ORIGINAL ARTICLE

Constructing and transient expression of a gene cassette containing edible vaccine elements and shigellosis, anthrax and cholera recombinant antigens in tomato

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Abstract

Shigella dysenteriae causing shigellosis is one of the diseases that threaten the health of human society in the developing countries. In Shigella, *IpaD* gene is one of the key pathogenic genes causing strong mucosal immune system reactions. Anthrax disease is caused by *Bacillus anthracis*. PA protective antigen is one of the subunits in anthrax toxin complex responsible for the transfer of other subunits into the cytosol of host cells. The 20 kDa subunit of PA (PA20) has the property of immunogenicity. CTxB or B subunit of *Vibrio cholerae* toxin (CT) is a non-toxic protein and has the function to transfer toxic subunit into cytosol of the host cells by binding to GM1 receptor. The aim of this study was to fuse *PA20, ipaD* and *CTxB* and transform tomato plants by this cassette in order to produce an oral vaccine against shigellosis, anthrax and cholera. CTxB was used for these two antigens as an immune adjuvant. *IpaD* and *PA20* genes were cloned in pBI121 containing the *CTxB* gene and Extensin signal peptide. In order to evaluate the transient expression of Shigellosis, Anthrax and Cholera antigens, agro-infiltrated tomato tissues were inoculated with *Agrobacterium tumefaciens* containing the gene cassette. Cloning was confirmed by PCR, enzymatic digestion and sequencing techniques. Expression of the antigens was examined by SDS-PAGE, dot blot and ELISA. Maturate green fruits demonstrated the highest expression of the recombinant proteins. The first phase of this study was carried out for cloning and expressing of CtxB, ipaD and PA20 antigens in tomato. In the next phase, we aim to analyze the immunogenicity of this vaccine candidate in laboratory animals.

Keywords Anthrax · Cholera · Tomato · Edible vaccine · Shigellosis · Agro-infiltration

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Background

Shigella is a genus from *Escherichia* order and the *Enterobacteriaceae* family that is a small, non-aerobic bacilli form, gram-negative, non-sedentary and non-sporulating bacterium. In terms of biochemical and serological properties Shigella is classified into four different categories including *Shigella dysenteriae*, *Shigella flexneri*, *Shigella boydii* and *Shigella sonnei* [1]. The main part of the mechanism of molecular pathogenesis of Shigella is the infection of host intestinal epithelial cells and intracellular survival in the epithelial environment. Pathogenesis controlling factors are coded by both plasmids and chromosomes of bacteria [2, 3]. *Ipa* operon contains *ipA, B, C* and *D* genes. The product of these genes are very important antigens, causing the host to show strong immune responses against Shigella. ipaD is a 37 kDa protein that recruits ipaB protein on the cell surface and triggers the invasion. This protein has been located on top of the third type of Shigella secretion system.

Bacillus anthracic is a rod-shaped Gram-positive bacterium with sporulation power. It is sedentary and grows rapidly at 37 °C [4]. Anthrax three main symptoms include skin, respiratory and digestive systems that 95% of cases reported worldwide is skin symptoms [5]. About 80% of skin type of anthrax is not an important issue and will spontaneously improve within a few weeks [6]. While 10–20% of cases, if not going to the clinical treatment, will lead to death [7].

Anthrax toxin consists of three proteins which are non-toxic in the monomeric form and when create a trimeric complex on the surface of mammalian cells, toxic form occurs. Edema factor (EF) and lethal factor (LF) enzymes are transported by the third unit called protective antigen protein (PA) to the cytosol of mammalian cells. EF is an adenylate cyclase causing Edema and impaired immune response to infection when combined with PA and injected to mammals [8, 9]. LF is a protease that cuts some of the mitogen-activated protein kinase kinases [10, 11]. Association of toxin subunits begins when the PA binds to a cellular receptor called receptor of anthrax toxin, a membrane protein with high expression. Then PA is cleaved into two fragments by a protease from the Furin proteases group. This enzyme removes the 20 kDa fragment of the N-terminal, while carboxyl end of the 63 kDa fragment remains attached to the cell receptors [12]. Unlike the native PA, the 63 kDa PA is oligomerized and comes in the form of a heptamer ring [13, 14]. While native PA remains on the cell surface the heptamer is transported into the cell with endocytosis [15]. Transferring non-toxic complexes to the low pH of endosomal vesicles causes 63 kDa heptamer to attach to the membrane to form a channel [16, 17]. EF and LF passing through the channel to reach the cytosol [18].

