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# Expression of recombinant HAO3 from an Iranian isolate of *Hyalomma anatolicum anatolicum* in *Pichia pastoris* and evaluation of its antigenicity

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ABSTRACT

*Hyalomma anatolicum anatolicum* tick is considered as one of the main problem of ruminants' productivity in endemic countries such as parts of Africa, the Middle East and India. The disease is economically important and hence, its control and eradication is a priority. This problem reinforces the need for alternative approach like vaccine to control tick infestations instead of continuous application of acaricide which led to the natural selection of the acaricide-resistant ticks. Therefore, the present study provided evidence for the construction of transformant containing the chromosomally integrated multicopy expression cassettes of *HAO3*, its successful and efficient expression in *Pichia pastoris* yeast and purification of the secreted protein by ultrafiltration (UF) system in a high level yield and purity.

The result of antigenicity assay for the rHAO3 protein pointed well toward its capability for the elicitation of antibody response in immunized rabbits. Interestingly, the results indicated that the expressed HAO3 protein reacted well with mid gut antigen (MGAg) and rBm86 (Gavac) antisera in ELISA and western blot assays making it evident that the epitopes present in expressed protein are well recognized by the antibodies against MGAg and rBm86 proteins. Moreover, the presence of cross-reactive epitopes between rHAO3 protein with its native antigen from mid gut cells was also determined.



## 1. Introduction

*Hyalomma anatolicum anatolicum* is one of the most important tick species with a wide range of host as well as geographical distribution that transmits *Theileria annulata*, the infectious agent of tropical theileriosis, which is mostly observed in South Europe, East Africa, Middle and South Asia, Middle East [1–3].

The most widely used method for the control of the tick species is the direct application of acaricides on host animals. However, use of acaricides has had limited efficacy in reducing tick infestations and is often accompanied by serious drawbacks, including the selection of acaricide-resistant ticks, environmental contamination and contamination of milk and meat products with drug residues [4]. Furthermore, development of new acaricides is a long and expensive process. All of these issues reinforce the need for alternative approach like vaccine to control tick infestations [4].

In the early 1990s, vaccines were developed that induced immunological protection of vertebrate hosts against tick infestations. These vaccines contained the recombinant *Rhipicephalus microplus* Bm86 gut antigen [5–9]. Two vaccines using recombinant Bm86 were subsequently registered in Latin American countries (Gavac) and Australia (TickGARD) during 1993–1997 [10]. These vaccines in combination with acaricide treatments demonstrated that an integrated approach resulted in control of tick infestations while reducing the use of acaricides [5,10,11].

To date, the recombinant Bm86 has been expressed in *Escherichia coli* [6,12], *Aspergillus nidulans* and *Aspergillus niger* [13] and *Pichia pastoris* [1,14–17]. Of these expression systems, *P. pastoris* has been shown to be the more efficient for protein secretion [16,18,19]. Furthermore, production of Bm86 in *P. pastoris* may increase the antigenicity and immunogenicity of the recombinant antigen [20,21].

However, the process previously reported for the production of recombinant Bm86 and Haa86 in *P. pastoris* is based on protein secretion [1,22], but the expression and vaccine formulation with recombinant Bm86 from local tick strains may be required for vaccine efficacy in some geographic regions. Available data suggest

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that the tick physiological differences in addition to the sequence variability in Bm86 antigen of various species can affect the efficacy of the vaccines [23,24]. In addition, molecular characterization of the recombinant HAO3 protein in the levels of nucleotide and amino acid showed a considerable variation in comparison with the Bm86 antigen that is commercially used as the standard tick vaccine [25].

Considering that no reliable field trials has been conducted yet to see the real potency of Bm86-based vaccines (TickGARD and GAVAC) in Iran, the results of the previous study [25], put the rationality of the application under the question and suggests that *H. a. anatolicum* controlling program in Iran could not rely on commercial TickGARD and GAVAC vaccines.

Hence, vaccine based on rHAO3 derived from local tick strains seems to be required for controlling ticks in Iran and its neighboring regions. For this reason, we in parallel to the relevance study in India [1], sought to construct a *P. pastoris* transformant bearing the chromosomally integrated multi-copy expression cassettes of *HAO3*, the Bm86 homolog from an Iranian strain of *H. a. anatolicum* tick, using the high concentration of antibiotic (zeocin). After that the antigenicity of the rHAO3 was also evaluated by immunofluorescence and quantitative indirect ELISA assays.

