Construction and expression of aspartic protease from *Onchocerca volvulus** as ompA fusion protein in a mutant strain of *Salmonella typhimurium*

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Abstract

Two constructions in pHS164 vector were designed to permit expression of OV7A and OV4A inserts encoding the N-terminal and C-terminal portion of an aspartic protease from *Onchocerca volvulus,* respectively. A novel 39 kD protein ompA-OV7A fusion protein was stably expressed as ompA fusion in a modified strain of *Salmonella typhimurium* strain SL5000 and *E.coli* strain JM109. Expression of the fusion protein in bacterial strains harboring the constructs were evaluated by western blotting. *E.coli* and *Salmonella* lysates were fractionated by 10% SDS-PAGE gel and then immobilized to nitrocellulose membrane by electroblotting. Primary polyclonal antibody generated in rats against the GST-OV7A fusion protein was used in the Western blots. It remains to be seen whether the fusion protein expressed *in vivo* will promote effective immune response.

Key words: filarial parasite, nematode, *Salmonella typhimurium*.

Introduction

The filarial parasite *O. volvulus* causes human onchocerciasis frequently leading to blindness and severe dermal as well as lymphatic pathology. Onchocerciasis affects approximately 40-50 million people in equatorial Africa, Yemen and Latin America (World Health Organization 1987).

Aspartic acid proteases are a relatively small group of enzymes which includes the mammalian pepsins, chymosins, cathepsins and renins. Structural data indicates that the enzymes have bilobal structures comprised mainly of β-sheets with a substrate-binding cleft containing the active site. Aspartic proteases represent attractive targets for immunoprophylaxis or the generation of protective immunity (McKerrow *et al.,* 1993).

To elicit a protective immune response to *O.volvulus,* antigens must be delivered and presented to the host in the correct fashion. The new generation of live *Salmonella* vaccines have been highly successful as carriers for the intracellular delivery of target antigens, usually on recombinant plasmids, into the *Salmonella* vehicle. To date, there are no publications describing the use of live vaccines for onchocerciasis. Recently, a novel construction of the avirulent *Salmonella* strain that expressed OvGST as a fusion to the N-terminal domain of the bacterial outer membrane protein-A with promising results was described (Catmull *et al.,*1999). Protein fusions of this kind are believed to enhance the immune response due to presentation of the foreign antigen to the outer surface of the bacteria. This initial study demonstrated that the application of this delivery system to *O. volvulus* antigens can induce antigen specific Th-1 like cellular immune responses (Yang *et al.,* 1990). This approach may also be useful in the study of other diseases for which intracellular antigen delivery and Th-1 cellular immune responses appear to be important for protection.

In this study we have made novel modifications to the mutant *Salmonella* strain that has expressed *Onchocerca* protease as fusion to only a small region of the N-terminal domain of the bacterial outer membrane protein-A (ompA) for enhancement of cell-mediated immune responses in animal models.

Materials and Methods

Bacterial strains and plasmid pHS164 structure

A modified strain of *S.typhimurium* derived from LT2 was identified with a r⁻ m⁺ phenotype, designated

strain SL5000. This strain of *Salmonella* not only allows the introduction of foreign DNA because it is restriction negative but also modifies the DNA because it remains modification positive. Thus SL5000 was used as an intermediate host to generate *Salmonella* compatible plasmid DNA from plasmids initially derived from *E.coli.* The bacterial strains were cultured in Luria broth (LB) and on LB agar. The media was supplemented with 50 µg/mL of ampicillin. The vector pHS164 (5448 bp) was used for the expression of foreign antigens as fusions with the N-terminal region of the *Shigella* outer membrane protein A (ompA; Pistor and Hobom, 1990). The outer membrane proteins (omp) of *Shigella dysenteriae,* which can be used as carrier proteins to present foreign peptide epitopes on the bacterial cell surface, has been used to construct a group of enterobacterial surface expression vectors for foreign genes protein (Lang, 2000). Secretory signals are located at the N-terminal region of ompA and therefore fusion proteins that include this signal peptide should be directed to the outer membrane of the *Salmonella* bacteria (Beher *et al.,* 1980). The plasmid can be chemically induced by IPTG. The presence of ampicillin resistant factor (*amp^r*) in this construct permitted selection of transformants on LB agar containing ampicillin.