Cholera is a lethal diarrheal disease caused by a Gram-negative bacterium of *Vibrio cholerae*. The main symptoms of cholera are mainly due to the secretion of cholera toxin called CT. The toxin is a pentameric protein with a molecular weight of 85 kDa composed of two subunits called A and B. The subunit B of cholera toxin (CTxB) with a molecular weight of 11.6 kDa binds to ganglioside GM1 receptor and facilitates the endocytosis of cholera toxin into the host cells. CTxB protein is known as an effective immunogen in the intestinal mucosa. This protein has a variety of applications including usage as an adjuvant in vaccines in which the mucosal immune response is important. It is also known as a suitable vaccine adjuvant for oral and inhalation [19, 20].

Tomato is a valuable vegetable in the Middle East and economically is in the second place in the world after potato. Original homeland of tomato is central and South America and more likely the west coast of South America. Tomato belongs to the *Solanum* Genus. Domestic tomato is *Lycopersicum* sp. Transgenic plants are a good alternative to the animal cells and prokaryotic expression systems.

Transgenic plants are produced with different purposes, such as to obtain higher yield, improved quality and resistance to pests and diseases. Therefore, similarly transgenic plants can be created to express synthetic and medicinal proteins including monoclonal antibodies, antigens, vaccines, therapeutic enzymes, blood proteins, cytokines, growth factors and hormones [21]. The most important advantage of using transgenic plants is healthy aspect of their products. Transgenic plants are not host to human pathogens. Thus human pathogens such as contaminated products to hepatitis, HIV, carcinogens and bacterial toxins, etc. will not exist in transgenic plants [22]. Peptide antigens that are expressed in the edible and delicious parts of plants have led to the idea of the synthesis of active edible vaccines [23].

With this background we aimed to conjugate three pathogenic bacteria antigens to express a conjugated vaccine against three diseases in tomato plants. For this purpose, after cloning of the genes in pBI121 binary expression vector, transient gene expression evaluated by agroinfiltrated tomato leaves and fruit inoculated with *Agrobacterium tumefaciens* containing the genes construct. Expression of the gene cassette was examined by dot blot and ELISA techniques.

Materials and methods

Vectors used

A plant binary expression vector, pBI121 containing the Extensin signal peptide and *CTxB* (Fig. 1) and a pET28 plasmid containing *ipaD* and *PA20* genes (both dedicated by University of Imam Hussain, Iran) were used in this study. These two plasmids were used for the PCR-based amplification of the three genes and further on for cloning in a single cassette and eventually, agroinfiltration experiments.

Cloning of ipaD

A 321 bp fragment of the N-terminal region of *ipaD* gene was amplified by PCR using the forward and reverse primers (Table 1) harboring the restriction sites of *Bam*HI and *Xho*I. Digestion of amplified *ipaD* and pBI_Ex-CTxB vector performed with *Bam*HI and *Xho*I (Fermentase). Gel extraction was carried out with GeneAll gel extraction kit. Ligation performed by T4 DNA Ligase (Fermentase). *E. coli* strain DH5α competent cells prepared [24] and transformed by the recombinant plasmid. Transgenic colonies selected from the selection medium containing 50 mg/l kanamycin. Individual colonies were picked up and processed for the presence of the *ipaD* fragments by sequencing and digestion.
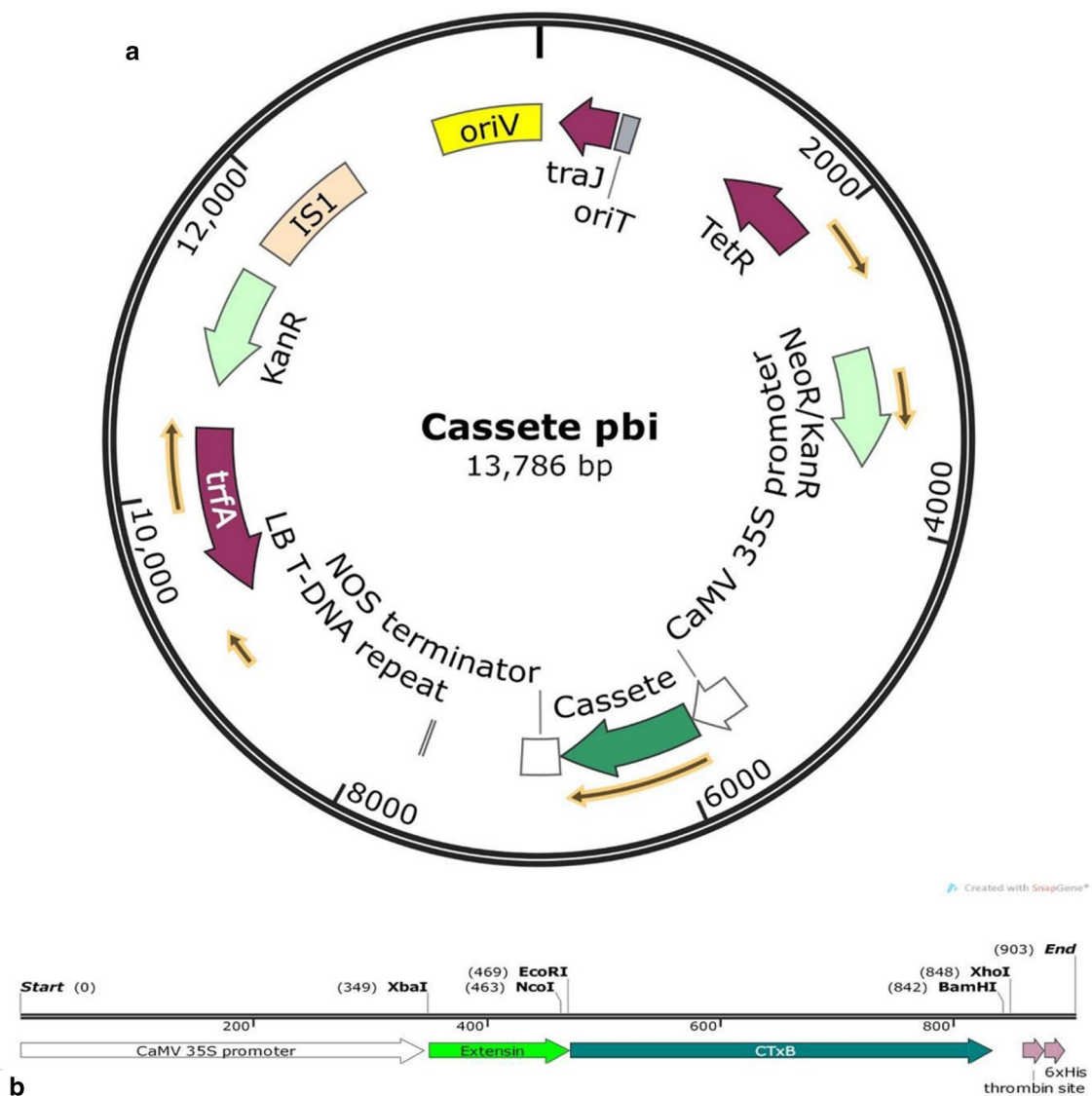
Fig. 1 Linear and circular map of pBI_Ex-CTxB plasmid

