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ORIGINAL ARTICLE

Preparation and immunogenicity of tag-free recombinant human eppin

Jie Zhang[1,*], Xin-Liang Ding[2,*], Zeng-Hui Bian[3], Yan-Kai Xia[4], Shou-Lin Wang[4], Ling Song[4] and Xin-Ru Wang[4]

Human epididymal protease inhibitor (eppin) may be effective as a male contraceptive vaccine. In a number of studies, eppin with an engineered His_6-tag has been produced using prokaryotic expression systems. For production of pharmaceutical-grade proteins for human use, however, the His_6-tag must be removed. This study describes a method for producing recombinant human eppin without a His_6-tag. We constructed plasmid pET28a (+)-His_6-tobacco etch virus (TEV)-eppin for expression in *Escherichia coli*. After purification and refolding, the fusion protein His_6-TEV-eppin was digested with TEV protease to remove the His_6-tag and was further purified by NTA-Ni^{2+} affinity chromatography. Using this procedure, 2 mg of eppin without a His_6-tag was isolated from 1 l of culture with a purity of >95%. The immunogenicity of the eppin was characterized using male Balb/c mice.
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Keywords: Eppin; immunogenicity; male contraception; recombinant protein preparation; tag-free

INTRODUCTION

Epididymal protease inhibitor (eppin; SPINLW1) is a serine protease inhibitor-like protein that has been proposed as a target for a novel male contraceptive method.[1] Eppin was discovered in 2001 as a cysteine-rich protein that contains both Kunitz-type and WAP-type four disulfide core protease inhibitor consensus sequences.[2] Subsequent studies showed that the protein is specifically expressed in the testis and epididymis in primates and rodents.[2,3] In both sets of species, two isoforms of eppin are present: one isoform is secreted, and the other lacks a signal sequence.[2] Functional studies of eppin have focused on the form that lacks a signal sequence.[4–7] The importance of eppin in male reproduction was confirmed by the demonstration of a reversible contraceptive effect in male monkeys immunized with recombinant human eppin and by the upregulation of *EPPIN* gene expression in non-obstructive azoospermia patients.[1,8] In further studies, we found that variants of the *EPPIN* gene affect the risk of idiopathic male infertility in the Han-Chinese population.[9,10] However, the molecular mechanism of eppin action is not presently understood.

The expression and purification of recombinant protein often represents a first step in the study of a new protein.[11] In previous studies, recombinant human eppin with an *N*-terminal poly-histidine tag (His_6-tag) was used to investigate the protein's function.[1,6,7,12,13] However, tags, whether large or small, have the potential to interfere with the biological activity of a protein,[14,15] and the His_6-tag cannot be used for the production of pharmaceutical-grade proteins.[16] Several strategies have been used to remove fusion tags from proteins after purification.[14,17] Cleavage by a specific endoprotease at a specifically recognized sequence inserted between the tag and the target protein is a feasible way to remove the fusion tag.[18] The endoproteases most commonly used for this purpose are factor Xa, enterokinase (enteropeptidase, EK) and thrombin.[15] These endoproteases do not exhibit stringent sequence specificity and often cleave at non-target sites.[19] Recently, viral proteases, including the tobacco etch virus (TEV),[18] have been used as endoproteases to remove fusion tags; such proteases offer the advantage of a high stringency of sequence recognition.

In this study, we expressed eppin as a recombinant protein with an *N*-terminal His_6-tag and an intervening TEV endoprotease recognition site to facilitate tag removal and removed the His_6-tag from the recombinant protein using TEV endoprotease. We immunized Balb/c mice with the purified eppin and detected high-titer antibodies to eppin in the serum. The production of tag-free eppin will facilitate the study of its structure–function relationship and may contribute to the development of potential clinical applications of this protein.

