2	0.016	0.52
7	25	TB	0.05	8.5	3.91	2.61	24	49.69	0.05	1.28
8	25	SB	0.15	7	3.22	1.96	24	40.77	0.03	0.78
9	25	32Y	0.1	8	3.68	2.02	24	26.69	0.02	0.52
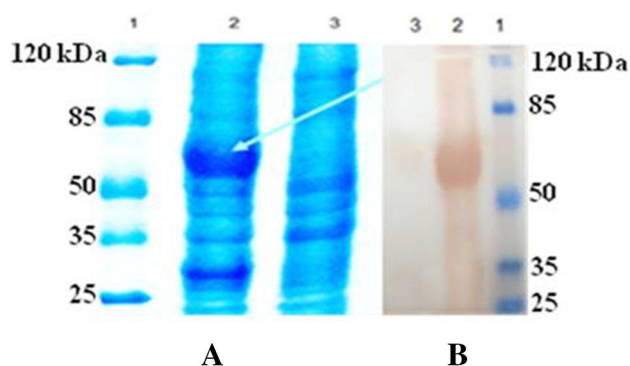
Fig. 2 Expression analysis of recombinant ER$_{2566}$ harboring of *pTX-V* using SDS-PAGE (**a**) and Western blot (**b**). (1) Protein size marker, (2) after induction and (3) before induction


Fig. 3 Purification of V antigen using intein tag. Expression at zero time (1) and after four hours of induction (2), protein size marker (3), cell lysis (4), the output after passing the cell lysis from column (5), output of washing column with buffer (6), initial output after induction by cleavage buffer (7), purified protein after 40 h (8, 9, and 10), the chitin beads after cleavage step (11), output after striping step (12)

the product was ligated into the pTXB1. Two colonies were randomly selected and pTXB1-V double digestion was performed using *PstI* and *NdeI* enzymes (we could not use *SapI* enzyme because this site was disrupted after cloning, and the restriction site of *PstI* is present on pTXB1 but not in the sequence of the cloned V antigen). According to Fig. 1b, the emergence of a 1772 bp band on 1% agarose gel electrophoresis confirmed the production of a new plasmid pTXB1-V (Fig. 1c).

The Fusion Protein (V-INT–CBD) Expression

The pTXB1-V plasmid was transferred into *E. coli* ER2566 and induced by IPTG. The protein expression was assessed by using SDS-PAGE and Western blotting at 0 and 4 h post-induction. A protein size of about 65-kDa confirmed the expression of V antigen together with INT and CBD (Fig. 2) in which the molecular weight of INT and CBD was about 34 kDa and the molecular weight of V antigen was about 33 kDa.

Purification of V Antigen

The V-INT–CBD fusion protein was purified using chitin beads affinity chromatography. To this end, the lysed cells were passed through a column packed with chitin beads. After washing and removal of non-specific proteins, most of the fusion precursors were attached to the resin due to the high affinity of CBD to chitin beads. A lysis buffer containing 50 mM dithiothreitol (DTT) as a cleavage disulfide bond was added into the column and incubated at room temperature for 16 h to release V antigen. In this step, INT and CBD were bound to chitin column. Finally, to remove INT–CBD from the resin, a 2% SDS stripping buffer was flowed through, and CBD and INT (34 kDa) were eluted from the chitin resin. Figure 3 shows the purification step of V antigen with an INT tag on SDS-PAGE. The results of recombinant protein optimization using the Taguchi method are shown in Table 3. Table 4 shows the analysis of variance (ANOVA) for the responses of recombinant protein production carried out according to the factors' contribution