Table 1 Primers used for amplifying and cloning of *PA20* and *ipaD*. The underlined sequence in *PA20* forward primer is relate to Linker and the red colored sequences is related to the added restriction site to the primers

Primer	Tm	Enzyme site	Sequence
ipaD-F	62	*Bam*HI	TG*GGATCC*CGTACCACCAACC
ipaD-R	62	*Xho*I	AG*CTCGAG*GGTGTAAGAAGACACCGCGTGC
PA20-F	59	*Bam*HI	AG*GGATCC*GAAGTTAAACAGGAGAACCGG
PA20-R	59	*Bam*HI	AG*GGATCC*GGTCCTGGTCCTCTTGAGTTC GAAGATTTTTGTTTTAATT CTGGC

Cloning of PA20

A 535 bp fragment of a-1 domain of PA20 was amplified by PCR with the forward and reverse primers (Table 1) containing *Bam*HI restriction sites. The amplified fragment and plasmid were digested by *Bam*HI. The linearized plasmid was treated with alkaline phosphatase (Fast-AP Fermentase, 1U for 1 µg plasmid) and extracted from gel (GeneAll gel extraction kit). Following transformation of the ligation product, kanamycin-resistant colonies were selected. To

confirm the presence of PA20 in pBI_Ex-CTxB-PA20-ipaD vector, sequencing, PCR amplification and restriction digestion were used (Fig. 2).

Agroinfilteration

The recombinant vector was transformed into competent *Agrobacterium tumefaciens GV-3101* strain by heat shock [25]. Kanamycin and rifampin were used to select colonies harboring recombinant plasmid and *Agrobacterium* (and not other possible bacteria contamination like transformed *E. coli*), respectively. A colony of *Agrobacterium* was first cultured in the LB medium containing 200 μM acetocyringone and 50 mg/l kanamycin overnight and the bacteria was pelleted at 3500 rpm for 10 min at room temperature. Pelleted bacteria were then resuspended in a 100 ml inoculation buffer and remained on the desk for 3–4 h. To evaluate the transient expression of the genes, agro-infiltration was carried out on the two-month-old tomato plant leaves, maturate green and completely red state fruits, grown in the green house. Leaves and fruits were inoculated with *Agrobacterium tumefaciens* via agro-injection method. In this assay inoculation medium containing 10 mM MgSo4, 10 mM MES and 200 μM acetosyringone (pH adjusted to 5.6) used for injection of *Agrobacterium* with a syringe. The *Agrobacterium* solution was injected smoothly with a needleless syringe to leaf and with a needle containing syringe to fruit pericarp tissues. Fortunately, in the injection area, *Agrobacterium* solution movement inside the leaf and fruit tissue was clearly seen. We tried to inject the parts (about 2 cm in diameter) of the leaf and fruit with *Agrobacterium* completely until saturated level, that we could not inject anymore. Finally, the areas where the solution completely injected, was carefully marked. For protein extraction assay, the places where *Agrobacterium* was injected and marked was precisely and carefully sampled to eliminate the non-*Agrobacterium* injected parts. The inoculated samples were kept in a growth chamber at 26 °C in a 16/8 h dark and light condition for 5–7 days.