MATERIALS AND METHODS

Materials

Human epididymal tissues were generously provided by patients at the Affiliated Hospitals of Nanjing Medical University (Nanjing, China), who gave informed consent for the use of tissues obtained by surgical resection. The *Escherichia coli* TOP10 competent cells and BL21 Star (DE3) competent cells were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). The expression vector pET28a (+) and TEV endoprotease were generous gifts from Dr Zi-Chun Hua, The State Key Laboratory of Pharmaceutical Biotechnology and Department of Biochemistry, College of Life Science, Nanjing

[1]School of Radiation Medicine and Public Health, Medical College of Soochow University, Suzhou 215123, China; [2]Department of Public Health, Wuxi Center for Disease Prevention and Control, Wuxi 214023, China; [3]Municipal Center for Disease Control and Prevention, Nanjing 210003, China and [4]Key Laboratory of Reproductive Medicine, Institute of Toxicology, Nanjing Medical University, Nanjing 201129, China
* These authors contributed equally to this work.
Correspondence: Dr XR Wang (xrwang@njmu.edu.cn)
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University, China. The gel extraction kit was from Qiagen (Valencia, Canada); plasmid extraction kits were purchased from Tiangen Biotech Co., Ltd (Beijing, China). Restriction enzymes, Taq DNA polymerase, dNTPs, M-MuLV reverse transcriptase, ribonuclease inhibitor and T4 DNA ligase were obtained from MBI Fermentas (Burlington, Canada). Primers were synthesized by Invitrogen (Shanghai, China). All other reagents were of research grade and were obtained from commercial sources.

Cloning of the *EPPIN* gene and construction of its expression plasmid

Total RNA was extracted from human epididymal tissue using TRIzol reagent according to the manufacturer's protocol. ***EPPIN*** cDNA (nucleotides 86–434) lacking part of the *N*-terminal secretory sequence was generated by reverse transcriptase polymerase chain reaction using total epididymal RNA as a template. Gene-specific primers were used to amplify ***EPPIN*** cDNA. The forward primer was 5′-CGGTCATATGGAAAACCTGTATTTTCAGGGCGTCCAGGGACCTGGTCTGA-3′; this primer contains four protective base pairs (CGGT), an NdeI site (CATATG) and a nucleotide sequence encoding the TEV endoprotease (GAAAACCTGTATTTTCAGGGC). The reverse primer was 5′-GTTCGAGCTCTCAGGGAAAGCGTTTATTCTTGCAG-3′, which contains four protective base pairs (GTTC) and a SacI site (GAGCTC). The amplified ***EPPIN*** cDNA fragment was double-digested by NdeI/SacI and cloned into the pET28a (+) vector, which was digested in the same manner, to produce the pET28a (+)-eppin plasmid. The plasmid was transformed into *E. coli* TOP10 competent cells, and positive clones were validated by DNA sequencing.

Time course expression study and protein expression analysis

The pET28a (+)-eppin plasmid was transformed into *E. coli* BL21 Star (DE3) competent cells for expression of the recombinant protein His_6-TEV-eppin. Single clones were inoculated into 5 ml of Luria–Bertani medium with 100 μg ml^{-1} of kanamycin and incubated at 37 °C with centrifugation at 280 *g* overnight. The next day, 200 μl of the overnight culture was used to inoculate 20 ml fresh Luria–Bertani, and the fresh culture was incubated at 37 °C until its optical density at 600 nm ($o.d._{600}$) was about 0.6 (about 2 h). Protein expression was induced by the addition of IPTG to a final concentration of 1 mmol l^{-1} and cultured at 37 °C at 280 *g* or various time durations. Two milliliters of bacterial culture were removed at time points 0, 1, 2, 3, 4 and 5 h after induction. The cell pellets were collected from the samples by centrifugation at 12 000 *g* or 10 min at 4 °C, resuspended in 30 μl of sodium dodecyl sulfate (SDS) sample loading buffer and analyzed by 15% SDS–PAGE. The Coomassie-stained SDS–PAGE gel was scanned with a UVP white/ultraviolet trans-illuminator, and the protein bands were quantified using Grab-it 2.5 and Gelwork software.