## 2. Materials and methods

### 2.1. Preparation of mid gut antigen (MGAg)

Mid gut antigen of *H. a. anatolicum* was prepared according to the method of Ghosh et al. [26]. In brief, mid gut was dissected from partially fed adult female ticks, homogenized in 0.1M PBS (pH 7.2) containing 1 mM mixture of protease inhibitors and 0.02% merthiolate. The homogenate was sonicated under cooling at 8 μm amplitude four times of 30 s each with interspersed breaks of 10 s, centrifuged at 14,000× *g* at 4 °C for 10 min and the supernatant was collected. The pellet was again dissolved in extraction buffer, sonicated, centrifuged and supernatant was collected, pooled, filtered through 0.45 μm filter (Sartorius) and used as mid gut antigen (MGAg) for antibody production in rabbits. Protein of this determined to be 1.2 mg/ml by Lowry-Peterson method [27].

### 2.2. Construction of expression plasmid

Recombinant plasmid was constructed according to our previous study [25]. In brief, HAO3, Bm86 homolog, coding region was excised from pTZHAO3 plasmid by the application of upstream (5′-CATATGGAATTCCAAGTTCTC-TATGCCCAACC-3′) and downstream (5′-CATAGGTCTAGACTTTCCGGGATCTGGATCTG-3′) specific primers that harbored *EcoRI* and *XbaI* restriction sites (underlined sequences), respectively. The C-terminal anchoring and putative signal sequences were removed from the *Hao3* gene fragment and additionally the mentioned restriction sites were created in its ends. It was then cloned into *P. pastoris* expression vector pPICZαA (Invitrogen) digested with *Eco*RI and *Xba* I. In this way, Bm86 homolog was cloned under the control of the alcohol oxidase (*AOX1*) promoter, in frame with the yeast alpha-factor secretion signal with the C-terminal c-myc/His tag. The expression construct was sequenced at both ends and that with correct sequences was named pPICHAO3 and used for transformation of *P. pastoris*.

### 2.3. Transformation of P. pastoris KM71H and selection of transformants

Electrocompetent cells of *P. pastoris* KM71H were prepared according to the instructions of Invitrogen Company (Invitrogen, USA). Briefly, the cells were washed in ice-cold sterile water for several times and finally resuspended in ice-cold 1 M sorbitol. The recombinant expression plasmid (~10 μg) containing *HAO3* gene from *H. a. anatolicum* was linearized with *PmeI* restriction enzyme (Fermentas, Lithuania) and transformed into competent *P. pastoris* cells (80 μl) by electroporation using a Micropulser system (Bio-Rad, USA) and the parameters of 1500 V, 25 μF and 5 ms pulse length in 2 mm cuvettes. Multi-copy transformants were selected on YPDS plates containing increasing concentrations (100, 200, 500, 1000, and 2000 μg/ml) of zeocin (Invitrogen, USA). After 3 days of incubation at 28–30 °C, the right colonies bearing the chromosomally integrated copies of *HAO3*, were selected by PCR amplification of purified genomic DNA using specific primers as mentioned above. Clones with putative multi-copy strains pPICHAO3 were retained for further expression studies.

### 2.4. Expression and selection of high producers

The transformed yeast cells were cultured in 500 ml baffled shake flask containing 100 ml of buffered minimal glycerol (BMGY) medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6, 1.34% yeast nitrogen base and 1% glycerol) until optical density at 600 nm reached 2–3. Cells were harvested by centrifugation, resuspended in 20 ml of buffered minimal methanol (BMMY) medium (similar composition to BMGY with 1% methanol instead of 1% glycerol) and cultured for 3 days. Methanol (100%) was added every 24 h, up to the final concentration of 1% to induce the expression. Then, 1.0 ml of the cell-free culture supernatants was collected daily and concentrated with 10% saturated trichloroacetic acid (TCA). The concentration of the total protein was obtained by Lowry-Peterson method [27]. The relative abundance of rHAO3 (%) as compared to the total protein present in the samples was evaluated by SDS-PAGE analysis with a GeneSnap scanning software, and determination of rHAO3 concentration was calculated by multiplying the two corresponding values obtained above. The highest expressing clone was then selected for further study.

### 2.5. Cell harvesting and purification of rHAO3 in P. pastoris

The BMMY culture was centrifuged at 5000× *g* for 30 min to separate cells. Supernatant was then collected and filtered through a tandem filtration system with a 20 μm cartridge (Sartorius, Germany), 5 μm and 0.45–0.22 μm cartridges (Millipore, USA) and checked for total and recombinant protein content using the Lowry-Peterson method with BSA as standard [27]. Recombinant protein was separated by size exclusion using a Millipore ultrafiltration (UF) system having a membrane with a molecular weight cut-off of 50 kDa (Sartorius, Germany).

For ELISA and western blot analysis of the expressed protein, the high pure and single band of rHAO3 was purified from polyacrylamide (SDS-PAGE) gel according to previous studies [28,29]. Briefly, excised gel pieces were placed in microcentrifuge tube and added 0.5 ml of elution buffer (50 mM Tris–HCl, 150 mM NaCl, and 0.1 mM EDTA; pH 7.5), so that the gel pieces were completely immersed and then incubated in a rotary shaker at 30 °C overnight. After centrifugation of tubes at 10,000× *g* for 10 min, supernatant was stored at −20 °C and used as a gel-purified and very high pure rHAO3.