Protein extraction

Inoculated leaves and fruits were sampled and used for protein extraction. The lysis buffer was prepared (Tris–HCL 0.1 M, pH = 8, Ascorbic acid 15 mM, DTT 2 mM and PVP 3%). 1 g of fine powdered samples in liquid nitrogen was poured in 3 ml of lysis buffer and completely vortexed and kept in 4 °C for 1 h. The lysate centrifuged in 4 °C for 20 min at 15,000 rpm. The supernatant retrieved and for every 1 ml supernatant 300 μl glycerol was added and stored in − 80 °C.

SDS-PAGE and dot blotting

To evaluate the transient expression of antigens in agro-injected and intact (as control) samples, SDS-PAGE and dot blot analysis were carried out. Anti-ipaD [26] polyclonal antibodies were used as primary antibody against ipaD and goat Anti-Rabbit HRP conjugated (thermofisher, 65-6120) were used as secondary antibody against FC segment of anti-ipaD antibody. Total proteins were extracted from agro-injected leaves and fruits. Recombinant proteins were purified by Co^{2++} column using His-Tag present at the C-terminal of the recombinant protein and analyzed by SDS-PAGE and dot blotting.

ELISA

To perform an ELISA assay Anti-ipaD Rabbit polyclonal and goat Anti-Rabbit HRP conjugated antibodies were used as primary and secondary antibodies respectively in the presence of TMB substrate. In this experiment the ipaD protein (one of our vaccine peptide) were used to detect indirectly by two antibodies. First, the recombinant protein was coated in ELISA wells and the unbounded sites were blocked by skim milk. Anti-ipaD and further anti-human (anti-FC) antibodies used respectively for detecting the ipaD and providing signal. TMB substrate added to the reaction and after color change, reaction stopped by NaOH. Results were analyzed by ELISA reader (BioTek USA).
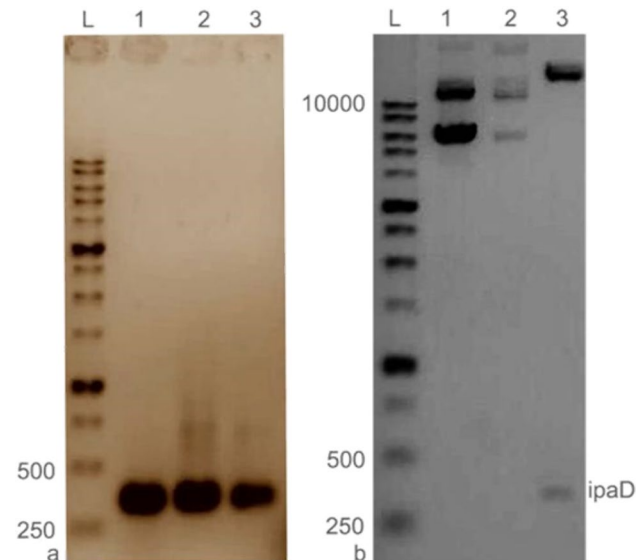

Fig. 2 Amplification of *ipaD* by PCR and digestion of the recombinant plasmid. **a** 321 bp length of *ipaD* amplified by PCR and **b** lane 1 and 2 recombinant plasmid extracted from *E. coli* after cloning and lane 3 digested recombinant pBI_Ex-CTxB-ipaD plasmid with *BamHI* and *XhoI* and separation of *ipaD* (321 bp) from plasmid

Results

Cloning of ipaD

A 321 bp fragment of N-terminal of *ipaD* was amplified using forward and reverse primers by PCR (Table 1) and analyzed on a 1% (w/v) agarose gel (Fig. 2). Figure 2b (lane 3) shows the 321 bp fragment related to *ipaD* that was pulled out from the recombinant pBI_Ex-CTxB-ipaD plasmid by digestion with *Bam*HI and *Xho*I restriction enzymes.
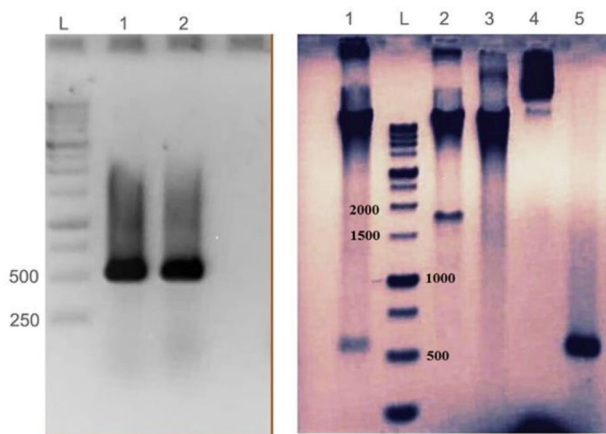