To determine whether the expressed protein was soluble or insoluble, cells collected from 5 ml of culture were resuspended in 0.5 ml of distilled water and lysed by sonication. Insoluble proteins were collected by centrifugation and dissolved in SDS sample loading buffer, while the supernatant with the soluble proteins was mixed with an equal volume of 2× SDS sample loading buffer; both fractions were analyzed by SDS–PAGE and Coomassie staining.

Large-scale protein expression and purification procedure

For large-scale protein expression, 10 ml of an overnight culture of pET28a (+)-eppin- transformed BL21 Star (DE3) was used to inoculate 1 l of Luria–Bertani medium containing kanamycin at 37 °C. Induction was initiated at mid-log phase ($o.d._{600}\approx0.6$) by the addition of IPTG to a 1 mmol l^{-1} final concentration. The cells were harvested after 4 h by centrifugation and stored at −70 °C until protein purification.

To purify the recombinant proteins, the cell pellet was resuspended and sonicated in lysis buffer (50 mmol l^{-1} Tris, 100 mmol l^{-1} NaCl, pH 8.0). After centrifugation, insoluble inclusion bodies were resuspended in binding buffer (50 mmol l^{-1} sodium phosphate, 300 mmol l^{-1} NaCl, 8 mol l^{-1} urea, pH 8.0) with 10 mmol l^{-1} reduced glutathione (GSH) and 2 mmol l^{-1} glutathione-oxidized form (GSSG) and dissolved overnight at room temperature. The dissolved liquid was centrifuged and the supernatant loaded onto a Ni-NTA agarose column. The column was washed with binding buffer and subsequently with wash buffer (50 mmol l^{-1} sodium phosphate, 300 mmol l^{-1} NaCl, 8 mol l^{-1} urea, pH 6.0). The bound protein was eluted with elution buffer (50 mmol l^{-1} sodium phosphate, 300 mmol l^{-1} NaCl, 8 mol l^{-1} urea, 250 mmol l^{-1} imidazole, pH 8.0).

The separated crude bacterial lysates (total, soluble and insoluble fractions) and purified His_6-TEV-eppin were analyzed by SDS–PAGE. The percentage of His_6-TEV-eppin expression was determined to be total induced His_6-TEV-eppin/total protein in crude bacterial lysates×100%.

Protein refolding by dilution and concentration by ultrafiltration

The eluted fraction (50 ml) was immediately diluted to 400 ml in refolding buffer (50 mmol l^{-1} Tris, 100 mmol l^{-1} NaCl, 0.4 mol l^{-1} *L*-arginine, 10 mmol l^{-1} GSH, 2 mmol l^{-1} GSSG, pH 8.0); the refolding buffer was added slowly by a constant flow pump at a velocity of 0.1 ml min^{-1}. After refolding by dilution, ultrafiltration in a stirred ultrafiltration cell (Millipore, Billerica, MA, USA) was performed to remove urea and imidazole from the protein solution. During the ultrafiltration process, the volume of the protein solution was reduced to 40 ml. Additional fresh buffer (50 mmol l^{-1} NaH_2PO_4, 100 mmol l^{-1} *L*-arginine, pH 7.4) was then added to bring the volume to 400 ml. This ultrafiltration process was repeated eight times to completely remove urea and imidazole. The purified protein was stored at −70 °C.

Time course of His_6-TEV protease digestion

To determine the optimal cleavage time, 0.5 mg of purified recombinant protein was incubated with 0.01 mg of His_6-TEV protease (TEV/substrate ratio=1 : 50) in 1 mmol l^{-1} DTT, 50 mmol l^{-1} Tris, pH 8.0, at 20 °C for 0, 2.5, 5 and 7 h, and the products were analyzed by 15% SDS–PAGE and Coomassie staining.