DNA sequence analysis

Sequences were obtained by the dideoxy-method (Sanger *et al.,* 1977) using T7 Sequenase kits (Pharmacia). The sequencing procedures was performed on double stranded, denatured plasmid DNA using the pHS164 sequencing primer.

Screening of recombinant clones using PCR

Colony picks were taken and resuspended in 12 µL water which was boiled for 5 min and then the cell debris was pelleted by a 5 min centrifugation at approximately 11,000 g. 10 µL of the lysis solution was added to 5 µL of PCR master mix resulting in a solution containing 1 x synthesis buffer, 2.5 mM $MgCl_2$, 200 mM of each dNTP, 0.5 U *Taq* DNA polymerase and 5 pmoles of each primer. Primers specific for inserts in conjunction with the vector forward sequencing primer were used.

Preparation of protein samples

Following induction, bacterial cells were recovered from 1 mL of culture by centrifugation at 12,000 *xg* for 30 s. The supernatant was discarded and the cell pellet resuspended in 0.1 mL of distilled water after washing once in 0.5 mL of ice cold 50 mM Tris-HCl pH 7.4. The protein samples were mixed with 25 µL of 5 x SDS reducing loading buffer (60 mM Tris-HCl pH 6.8/25% glycerol/2% SDS/ 14.4 mM 2-β mercaptoethanol/0.1% bromophenol blue) and chromosomal DNA sheared by sonication. After centrifugation at 12,000 *xg* for 5 min, the supernatants were incubated for 2 min in boiling water prior to loading on the gel. Prestained molecular weight markers were used to establish the apparent size of proteins

Western blot analysis

After SDS-PAGE, the proteins were transferred electrophoretically to nitrocellulose membrane using a BioRad minitrans-Blot apparatus. In a gel holder a piece of Whatman paper saturated in transfer buffer was placed on a pre-soaked Scotch-Brite pad and then the cathode side of the pre-equilibrated gel was located on the paper. A piece of Hybond-C membrane (Amersham), was cut to the same size as the gel and placed directly onto the anode side of the gel, removing air bubbles by rolling a glass pipette over the membrane. The gel-membrane sandwich was completed by overlaying saturated filter paper and scotch brite on top of the membrane before closing the gel holder. Electroblotting was carried out by applying a constant voltage of 80 V for 1 h at 4 °C.

The blocked membrane was probed with primary antibody in blocking buffer followed by rinsing the membrane three times in PBS/0.1% Tween-20 (v/v) for 5 min with gentle agitation. The membrane was transferred to an appropriate dilution of secondary antibody conjugate in blocking buffer and incubated for 1 h at room temperature. The enhanced chemiluminescence (ECL) method (Mathews *et al,* 1985) was used to detect secondary antibody-HRP conjugates, following the manufacture's (Amersham) instructions.

Results

Two strategies were devised to clone the OVAP N-terminus and C-terminus (Jolodar *et al.,* 1998) in frame with the ompA N-terminus in the pHS164 expression vector. The summary of constructions are listed in Table I.

Construction of pHS-OV4A and pHS-OV7A

The cDNA OV4A clone encodes the OVAP C-terminus and includes the 364 bp 3' untranslated region. An aliquot of the OV4A clone in pGEM-T vector was digested

Table I - Summary for subcloning in pHS164.

Construction	Termini	Base pair	Amino acid	Domain	Vector
pHS-OV7A	Eco RI	706	235	N-terminal	pHS164
pHS-OV4A	NsiI, ApaI	958	198	C-terminal	pHS164