Fig. 3 Amplification and cloning of *PA20*. **a** 535 bp of *PA20* gene that amplified with PCR and **b** digestion of pBI_Ex-CTxB-PA20-ipaD with *Bam*HI (lane 1) and *Hin*DIII (lane 3). In lane 1, 535 bp of *PA20* fragment separated from recombinant plasmid. In lane 3, 1800 bp fragment separated from recombinant plasmid because there was two recognition site for *Hin*DIII, one site in PA20 and another in plasmid. Lane 4, undigested recombinant plasmid and lane 5, 535 bp *PA20* amplified with PCR loaded as control

Fig. 4 Amplification of different fragments of gene cassette. **a** 321 bp of *ipaD* (lane 1), 535 bp 0f PA20 (lane2), **b** PA20-ipaD 856 bp (lane 1–4), and **c** 1692 bp of promoter to *ipaD* (lane1), 1372 bp of promoter to *PA20* (lane2) and 535 bp of *PA20* as control (lane3)
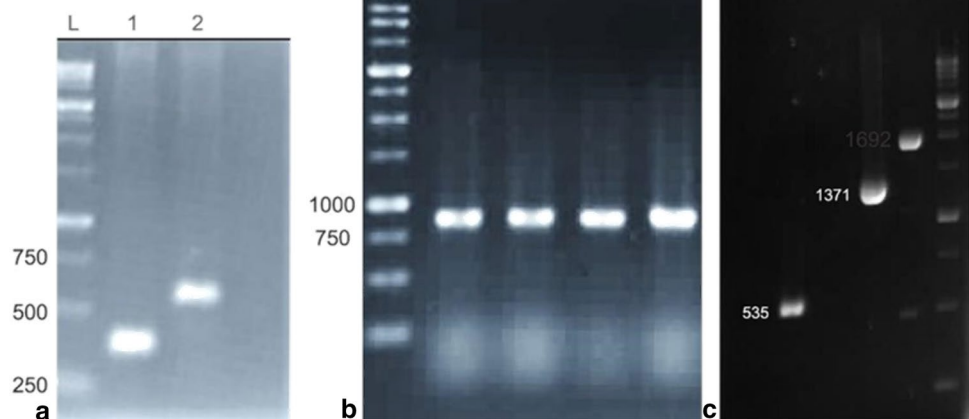
Cloning of PA20

The PCR-based amplified fragment with the length of 535 bp related to *PA20* gene was inserted into pBI_Ex-CTxB-ipaD plasmid between the *CTxB* and *ipaD* genes using *Bam*HI restriction site (Fig. 3a). In order to prevent self-ligation (due to single digestion), the 5′ phosphate was removed by the alkaline phosphatase treatment. Correct cloning of *PA20* in pBI_Ex-CTxB-ipaD was confirmed by sequencing and digestion (Fig. 3b). Figure 3b lane 1 shows the result of digestion of the new engineered plasmid (pBI_Ex-CTxB-PA20-ipaD) with *Bam*HI and the presence of 535 bp fragment (*PA20*). Since the cloning was carried out with the single digestion, checking the orientation of insertion was necessary. Therefore, plasmids were digested with *Hin*dIII. There are two *Hin*dIII restriction sites, one in *PA20* and the other in the plasmid backbone. The digestion of the recombinant plasmid led to the appearance of a 1800 bp fragment and the linearized plasmid on the agarose gel. This digestion procedure shows that the fragment had inserted into the plasmid in a correct orientation (Fig. 3b lane 2). However, digestion of non-recombinant plasmid with *Hind*III, led only to the conversion of circular plasmid to a linear form (Fig. 3b lane 3).
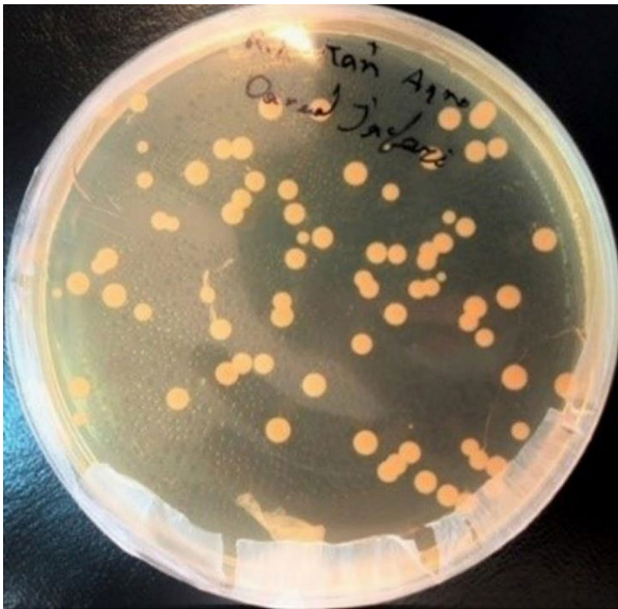
In this experiment each steps of cloning were checked and confirmed by PCR. Based on the agarose gel electrophoresis results, all fragments were exactly equal in size to predicted ones (Fig. 4; Table 2). The results on the agarose gel shows the right assembly of all inserts in the gene cassette. List of primer sequences used in this study were shown in Tables 1 and 2.