Cleavage of His_6-tag and purification of eppin

Purified, renatured and ultrafiltered His_6-TEV-eppin was digested using His_6-TEV protease as described above. In brief, 12 mg of substrate was incubated at 20 °C for 5 h with 0.24 mg of His_6-TEV protease in 1 mmol l^{-1} DTT, 50 mmol l^{-1} Tris, pH 8.0. The incubation products were then loaded onto a Ni-NTA agarose column. The cleaved eppin flowed through the column, while His_6-TEV-eppin and His_6-TEV protease bound to the column. The cleaved eppin was collected and stored at −70 °C until the immunogenicity study. All fractions obtained in the isolation process were analyzed by 15% SDS–PAGE followed by staining, scanning and protein band quantification as described above. Purity was expressed as cleaved eppin/total protein in fraction×100%.

Western blot

Protein samples were separated on 15% SDS–PAGE gels and electrotransferred to polyvinyldifluoride membrane using a semidry electrophoretic transblotter (Bio-Rad, Hercules, CA, USA). The membrane was blocked with 5% non-fat milk in Tris-buffered saline (pH 7.4) supplemented with 0.1% (v/v) Tween-20 (TBST) for 2 h and then incubated at 4 °C overnight with rabbit eppin antibody (1 : 1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted in blocking solution. The membrane was washed three times in TBST and probed with peroxidase-conjugated goat anti-rabbit IgG (1 : 5000; Beijing ZhongShan Biotechnology Co., Beijing, China) for 1 h at 37 °C. After washing, specific signals were detected using enhanced chemiluminescence Western blotting detection reagents (Amersham Life Science Ltd, Little Chalfont, UK).

Protein determination

Protein concentration was measured as described by Bradford[20] with bovine serum albumin as a standard.

Immunogenicity study of eppin

Purified recombinant eppin was used in the immunogenicity study. Twenty specific pathogen-free Balb/c male mice (6–8 weeks old) were obtained from Shanghai Laboratory Animal Research Center (Shanghai, China) and randomly divided into three groups (phosphate-buffered saline (PBS) group, eppin group and eppin-alone group). The mice were maintained with food and water *ad libitum* in a temperature-controlled room with a 12-h light/12-h dark cycle. Mice of the eppin group were immunized by subcutaneous injection on the hindlimb of 100 μg of eppin diluted in 100 μl of PBS, pH 7.2, and emulsified with an equal volume of complete Freund's adjuvant for the primary immunisation (day 1). Freund's incomplete adjuvant was used in the boost immunisation on day 17. Animals of the PBS group and the eppin-alone group received the same injections as the eppin group but without eppin or adjuvant, respectively. Serum samples were taken from mice of all groups on days 0, 15, 31, 45 (weeks 0, 2, 4 and 6 after the first immunisation) and assayed for anti-eppin IgG.

Antibody titers were determined by enzyme-linked immunosorbent assay in 96-well microplates. The plates were coated with 100 μl per well of purified recombinant eppin at a concentration of 0.001 mg ml^{-1} in coating buffer (50 mmol l^{-1} sodium bicarbonate, pH 9.6) at 4 °C in a humidified atmosphere overnight. The plates were washed three times with TBST, and 200 μl of blocking buffer (5% non-fat milk in TBST, pH 7.6) was added to each well. The plates were incubated at 37 °C for 2 h and washed three times with TBST. After washing, 100 μl of appropriately diluted mouse serum was added to the wells of the blocked plates. The plates were incubated for 2 h at 37 °C and washed as previously described. Horseradish peroxidase-conjugated goat anti-mouse IgG was diluted to 1 : 5000 with 5% non-fat milk in TBST, added (100 μl per well) to each well and incubated at 37 °C for 1 h. The plates were washed three times with TBST. One hundred microliters of TMB substrate (Tiangen, Beijing, China) were then added to each well, and the plates were incubated in the dark at 37 °C for 15 min. The reaction was stopped with 50 μl per well of 2 mmol l^{-1} H_2SO_4 solution, and the absorbance was measured at 450 nm (Bio-tek, Winooski, VT, USA). The end-point titer of the individual plasma samples was considered positive if the absorbance was at least twofold higher than the background. The mean±s.e.m. of antibody titers was calculated for each group at each serum collection.