### 2.6. Rabbit immunization with rHAO3, MGAg and commercial Bm86 antigens

New Zealand white rabbits, weighing approximately 1–1.5 kg were obtained from Razi Vaccine and Serum Research Institute, Tehran. They were maintained in disinfected cages at small animal

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house of the Biotechnology department and were fed *ad libitum*. Rabbits were used for raising specific antibodies.

Prior to adjuvation of the antigens, UF-purified rHAO3 and MGAg solutions were adjusted to concentrations of 500 μg/ml and 2000 μg/ml, respectively. They were filtered through 0.45 and 0.22 μm cartridges (Sartorius, Germany) under sterile conditions in a laminar flow to obtain sterile antigen solutions. Two New Zealand White rabbits per group was each immunized with 2 doses (days 0 and 14) of UF-purified rHAO3, MGAg and rBm86 (Cuban Camcord strain) vaccine (Gavac, revetmex, Mexico). The first injections were given subcutaneously with 120 μg of purified rHAO3 and 700 μg of MGAg in 500 μl sterile PBS emulsified with an equal volume of Freund's complete adjuvant, CFA, (Sigma, USA) and the second one was given subcutaneously with the same concentration of antigens in 500 μl PBS in equal volume of incomplete Freund's adjuvant (IFA). Two rabbits were kept as positive control and were injected subcutaneously with two doses of 0.5 ml containing 50 μg of commercial Bm86, rBm86, (Gavac). Two rabbits were kept as negative control and were injected subcutaneously with equal volume of PBS and CFA/IFA.

Two weeks after the last immunization (on day 28), blood samples were collected from each rabbit into tubes and maintained at 4 °C until arrival at the laboratory. Serum were then separated after centrifugation and stored at −20 °C until use. Antibody titer was measured by ELISA.

### 2.7. Determination of serum antibody levels by ELISA

Gel-purified rHAO3 antigen (1 μg/well) was used to coat ELISA plates overnight at 4 °C. Sera against rHAO3, MGAg and rBm86 were serially two-fold diluted from 1:250 to 1:16000 in PBST. The plates were incubated with the diluted sera for 1 h at 37 °C and then incubated with 1:5000 goat anti-rabbit IgG-HRP conjugates (Sigma–Aldrich), for 1 h at 37 °C. The color reaction was developed with 3,3′,5,5′-tetramethylbenzidine, TMB, (Pishtazteb, Tehran, Iran), at OD 450 nm.

### 2.8. SDS-PAGE and western blot analysis

Protein samples, which were collected daily and concentrated with 10% saturated trichloroacetic acid (TCA), were separated on 12% SDS-PAGE according to the method of Laemmli [30]. The gel was stained with coomassie brilliant blue R-250 and a broad-range marker (Fermentas, Lithuania), was used for the estimation of protein size.

For western blot, gel-purified containing single band of rHAO3 as described above was transferred onto polyvinylidene difluoride (PVDF) membranes and blocked by 3% bovine-serum albumin (BSA) at room temperature, washed three times in TBST (25 mmol/L Tris–HCl, 150 mmol/L NaCl, 0.1% (w/v) Tween 20, pH 7.2). Subsequently, membranes were separately probed with sera from rabbits immunized with rHAO3 (1:1000 dilution), MGAg (1:500 dilution), and rBm86 (1:1000 dilution) as described above. Membranes were then washed three times with TBST and incubated separately with anti-rabbit IgG horseradish peroxidase (HRP) conjugate (Sigma–Aldrich) diluted 1:2000 in TBS. after washing the membranes again, color was developed using diaminobenzidine (DAB) substrate for HRP.

### 2.9. Mid gut cell culture and indirect immunofluorescence (IF) assay

Mononuclear cells of mid gut from *H. a. anatolicum* ticks were washed several times with hanks' balanced salt solution (HBSS), homogenized through a sterile tissue grinder and cells were then released from mid gut tissues by enzymatic digestion with 100 U/ml collagenase (Sigma, USA) for 1 h at 37 °C. After centrifugation, the pellet was washed three times in HBSS and then resuspended in complete TC-100 (Sigma, USA) medium (TC-100 supplemented with 5% FCS, 1% L-glutamine, 1% HEPES, 0.1% 2ME, 100 U/ml Penicillin, 100 μg/ml Streptomycin, 2.5 μg/ml Amphotericin B). Cells were then seeded at a density of $3–5 \times 10^5$ cells per cell culture flask (Nunc, Denmark). The plates were incubated for 3 days at 37 °C in 5% $CO_2$ humidified atmosphere. Indirect immunofluorescence was carried out according to our previous studies [31,32]. Briefly, after fixation of dissociated mid gut cells on glass cover-slips by 4% paraformaldehyde, HAO3, which is an extracellular membrane-bound glycoprotein of mid gut cells, was detected by home-made pooled polyclonal rabbit antisera for rHAO3, MGAg, and rBm86 (Gavac), respectively. Following antibody removal and washing steps for three times with PBS, the cells were treated with FITC-labeled goat anti-rabbit IgG (Sigma–Aldrich).