Agrobacterium transformation

Recombinant plasmid was transferred into *A. tumefaciens* strain *GV-3101* by heat shock method (Fig. 5).

Table 2 The primers used in PCR reaction

Primer	Fragment to be amplified	Size (base pair)
ipaD F		
	ipaD	321
ipaD R		
PA20 F		
	PA20	535
PA20 R		
PA20 F		
	PA20-ipaD	856
ipaD R		
CAMV35s F		
	CAMV-Ex-CTxB-PA20	1371
PA20 R		
CAMV35s F		
	CAMV-Ex-CTxB-PA20-ipaD	1692
ipaD F		

Fig. 6 Injected leaves of tomato plant with *Agrobacterium tumefaciens* harboring the recombinant plasmid

Fig. 7 The method of sampling of inoculated region (pericarp) of tomato fruits for protein extraction

Recombinant protein analysis

Protein extraction

Total protein was extracted from the *Agrobacterium* injected tomato leaves and fruits after five days from the injection (Fig. 7).

SDS-PAGE and dot blotting

Total protein was extracted from injected and intact (as control) tissues, then recombinant proteins were purified by Co^{2++} column and analyzed by SDS-PAGE. The 50 KD protein on the SDS-Page shows the purified recombinant protein (Fig. 8). Figure 9 shows the nitrocellulose paper with the blotted protein. The maximum coloring was detected for F. Green (green fruit). In the red fruit sample, the coloring was lower than the green fruit. This experiment revealed that recombinant protein production rate is much higher in green fruits than leaves and red fruits.
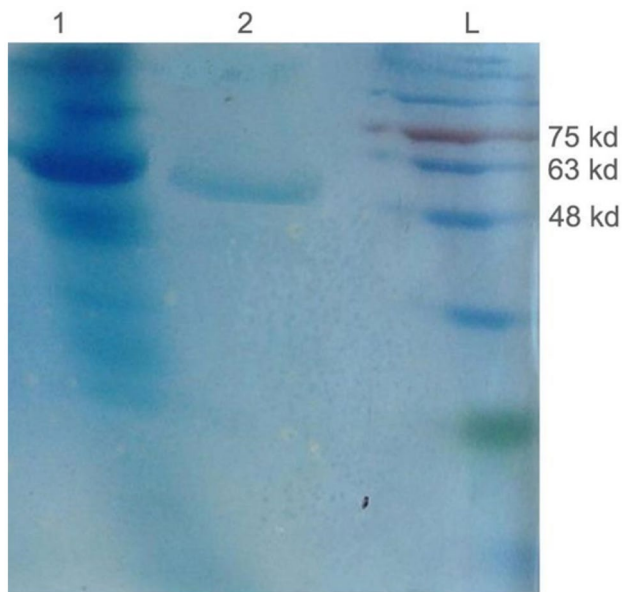

Fig. 5 *Agrobacterium tumefaciens* colonies containing recombinant plasmid grown in Kanamycin (50 mg/l) and riphampin (50 mg/l) containing medium

Transient expression of recombinant protein

Greenhouse grown tomato plants were used to inject *Agrobacterium* into their leaves and fruits (Fig. 6).