RESULTS

Construction of expression plasmid pET28a (+)-His_6-eppin

The coding sequence of eppin lacking part of the *N*-terminal secretory sequence was cloned into the T7 promoter-driven fusion expression vector pET28a (+) (**Figure 1**). The resulting plasmid, pET28a (+)-His_6-eppin, was analyzed by polymerase chain reaction and restriction enzyme digestion and confirmed by DNA sequencing (data not shown).

Expression of His_6-TEV-eppin in *E. coli*

The prokaryotic expression vector pET28a (+)-His_6-eppin was transformed into the *E. coli* BL21 Star (DE3) expression host strain for protein expression. A small-scale time course experiment was performed to determine the optimal induction time for protein expression by the bacterial culture. The largest amount of correctly sized (17 kDa) His_6-TEV-eppin protein was present 4 h after IPTG induction; therefore, the cells were harvested at this time point (**Figure 2a and b**). The expressed protein was present predominantly in the insoluble fraction as inclusion bodies (**Figure 2c**). Inclusion bodies are useful because their formation generally leads to high protein expression levels and because they protect the protein from protease activity within the cell.[21] The presence of proteins in inclusion bodies also limits the number of purification steps needed because few contaminants are present within the inclusion bodies.[22] The results presented here show that recombinant His_6-TEV-eppin can be highly expressed in the inclusion bodies of the host cells.

Purification of His_6-TEV-eppin from inclusion bodies

For large-scale protein expression, about 30 mg of His_6-TEV-eppin was obtained from 1 l of culture (**Figure 2d**). For successful renaturation of the protein, it was necessary to completely remove the urea and imidazole from the protein solution. To improve the solubility of the His_6-TEV-eppin during dilution and refolding, we added the redox components GSH and GSSG and 0.4 mol l^{-1} *L*-arginine to the refolding buffer. Ultrafiltration was subsequently performed to remove the urea and imidazole and to concentrate the refolded protein. A refolding efficiency of approximately 40% was achieved with this method,

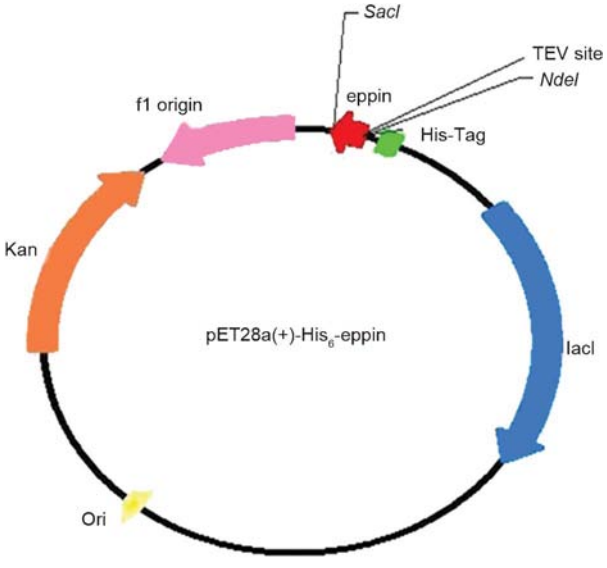

Figure 1 Diagram of the recombinant expression plasmid pET28a (+)-His_6-eppin. eppin, epididymal protease inhibitor; TEV, tobacco etch virus.