## 3. Results

### 3.1. Construction of recombinant plasmids carrying HAO3 (pPICHAO3)

The recombinant expression vector containing *HAO3* gene from *H. a. anatolicum* was constructed in order to secrete the 622-amino acid Hao3 protein to be used for the future expression of recombinant HAO3 in *P. pastoris* yeast (Fig. 1). The accuracy of the recombinant vector, pPICHAO3, was confirmed by molecular analysis and sequencing reactions which revealed a complete homology with related isolate deposited in Gen-Bank (Accession No. FJ160586.1), as described in our previous study [25].

### 3.2. Expression of recombinant HAO3 (rHAO3)

After electroporation of electrocompetent cells of *P. pastoris* KM71H, chromosomal integration of HAO3/Cmyc/6xHis gene in *P. pastoris* cells was evidenced by PCR and gene-specific primers (Fig. 2).

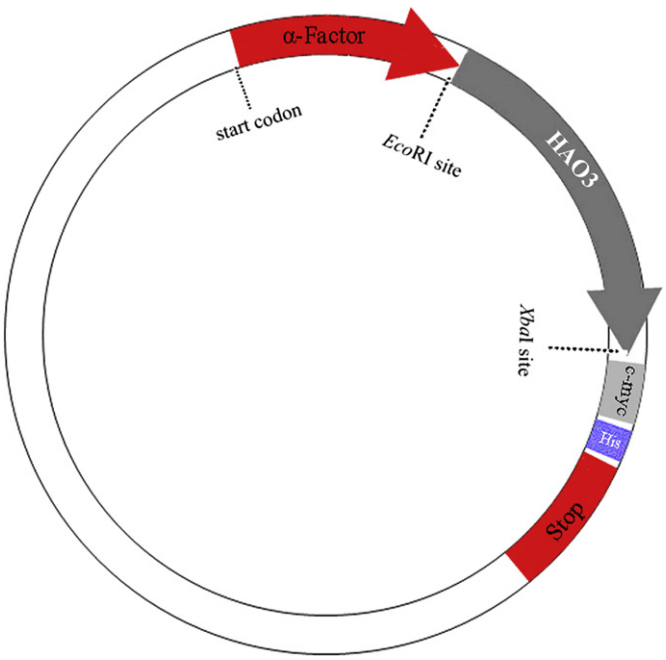


**Fig. 1.** Schematic representation of the pPICHAO3 yeast expression vector. The *HAO3* gene was cloned in the *Eco*RI/*Xba*I sites, in frame with the vector-derived secretory signal of α-factor and c-myc/His C-terminal epitopes.

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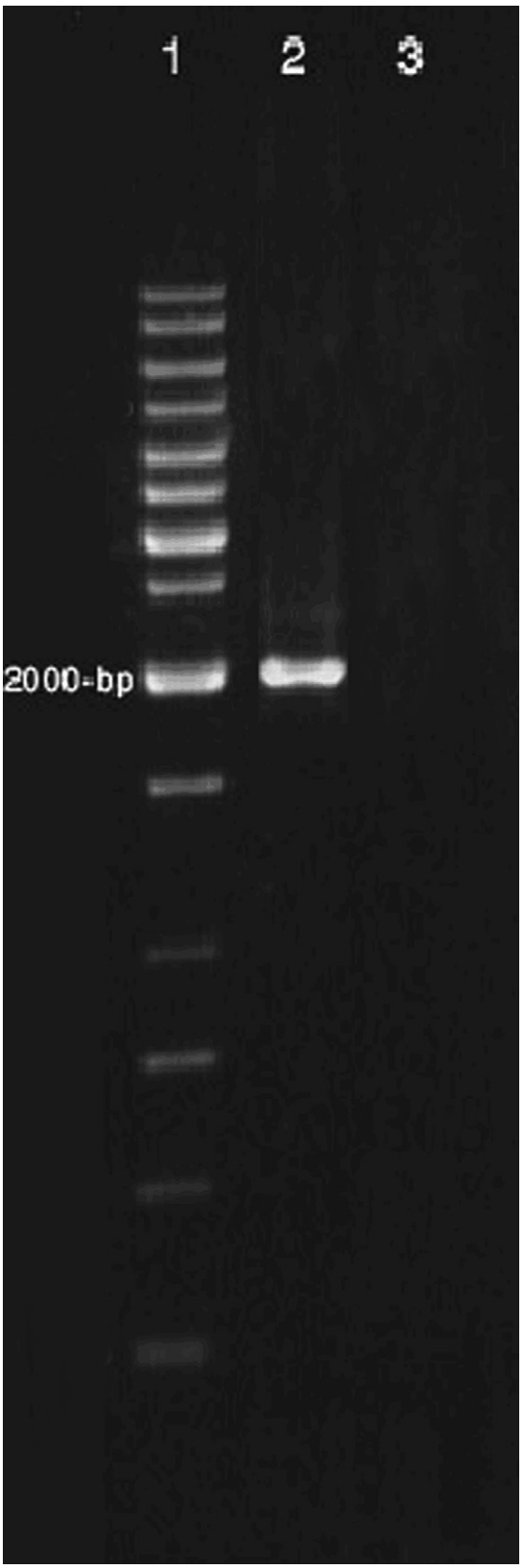