**Table 4** Analysis of variance for the obtained results of recombinant protein optimization using Taguchi method

Factor	DOF (f)	Sum of Sqrs. (S)	Variance (V)	F ratio (F)	Pur Sum (S′)	Percent P (%)
Temperature	2	0.412	0.206	11.207	0.375	41.626
Medium	2	0.316	0.158	8.584	0.279	30.928
IPTG	2	0.137	0.137	3.73	0.1	11.133
Others	2	0.035	0.017			16.313
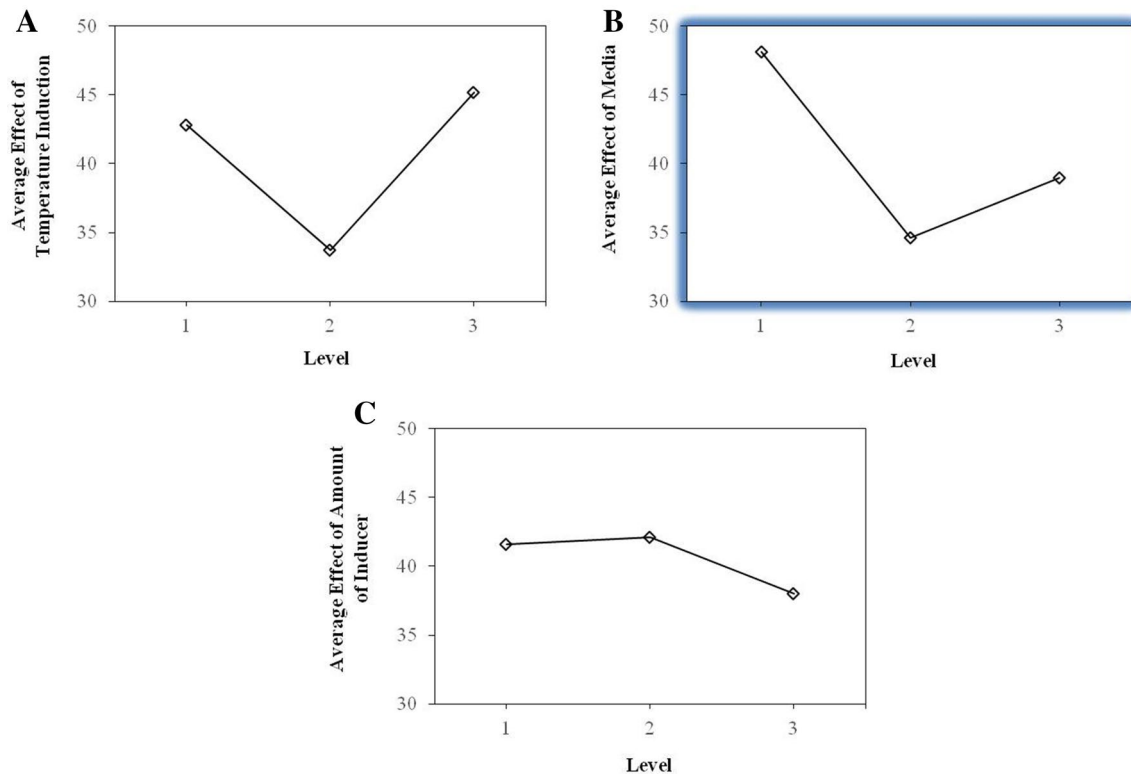
Fig. 4 The mean effect of temperature induction (**a**), media (**b**), and inducer concentration (**c**) at different levels on the recombinant fusion protein production. To compute the average performance or mean effect of any factor, amount of recombinant protein of experiments related to any level of each parameter are added and divided by the number of such trials

by the Taguchi method. In addition, the optimal conditions were obtained by analyzing the final concentration of the recombinant fusion protein and the variance of productivity (ANOVA).

The average effect of the investigated four factors has been plotted in Fig. 4 for visual inspection. The higher the slope of the graph of each factor in these figures indicates the amount of factor effect on the amount of produced protein. Hence, it can be concluded that the type of medium had the greatest influence on the production of the recombinant fusion protein. Also, the induction temperature had the greatest effect impact on the protein production, while IPTG concentration had the least effect (Fig. 4). Furthermore, the results showed that test 7 had the optimum conditions for the production of an INT-V antigen fusion protein in *E. coli*. Figure 5 shows the effect of different conditions including
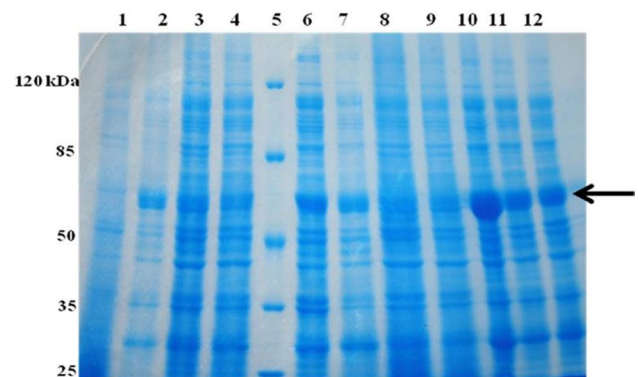
Fig. 5 Coomassie brilliant blue staining of protein, expressed in different temperatures, IPTG concentrations and culture medium by Taguchi statistical method. Time zero before induction (1, 9), test level one (2), two (3), three (4), protein size marker (5), test level four (6), five (7), six (8), seven (10), eight (11) and nine (12)
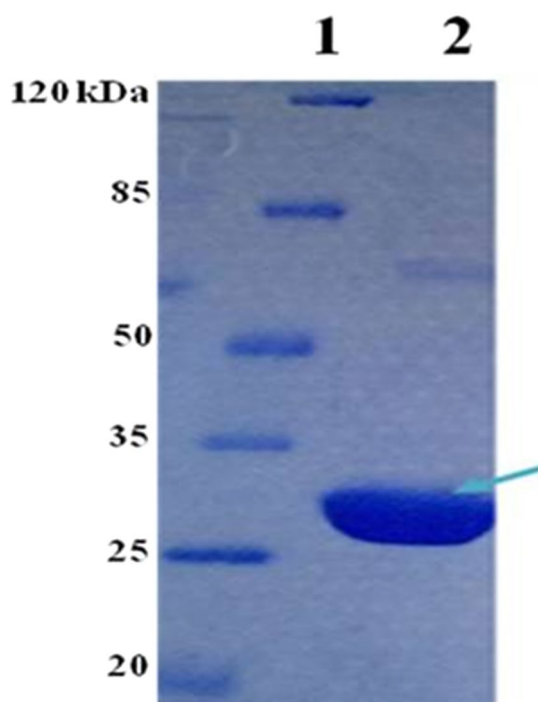
Fig. 6 Purified protein obtained from optimizing the V antigen production using INT after 16 h incubation at 4 °C in cleavage buffer. Protein size marker (1), purified protein (2)