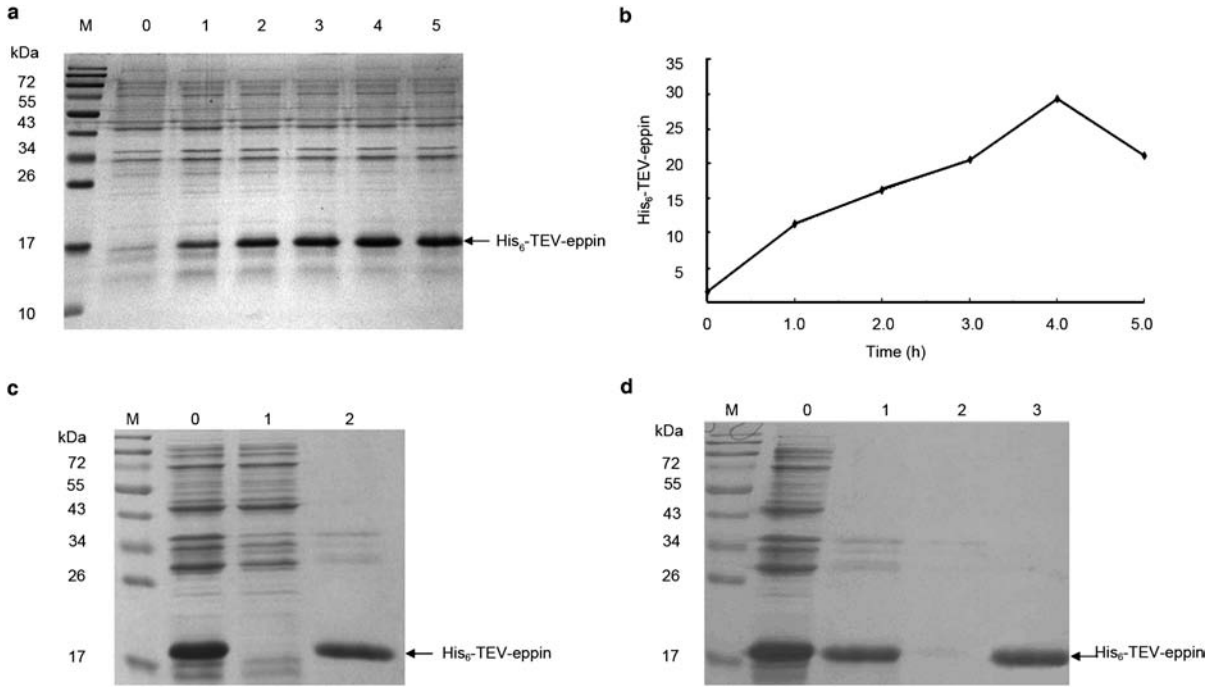

Figure 2 Analysis of the expression and purification of His_6-TEV-eppin. (**a**) SDS–PAGE analysis of His_6-TEV-eppin induced with 1 mmol l^{-1} of IPTG at different times (representative of three independent experiments). M, protein marker; 0, untreated; 1–5, treated for 1–5 h. (**b**) Quantitative analysis of SDS–PAGE (representative of three independent experiments). The time course of IPTG induction of His_6-TEV-eppin expression, as determined by quantitative analysis of the gel after SDS–PAGE, is plotted. (**c**) SDS–PAGE analysis of the localisation of His_6-TEV-eppin induced by IPTG (representative of three independent experiments). M, protein marker; 0, total cellular proteins from induced cells harboring His_6-TEV-eppin; 1, cellular supernatant fraction; 2, cellular insoluble fractions. (**d**) SDS–PAGE analysis of the purification of the recombinant protein His_6-TEV-eppin (representative of three independent experiments). M, protein marker; 0, total cellular proteins from induced cells harboring pET28a (+)-eppin; 1, inclusion bodies dissolved in 8 mol l^{-1} urea; 2, wash fractions from affinity chromatography; 3, eluted fraction from affinity chromatography. eppin, epididymal protease inhibitor; SDS, sodium dodecyl sulfate; TEV, tobacco etch virus.

yielding approximately 12 mg of refolded His_6-TEV-eppin from 1 l of culture.