**Fig. 2.** Chromosomal integration of HAO3 gene in *P. pastoris* cells was evidenced by PCR using gene-specific primers on 1.2% (w/v) agarose gel. 1: size marker 1 kb (Fermentas, Lithuania lane); lane 2: PCR amplification of insert on pPICHAO3 template using specific primers; lane 3: negative control.

The clone with the best activity on YPDS plates containing 2000 μg/ml zeocin after 3 days of incubation at 30 °C was chosen for up-scaled protein production. After cultivation, *HAO3* gene was expressed in transformants and the culture supernatant was daily sampled for the final analysis by SDS-PAGE.

As shown in Fig. 3A, the recombinant HAO3 protein was successfully expressed in *P. pastoris* strains as a secreted protein. The secretion of recombinant protein was detectable on day 1 and reached a maximal level after 3 days. The expressed recombinant HAO3 was about 95–100 kDa (Fig. 3A), which is larger than the theoretical size of the unglycosylated (72 kDa) protein. This indicated that the recombinant HAO3 protein was glycosylated after the post-translational modification. Under the optimized culture parameters of pH value, methanol daily addition concentration, and induction time length, the production of rHAO3 reached up to 109 mg/L with 72% purity for KM71 transformants.

### 3.3. Purification of rHAO3

Attempt to purify the protein by Ni-affinity chromatography was unsuccessful. Different denaturation conditions (8 M urea and 6 M guanidine HCl) and mild reducing conditions (1–2 mM 2-mercaptoethanol) were tried in the purification process according to previous study [31,32], but all were ineffective. Purification of rHaa86 protein by an alternative strategy such as size exclusion and diafiltration through a 50 kDa cut-off membrane followed by SDS-PAGE resolved the protein at 95–100 kDa (Fig. 3A).

The GeneSnap scanning analysis demonstrated that the purity of rHAO3 on days 3, gel-purified rHAO3 and UF-purified rHAO3 reached up to 42%, 98% and 72%, respectively.

### 3.4. Western blot analysis

For detection of the recombinant HAO3, western blot analysis was used under reducing conditions. As shown in Fig. 3B, the western blot by home-made pooled polyclonal rabbit antisera against UF-purified rHAO3, MGAg and rBm86, resulted in only one band on the membrane, revealing that rabbit antibodies bound specifically to the gel-purified rHAO3. The rHAO3 protein was not detected by the antihistidine antibody and it could be attributed to the complex conformational feature of this protein. Accordingly, anti-rHAO3 antibody reacted stronger than anti-MGAg and anti-rBm86 antibodies with gel-purified rHAO3 coated on FVDF membrane. These results demonstrate the cross-reactive nature of *P. pastoris*-expressed rHAO3 with its native glycoprotein extracted from mid gut of *H. a. anatolicum* tick and the rBm86 of *Bovian microplus*.

### 3.5. Monitoring of antibody response in rabbits by ELISA and IF

To find the antigenic potency of the purified protein, the obtained antisera were analyzed using the qualitative immunofluorescence and quantitative indirect ELISA assays.

By means of ELISA assay the raised antisera were separately quantified. The absorbance value of rHAO3, MGAg, and rBm86 immunized rabbits was determined after second immunization. Significantly high antibody response in comparison to control was recorded in all the immunized rabbits; although, the highest absorbance was related to rHAO3-immunized rabbits (Fig. 4).

As immunofluorescence assay evidenced, strong comparable fluorescence emissions were detected, when home-made rabbit antisera against rHAO3, MGAg, and rBm86 were separately applied (Fig. 5A, B and C, respectively). However, the absence of fluorescence emission in negative control showed the specificity of the experiment (Fig. 5D).