Fig. 8 SDS-PAGE of protein. Total protein (lane 1) and recombinant protein purified with Co^{2+} column (lane 2) and extracted of tomato fruit, *L* molecular weight marker
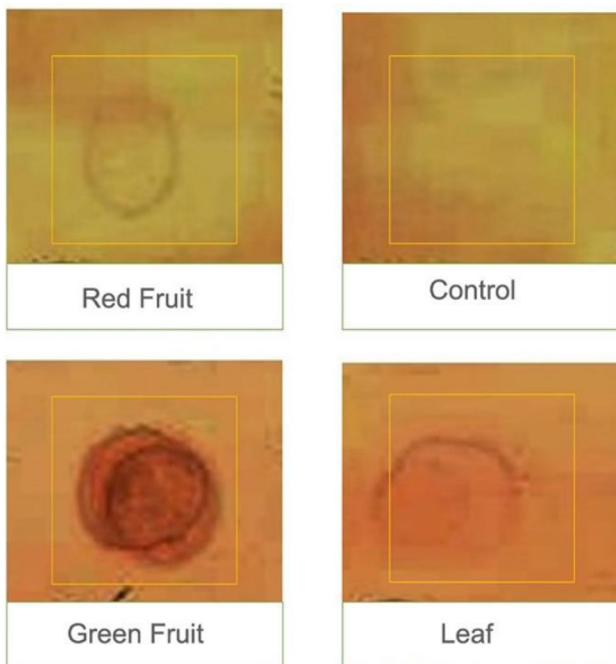

Fig. 9 Dot blotting of extracted protein of tomato tissues with anti-ipaD antibody

ELISA assay

The highest expression (signal) was related to the conjugation of antibody to antigen occurred in tomato green fruit in the 1/100 dilution (Fig. 10). Expression of recombinant protein in tomato red fruit and leaves are the same. No signal can observe in the control sample of non-inoculated leaves and fruits (Fig. 10).

Discussion

Undoubtedly vaccination is an integral part of public and individual health of human in the present day. According to the pandemic and epidemic diseases like shigellosis, anthrax and cholera, vaccination against these diseases is essential for their eradication.

To produce a vaccine for these diseases, the first step is to produce the antigen. Many studies have shown that plant expression systems are quite suitable for the production of proteins. Edibility, nature friendly and no disadvantages to humans, surpasses plants compared to other expression systems such as bacteria and animal. In the present study, first ipaD and PA20 were conjugated and attached to an antigen called CTxB with adjuvant properties. Plants such as tobacco, tomato, and lettuce have been used as model plants to express various recombinant proteins. Tomato is one of the most consumed vegetables in the world with high biomass per hectare that make it a good option for production of high-scale recombinant proteins or antigens.

The reason for using tomato in this study was edibility of its fruit. Undoubtedly, the importance of the prevention of acute bloody diarrhea or shigellosis is not a secret. Strains of *Shigella dysentery* affect many people every year, especially childrens in developing countries.

Here we used ipaD protein that used in various studies and its immunogenicity have been demonstrated. Since Shigella have the ability to attack only to epithelial cells [27], therefore, one can immunize epithelial system by oral administration of this bacterial antigen. Studies showed that ipaD protein has the immunogenicity property and also the potential of vaccine candidate and evaluated its in-vivo immunogenicity by orally administration [28, 29].

Various studies have examined the effect of adjuvant fusion to ipaD. For example, RTB is a plant-derived adjuvant and it was used to promote the immunogenicity of ipaD [30]. RTB specially delivers the ipaD to the mucosal associated lymph tissues (MALTs). The B subunit of cholera toxin or CTxB is a pentameric, non-toxic unit of cholera toxin responsible for binding of CT to its receptor, GM1. Studies revealed that the CTxA1 subunit of CT is responsible for CT toxicity. Binding of CT to CTxB subunit makes a route that CTxA1 can move via translocation through the central channel of CTxB in the clatrin and caveolin coated vesicles to the cytosol of host cells. Most of the mammalian cells, like red blood cells and leucocytes expresses the GM1 receptor on their surface [20]. It has been shown that the CTxB acts

Fig. 10 Data diagram obtained from ELISA reader
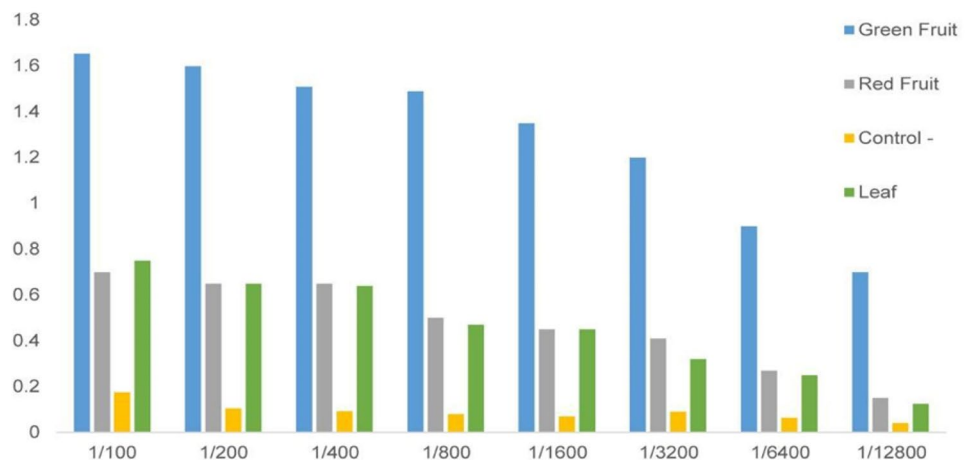
as an adjuvant for immune system and genetically fusing this protein to an antigen improves the immune response of the host to that antigen [20, 31]. Therefore, CTxB can deliver the antigens to MALTs and facilitate its uptake by APC (antigen presenting cells). The second role for CTxB is that it can immunize the host to *Vibrio cholerae*. The anthrax toxin produced by *Bacillus anthracic* has three subunits that are not toxic to the host cells.