Cleavage of the His_6-tag and purification of eppin

After the recombinant fusion protein had been successfully purified, renatured, filtered and concentrated, the next step was to remove the His_6-tag. As shown in **Figure 3a**, the optimal time for the cleavage of the His_6-tag of His_6-TEV-eppin by TEV was 5 h. Following TEV treatment, the digested sample was again loaded onto the Ni-NTA agarose column. The nickel beads bound the cleaved His_6-tag fragment and the TEV endoprotease, whereas the eppin did not bind to the column (**Figure 3b**). Approximately 2 mg of eppin was obtained from

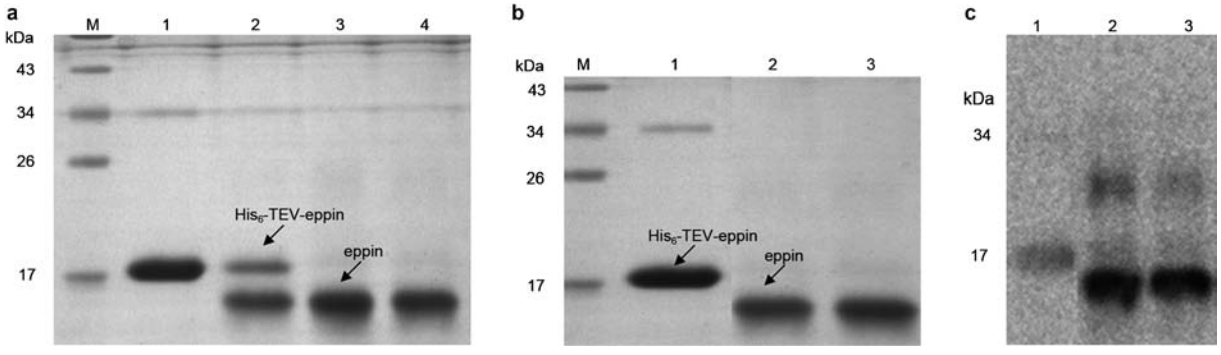

Figure 3 Analysis of His_6-TEV-eppin digested by His_6-TEV protease. (**a**) SDS–PAGE and time course of His_6-TEV-eppin digested by His_6-TEV protease (representative of three independent experiments). His_6-TEV-eppin (0.5 mg) was incubated with 0.01 mg His_6-TEV protease (TEV/substrate ratio = 1 : 50) at 20 °C for different times (lanes 1–4: 0, 2.5, 5 and 7 h, respectively) in 1 mmol l^{-1} DTT, 50 mmol l^{-1} Tris, pH 8.0. All samples were analyzed by 15% SDS–PAGE. Cleavage of the 17 kDa His_6-TEV-eppin produced a ~14-kDa eppin product. (**b**) SDS–PAGE analysis of His_6-TEV-eppin digested by His_6-TEV protease (representative of three independent experiments). His_6-TEV-eppin (12 mg) was incubated with 0.24 mg of His_6-TEV protease (TEV/substrate ratio = 1 : 50) at 20 °C for 5 h in 1 mmol l^{-1} DTT, 50 mmol l^{-1} Tris, pH 8.0. All samples were analyzed by 15% SDS-PAGE. Cleavage of the 17-kDa His_6-TEV-eppin produced a ~14-kDa eppin product. Lane M: protein marker; lane 1: His_6-TEV-eppin before digestion; lane 2: digested sample without purification; lane 3: purified eppin. (**c**) Western blot analysis of His_6-TEV-eppin digested by His_6-TEV protease. Lane 1: His_6-TEV-eppin protein before digestion; lane 2: digested sample without purification; lane 3: purified eppin. Molecular weight markers (kDa) are indicated to the left of the blots. eppin, epididymal protease inhibitor; SDS, sodium dodecyl sulfate; TEV, tobacco etch virus.

the starting amount of 12 mg of His_6-TEV-eppin using this process. As analyzed by SDS–PAGE followed by Coomassie blue staining, the purity of the eppin was about 95%.

Western blotting of recombinant eppin demonstrated that a 17-kDa monomer and a 34-kDa dimer were present before cleavage; a predominant immunoreactive band of ~14 kDa and minor bands of ~28 kDa were detected after the fusion protein was cleaved (**Figure 3c**).