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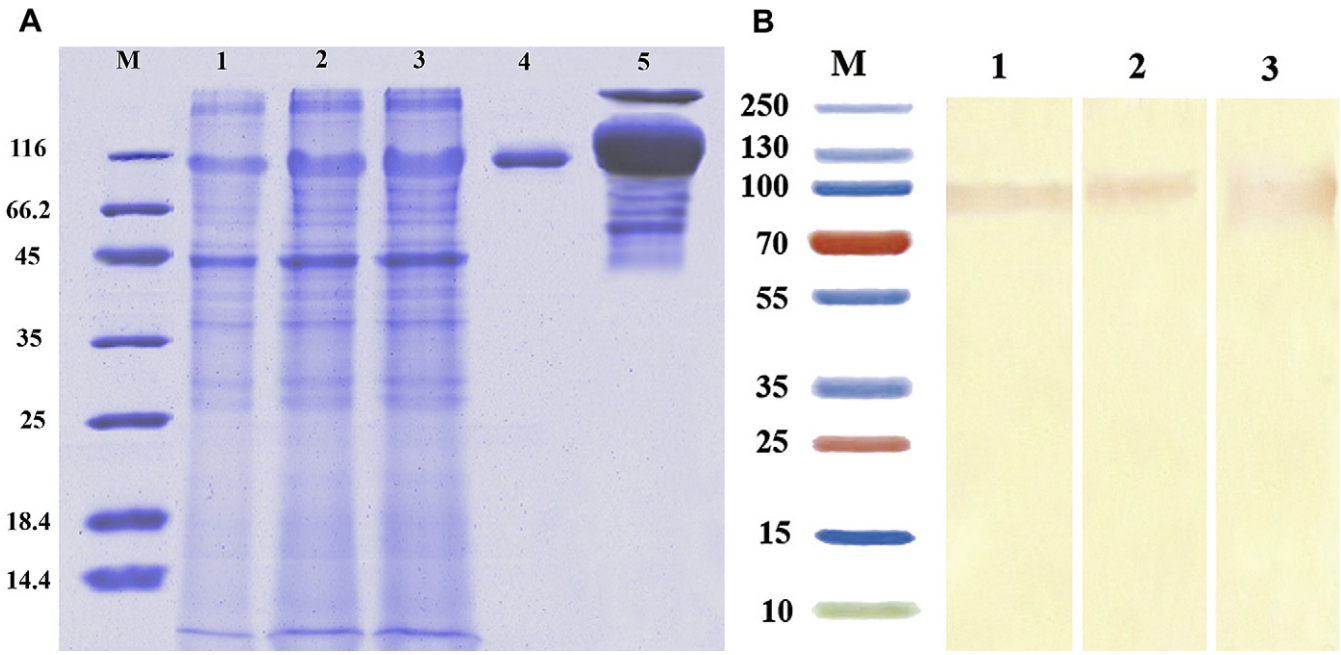


**Fig. 3.** Analysis of recombinant HAO3 protein in the culture media by SDS-PAGE and Western blot. (A) Samples of culture media were removed at 24 h intervals after methanol induction and separated by 12% SDS-PAGE; lane M: Unstained protein molecular weight marker (Fermentas, Lithuania), lanes 1–3: Samples from HAO3/Cmyc/6xHis-expressing *P. pastoris* after 1–3 days of methanol induction; lane 4: The high pure and single band of gel-purified rHAO3; lane 5: UF-purified rHAO3 using a membrane with a molecular weight cut-off of 50 kDa. (B) Gel-purified rHAO3 protein was transferred onto a PVDF membrane and identified by immunoblotting using HRP-conjugated goat anti-rabbit IgG antibody. Lane M: Schematic representation of the broad-range prestained protein marker (Fermentas, Lithuania); lane 1: Membranes was probed with sera from rabbits immunized with rHAO3 (1:1000 dilution); lane 2: Membranes was probed with sera from rabbits immunized with rMGAg (1:500 dilution); lane 3: Membranes was probed with sera from rabbits immunized with rBm86 (1:1000 dilution).

## 4. Discussion

Considering the structural features of the HAO3 protein and its requirement to post-translational modifications, in this study we used *P. pastoris* yeast as an alternative recombinant expression host for the expression of rHAO3 protein. *P. pastoris* has several advantages including high production yields, genetically stable expression strains, potential of secretion of recombinant proteins into the culture medium and simple inexpensive culture conditions, while recombinant proteins expressed in this organism are not encountered with the endotoxin contaminations [17].