with <u>Apa</u>I, cutting the plasmid construct once only, within the vector polylinker, resulting in a 5GGCC overhang. The 3' recessed ends of <u>Apa</u>I were filled using Klenow fragment. The linearised pGEMT-OV4A plasmid was then digested with <u>Nsi</u>I to generate a cohesive 5' end. The OV4A fragment was gel-purified and directionally subcloned into pHS164 that had 5' <u>Nsi</u>I and 3' <u>Sma</u>I ends. Although the pHS-OV4A construct contained 62 bp of pGEM-T vector polylinker at the 5-end of OV4A its sequence did not encode any stop codons to disrupt the expression process. The pHS-OV7A construct was designed to express the OV7A (Jolodar *et al.,* 1997) insert encoding the N-terminal portion of the aspartic protease from *O.volvulus*. The subcloning procedure of the OV7A fragment from pUC18 to pHS164 was facilitated by digestion with *E*coRI. The 3' recessed ends of the OV7A fragment were end filled using Klenow fragment to allow ligation with the blunt ends of StuI-digested pHS164 vector (Figure1). Recombinant plasmids were transformed into *E.coli* JM109 and bacteria containing the plasmids were isolated and plasmid prepared from these for DNA sequencing and for transformation into *Salmonella.* The predicted open reading frame was determined by analyzing the sequences, particularly around the cloning site. Putative recombinants were screened by PCR using primers specific for OV7A in conjunction with the vector forward sequencing primer followed by the DNA sequencing. This method allowed the presence of one or more copies of insert and their orientation to be determined. The plasmids were introduced into *S.typhimurium* SL5000 by the Lederberg and Cohen method (Lederberg *et al.,* 1974); however, due to the low efficiency of transformation into *Salmonella*, 5 to 50 μg of plasmid was used per transformation resulting in no more than 100 transformants.
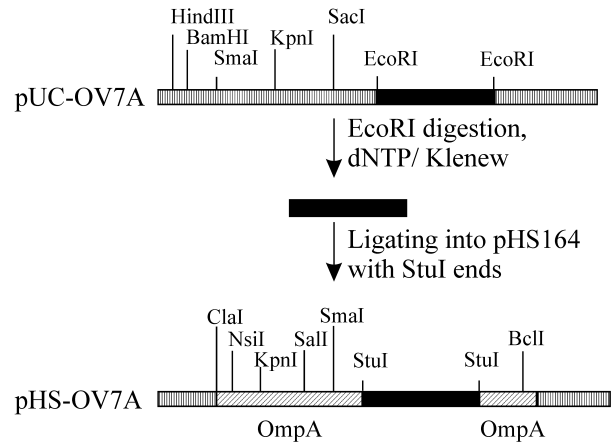

Figure 1 - The subcloning strategy used to generate the pHS-OV7A construct with an open reading frame of OV7A continuous with the 5' end of the OmpA gene in the pHS164 vector.

Detection of ompA fusion proteins by western blotting

Liquid cultures of pHS-OV7A transformants were grown to an OD600 nm of 0.5 before addition of IPTG to a final concentration of 0.2 mM to induce ompA transcription. After 2 h of induction the *E.coli* and *Salmonella* bacteria were lysed and insoluble debris was separated from the soluble protein. The supernatant recovered from the bacterial lysates was further fractionated by SDS-PAGE and the results are illustrated in Figure 2. Comparisons of IPTG induced verses uninduced pHS-OV7A and the pHS164 non-recombinant control lysates revealed a novel 39 kD protein in the induced extract of pHS-OV7A. This novel
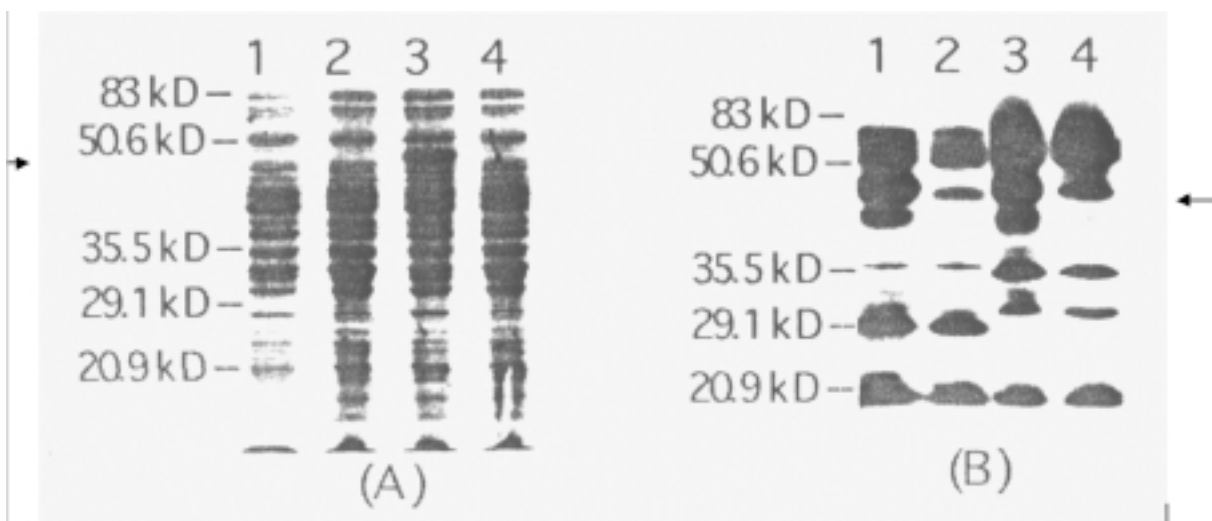