temperature, IPTG concentration, and medium on the V antigen expression. After optimizing the production conditions, the cells were incubated in the cleavage buffer after incubation at 4 °C for 16 h to obtain a protein with a high purity of 90% and a suitable efficiency, as shown in Fig. 6.

Discussion

There is no safe or effective plague vaccine. The World Health Organization classifies the plague as a recurrent disease and as a class A pathogen causing an epidemic disease [26]. Killed whole-cell vaccines can only prevent pests and live attenuated vaccines, making guinea pigs and mice immune to plague, although this vaccine has health problems for humans [27, 28]. Today, researchers emphasize subunit vaccine development. For decades, humans have been focusing on two recombinant proteins, *Yersinia pestis*, F1 and V proteins, as vaccine candidates [9, 10]. In order to facilitate the purification of recombinant proteins, soluble and affinity tags, such as schistosomiasis glutathione S-transferase (GST), Escherichia coli maltose-binding protein (MBP), transcription termination anti-termination factor (NusA), and peptide tags, such as poly His and poly Arg, are often used [29-32]. Since the affinity tag can affect the activity and/or structure of the target protein, it is typically cleaved from the target protein by a protease. Therefore,

the isolation of protease and target protein fusion markers requires some additional purification steps [33]. In addition, proteases are generally inefficient and non-specific, resulting in partially cleaved products. In an intein (INT)-based purification system, a modified INT fusion that induces a cleavage reaction at one end is catalyzed between the chitin-binding domain (CBD) and the recombinant target protein [34]. Using the natural self-splicing mechanism of INT, genetic engineers are able to produce commercial INT vectors to obtain recombinant proteins that do not require enzymatic or cofactor removal labels [35]. The most important feature of INT tag in recombinant protein production is its self-cleavability with the addition of amino acids at the end of the target protein [36]. Furthermore, this method does not require proteases enzyme and time consuming steps for the removal of target proteins. Wood and co-authors believed that this method combined with other methods not only reduces the time and cost of recombinant protein production but also improves the efficiency of the final product [17, 37, 38]. Co-expression of V antigen with a suitable carrier protein results in the protease being less susceptible to degradation during purification [39]. For this purpose, different expression systems are used to increase the production of V antigen. V antigen with GST and INT–CBD was produced in a relatively fused form in 2000 using three expression systems and after cleavage and purification of the target protein, its final concentration was 25 (pGEX-5X-2), 31 (pGEX-6P-2), and 0.75 mg/l [40]. In the present study, we first cloned and optimized the expression and purification of V antigen in the pTXB1 vector containing the 28-kDa-INT tag and 6-kDa-CBD. V antigen production was examined using the INT tag to assess the self-cleavage properties of INT and to optimize culture conditions to increase protein production. The results of the Taguchi method showed that the yield increased to 40% (1.28 g/l). Recent studies have also shown that the molecular weight of hybrid proteins increases by 34 kD compared to the native protein, but it has no negative effect on protein expression. On the other hand, INT-thiol induction demonstrated suitable V antigen purification (Fig. 5) and this strategy was used for large-scale production of proteins and INT self-cleavage can be performed at 4 °C without DTT. In summary, the pTX-V expression system can be used as a suitable substitute for the industrial production of vaccines against sub-units of plague.

Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest.

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