Moreover, *P. pastoris* is a eukaryote, and thereby provides the potential for producing soluble, correctly folded recombinant proteins that have undergone all the post-translational modifications

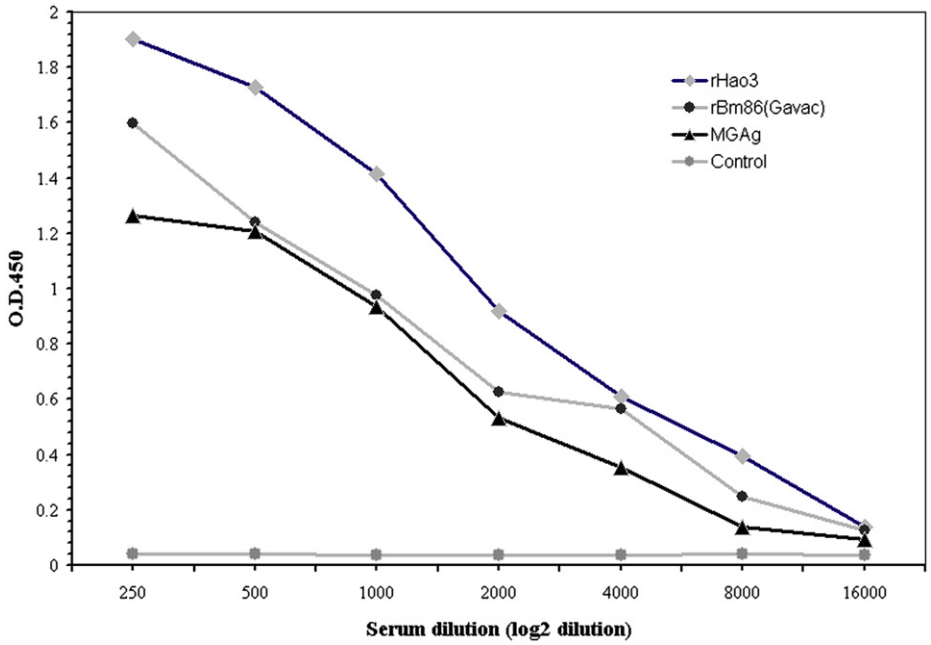


**Fig. 4.** Indirect ELISA for *P. pastoris*-expressed HAO3, in which gel-purified rHAO3 antigen (1 μg/well) was used to coat ELISA plates overnight at 4 °C. After further treatment with HRP-conjugated goat anti-rabbit IgG and TMB substrate, the obtained signals in accordance to the serially diluted antisera (prepared on days 28 after first immunization) were plotted at OD 450 nm.

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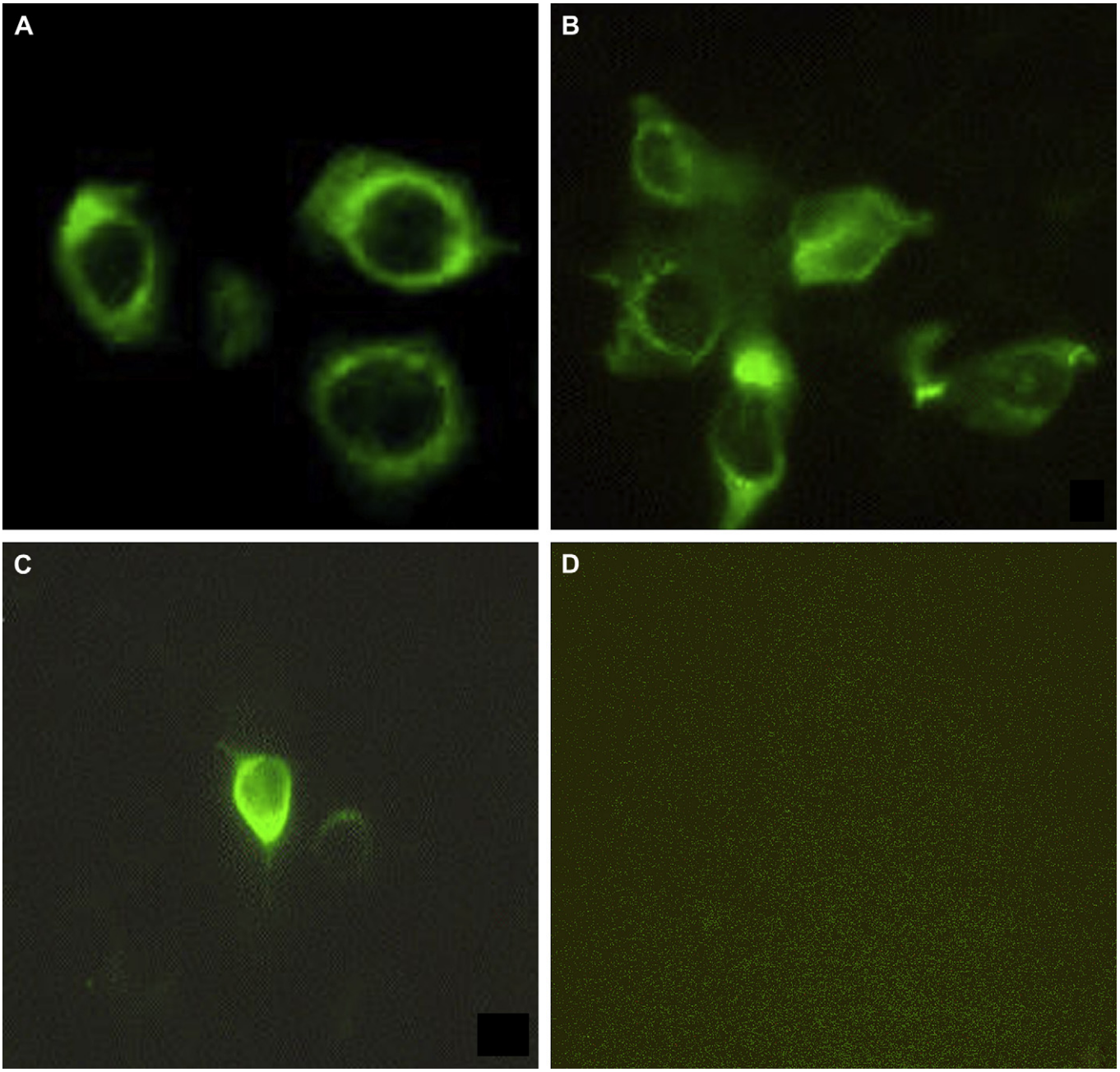