Figure 2 - Expression of ompA-OV7A fusion protein as determined by SDS-PAGE and Western blotting. (A) Protein from the pHS-OV7A *Salmonella* transformants post- and pre-induction are shown in lanes 3 and 4, respectively. Non-recombinant pHS post- and pre-induction are shown in lanes 2 and 1, respectively. (B) The expression of pHS-OV7A construct in E.coli JM109 and *S.typhimurium* SL5000 was examined by western blotting. The gel was blotted onto nitrocellulose, and probed with a rat antibody to GST-OV7A fusion protein. Lanes 1 and 2 show the induced and non-induced patterns of the pHS-OV7A *E.coli* transformants. Lanes 3 and 4 show the induced and non-induced patterns of pHS-OV7A *S.typhimurim* transformants.. Enhanced chemiluminescence (ECL) was used to detect the secondary antibody.

protein was similar to the size predicted for the ompA-OV7A protein, a fusion approximately 13 kD and 26 kD. There were no discernible differences between the pHS-OV4A and non-recombinant vector lysates. Due to the hydrophobic nature of this portion of the OVAP protein it is possible that the fusion product was insoluble, but it is also possible that the level of expression of the ompA-OV4A fusion protein was too low to allow visualization by Coomassie staining. Expression of the fusion protein in bacterial strains harboring the constructs was evaluated by western blotting. *E.coli* and *Salmonella* lysates fractionated by 10% SDS-PAGE gel were immobilized to nitrocellulose membrane by electroblotting. Primary polyclonal antibody used in the Western blots was generated in rats against the GST-OV7A fusion protein. Detection of the primary antibody was with a HRP-conjugated goat anti-rat immunoglobulin (DAKO, High Wycombe, Bucks, UK), combined with a peroxidase induced chemiluminescence (ECL) detection system. It should be noted that OV4A includes approximately 80 amino acids of the OV7A. Thus, this antiserum is also likely to recognize OV4A.

Discussion

It has been suggested that the aspartic proteases which are predicted to be secreted by parasite may be valuable for immunoprophylaxis against onchocerciasis (Lizotte-Waniewski *et al.,* 2000). Vaccine efficacy of recombinant cathepsin D aspartic protease from *Schistosoma japonicum* (Verity *et al.,* 2001) and *Haemonchus contortus* (Longbottom *et al.,* 1997) has been reported. Immunolocalisation experiments performed by the authors (Jolodar *et al.,* unpublished data) indicated that the recombinant OV7A protein encoding an aspartic protease was predominantly associated with the hypodermis and inner membrane zone of the uterine wall of adult females. The patterns of immunolabelling were highly specific when sections were probed with antibodies to the recombinant protein.

The expression of OV7A as a fusion with ompA was similar in both strains of bacteria and a single 39 kDa protein of equal intensity was detected. This indicated that the ompA-OV7A fusion protein was stably expressed in two hosts with different genetic backgrounds. However, the ompA-OV4A fusion protein in the lysates generated from IPTG induced bacteria transformed with the pHS-OV4A construct was not detected by the methods used here. It is possible that the polyclonal antibody generated to GST-OV7A did not contain any idiotypes that recognized OV4A epitopes or the ompA-OV4A protein was absent. The literature implies that the omp is an important outer membrane protein partially responsible for membrane integrity. They all have a common structural motif of a β-barrel that is composed of a variable number of transmembrane β-strands connected at the periplasmic side with short turns

and at the outside with long surface-accessible loops (Hobom *et al.,* 1995; Lang H. 2000).

This study demonstrated that the recombinant *Salmonella* construct pHS-OV7A expressed well *in vitro*. It remains to be seen whether the recombinant *Salmonella* construct pHS-OV7A expressed *in vivo* will promote an effective immune response. To determine this there will have to be a number of vaccine trials in animal models. Several of these animal models for onchocerciasis are recognized by the WHO and include mice, jirds, birds, cattle and chimpanzees. Whether the immune response to OV7A, and perhaps OV4A, delivered by *Salmonella* elicits the desirable Th-1 like immune response in these models and or offers protection is a question I hope will be answered positively in the near future.

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