Protective antigen (PA) translocate Edema and lethal factors to host cell cytosol [8, 9]. PA is cleaved into two parts, including PA20, by the cell surface proteases. After this cleavage, PA20 releases into the plasma and it seems not to have any roles in disease pathogenesis or bacterial virulence. However, this protein has a diagnostic value for anthrax and can be used to induce immunity against *Bacillus anthracis* [32]. PA20 immunogenicity was confirmed through multiple studies in-vivo in laboratory animals [33–35]. Reason et al. showed that more than 62% of antibodies against PA antigen were specific for a-1 domain of PA or PA20 fragment and this suggest the potential of this fragment to develop as a vaccine candidate [36] even in treatment of cancer due to its capability to induce apoptosis in host cells [37] We expressed these three mentioned antigens in tomato to achieve an edible vaccine. Various studies in this field have been carried out. Demurtas et al. expressed two corona virus antigens responsible for pandemic sever acute respiratory syndrome or SARS in tobacco [38]. Chowdhury and Bagasra presented a theory that could express different life cycle antigens of malaria parasite (about 12 antigens) in tomato plant. They introduced tomato as a healthy and natural friendly plant. The suitability of the use of plants for expressing antigens and the production of edible vaccines by plant expression systems shows a bright future [39]. The first studies that were conducted on potato and tobacco and now other plants such as tomato, banana and lettuce have been put into research on the edible vaccines. Due to the short lifetime for consumption, these plants have

a considerable disadvantage. The way suggested is not so complicated; drying and powdering the fresh fruits or plant tissues. In this regard, the tomato fruits expressing a particular antigen could be kept for 1 year without any decrease in the activity of the antigen [40]. Nowadays, bioinformatics analysis of proteins and antigens became an integrated steps in proteins and antigens identification [41]. Prior to experimental approaches, in a study using bioinformatics tools, we also, determined the most stable chimeric form of these three antigens (ipaD, PA20 and CTxB) in different eukaryotes and prokaryotes cells [42]. In the experimental phase of our study, in the first step, a 321 bp fragment of *ipaD* was inserted into plant expression binary plasmid. The second fragment (*PA20*) was inserted into the plasmid between *ipaD* and *CTxB*. Reverse primer of *PA20* included a sequence of 12 nucleotides (proline and glycine) as a linker that was added to the C-terminal of *PA20* through PCR amplification. Various investigations have used the proline-glycine linker to form a separator between two fused proteins. This sequence (linker) could be identified as the site of cleavage by the cell surface proteases like furin proteases. Delivering the fused antigens to the host cell surface by CTxB protein, makes the linker accessible to the cell surface proteases. Cleavage of this site by the proteases leads to the separation of PA20 and ipaD antigens. After the cloning procedures, we confirmed the correct sequence of recombinant plasmid by sequencing. We expressed the recombinant protein in tomato different tissues through an agro-infiltration assay. Based on the ELISA results, the expression of recombinant proteins in green fruits was 4 times higher than leaves and red fruits. Two assumptions can be made for this result. First, obviously, tomato fruits are more suitable than leaves for agro-injection by syringe, i.e. greater amounts of *Agrobacterium* can be injected into its pericarp tissues than leaves. Secondly, green tomato pericarp tissue is likely to be very active in protein biosynthesis. This is due to the fast growth of fruit in the green stage. In the end of fruit biomass production of

tomato, development of fruit stops and ripening begins. At the ripening state protein metabolism decreases in fruits. However, our results showed that tomato fruit is a suitable choice for the expression of recombinant proteins. Since the maximum expression of antigens was observed in the green tomato fruits. The noteworthy point is that tomato fruit is consumed in a ripe and red state. the production of recombinant proteins in green fruit or leaves will not be so useful. However, it should be noted that in our gene construct a general CaMV35S promoter was used. Thus, as a suggestion for the specific expression of these proteins, a tomato fruit-specific promoter involved in ethylene biosynthesis can be used, so that these genes are activated and expressed at the time of fruit ripening.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval Ethical approval and informed consent were not required for this type of study.

Research involving human participants or animals This article does not contain any studies with human participants or animals performed by any of the authors.

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