**Fig. 5.** Immunofluorescence analysis of dissociated *H. a. anatolicum* mid gut monolayer tissue to cells by enzymatic digestion with 100 U/ml collagenase. After fixation of dissociated mid gut cells on glass cover-slips by 4% paraformaldehyde, Hao3 was detected by home-made pooled polyclonal rabbit antisera for rHAO3 (A), MGAg (B), rBm86 (C), and negative control (D), respectively. The cells were then treated with FITC-labeled goat anti-rabbit IgG.

(e.g., protein processing, folding, and post-translational modifications) required for functionality [33–35].

Recombinant HAO3 secreted in *P. pastoris* appeared in SDS-PAGE and western blot with a size range of 95–100 kDa. The homologous of HAO3 protein, rBm86, previously expressed in *P. pastoris* also had a size ranging from 90 to 100 kDa [16], 100–110 kDa [20], and 120–140 kDa [1].

These differences in estimated molecular weight of the proteins may be due to strain differences in glycosylation, which is responsible for the wide appearance of the protein band in the SDS-PAGE and western blot [7].

The concentration of recombinant HAO3 reported herein after protein secretion and a simple centrifugation-filtration purification process was higher than that obtained for membrane-bound Bm86. Additionally, the purity of this secreted protein was considerable; however, there was some contaminants in the supernatant after cell separation. These contaminants suggest the probability of cell lysis during the stationary phase of the culture process due to suboptimal growth conditions [16], and secretion of autologous proteins by *P. pastoris* [36].

Purification under non-denaturing conditions such as UF system avoids arduous work of re-folding of denatured protein. This is advantageous to retain conformational epitopes on the protein and thereby facilitates better reactivity with antibodies. It also helps to retain the immunogenicity of recombinant protein, as was reported earlier in Ref. [37].

The result of antigenicity assay for the rHAO3 protein pointed well toward its capability for the elicitation of antibody response in

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immunized rabbits. Interestingly, the results indicated that the expressed HAO3 protein reacted well with MGAg and rBm86 antisera in ELISA and western blot assays making it evident that the epitopes present in expressed protein are well recognized by the antibody.

Finally, the preliminary expression of the rHAO3 and evaluation of its immunogenicity evidenced the presence of cross-reactive epitopes between this protein with and MGAg. This result suggests that this recombinant antigen can be used for the development of vaccine for the control of tick infestations in Iran and neighboring regions. However, further research will be needed to evaluate the immunity of the expressed HAO3 protein in its host, cross-bred Calve, by challenging rHAO3-immunized Calve with unfed adults of *H. a. anatolicum*.

In this study, we purified the expressed HAO3 protein according to the method of previous study [16]. With this in mind, the number of integrated copies of the expression cassette can affect the amount of protein expressed; therefore, we used a transformant resisting to 2000 μg/ml instead of 100 μg/ml of zeocin.

In a study reported by Vassileva et al., the selection effects for hyper-resistance to 100, 500, 1000 and 2000 μg/ml of zeocin were examined with the transformants analyzed for copy number. It was found that transformants resistant to 100 μg/ml of zeocin generally contained one copy, those resistant to 500 μg/ml had two copies, those resistances to 1000 mg/ml had three copies and transformants resistant to 2000 μg/ml had four copies [2]. In contrast, other studies by Sarramegna et al. have found that transformants resistant to 1000 μg/ml zeocin had copy numbers of 15–25 [38].

In conclusion, the present study provided evidence for the construction of transformant containing the chromosomally integrated multi-copy expression cassettes of *HAO3*, its successful and efficient expression in *P. pastoris* yeast and purification of the secreted protein by ultrafiltration (UF) system in a high level yield and purity. The purified protein completely retained its structural conformation so that the induced antibodies against MGAg and rBm86 proteins were able to recognize and bind to the rHAO3.

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