Immunogenicity study of eppin

Serum samples from the vaccinated mice were titered for IgG specific to purified eppin by serial dilution with a standard enzyme-linked immunosorbent assay. Serum from the group of mice injected only with PBS was used to determine the background level of the immunoassay. The mean reciprocal of end-point titers for each group of animals at different times is shown in **Figure 4**. Two weeks later after the initial immunisation, an antigen-specific IgG antibody response was detected in the eppin protein group; however, the mean reciprocal end-point dilution titer did not exceed 1000 until after the boost immunisation (**Figure 4**). By day 31 (4 weeks), animals in the eppin group showed a strong antibody response; the mean reciprocal end-point dilution titer±s.e.m. was 13 733±2554, and the high titer IgG was maintained for at least 2 weeks (the end-point dilution titer was 16 267±1392 at day 45 (6 weeks)). These results indicate that purified eppin can stimulate powerful humoral immunity.

DISCUSSION

Although affinity tags provide highly efficient tools for protein purification, the presence of an affinity tag in a recombinant protein may be unnecessary or represent a disadvantage for the clinical use of the protein.[23] In this study, we present a method for producing tag-free eppin.

We constructed the pET28a (+)-His_6-eppin plasmid with an intervening TEV endoprotease recognition site, followed by transformation into the *E. coli* BL21 Star (DE3) expression host strain for protein expression. Because most of the recombinant His_6-TEV-eppin produced in *E. coli* formed inclusion bodies, we purified the fusion protein under denaturing conditions.

To cleave the His_6-tag with TEV protease, it was necessary to remove the urea and imidazole and refold the proteins. The use of additives during the refolding process often helps to improve the yield of biologically active protein from inclusion bodies.[24] *L*-Arginine is known to reduce the aggregation of a number of proteins.[25–27] In addition, eppin has 14 cysteine residues (10.5% cysteine content), and the correct refolding of this protein requires that a redox system be present in the refolding solution. Therefore, during the dilution refolding procedure, *L*-arginine and GSH/GSSG were added to improve the solubility of His_6-TEV-eppin. A refolding efficiency of about 40% was achieved. After cleavage and separation on a Ni-NTA agarose column, 2 mg of eppin without the His_6-tag was isolated from 1 l of culture with a purity of >95%.

The purified eppin exhibited excellent immunogenicity. After immunisation of male Balb/c mice with purified eppin mixed with Freund's adjuvant, the highest end-point dilution titer in serum was above 10 000, and the high titer of IgG could be maintained for at least 2 weeks.

This paper describes a novel method for the production of recombinant human eppin without a His_6-tag. The method described in this work should facilitate further studies of the structure and function of eppin and may also permit the large-scale production of biologically active recombinant human eppin suitable for use in a contraceptive vaccine for men.

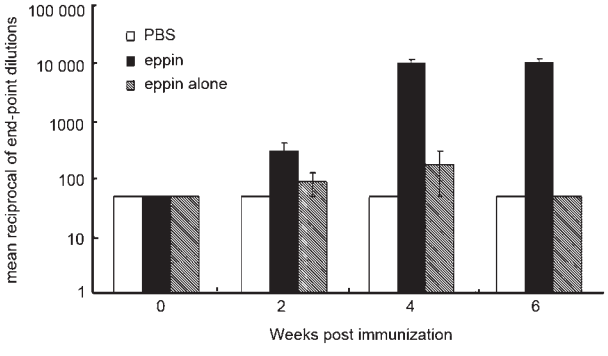

Figure 4 Specific immune response in Balb/c mice injected with eppin. The eppin group received eppin mixed with complete Freund's adjuvant, and Freund's incomplete adjuvant was used in the booster. As controls, the eppin-alone group received eppin without adjuvant, while the PBS group received two injections containing only adjuvant and PBS. eppin, epididymal protease inhibitor; PBS, phosphate-buffered saline.

AUTHOR CONTRIBUTIONS

JZ and XLD designed and performed experiments, analyzed data and wrote the paper; ZHB performed experiments; YKX, SLW, LS and XRW supervised the project.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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