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Identification of a 24 kDa excretory secretory protein in *Anisakis simplex*

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ABSTRACT

A gene coding for a 24 kDa protein (22U homologous; As22U) was isolated from the *Anisakis simplex* third-stage larvae cDNA library during expressed sequence tag analysis. As22U was 636 bp long, and was found to code for 212 amino acid residues with a calculated mass of 23.5 kDa and a PI of 9.06. The As22U deduced amino acid sequence harbored a signal peptide region and 16 highly conserved cysteine residues, and it was identified in both the total extracts and excretory secretory (ES) protein of *A. simplex*. Its molecular weight was measured at 24 kDa via western blot analysis. The expression levels of thymic stromal lymphopoietin, IL-25, and CXCL1 (Gro-α) genes were increased at 6 h after recombinant As22U treatment in mouse intestinal epithelial cells. Additionally, thymus and activation-regulated chemokine gene levels were increased at 14 h after treatment. Although we do not currently have sufficient evidence to determine whether As22U plays a role as an allergen, this remains possible. Further *in vivo* studies may provide some insight as to the allergenic properties of As22U.

1. Introduction

Anisakidosis refers to a human infection caused by the larval stages of nematode members of the families Anisakidae or Raphidascarididae. *Anisakis* and *Pseudoterranova* are the two genera associated most frequently with anisakidosis. Any fish or cephalopod species can be parasitized by the 3rd stages of those larvae (L3). The ingestion of fish infected with L3 can induce anisakidosis in humans (Moreno-Ancillo et al., 1997). Symptoms of anisakidosis arise when the nematode penetrates the gastric mucosa, which results in acute epigastric pain, occasionally accompanied by nausea and vomiting. The other common manifestation of human anisakidosis is an IgE-mediated reaction that sometimes arises in sensitized individuals. These allergic symptoms may include urticaria, angioedema, and anaphylaxis, and arise a few hours after the ingestion of infected fish (Alonso et al., 1997; Esteve et al., 2000; Fraj Lazaro et al., 1998).

Diagnosis of an *Anisakis simplex* (whale worm) allergy is currently carried out by tests based on IgE reactivity to a complete extract of L3 *A. simplex* larvae. However, some healthy people have scored positive on these tests, which indicates that these tests are not sufficiently specific (Caballero et al., 2011; Del Rey Moreno et al., 2006). The best alternative to improve diagnostic specificity has involved the use of purified recombinant *A. simplex* allergens with the same immunological properties as their natural counterparts.

In order to resolve these problems, many scientists have struggled to identify new specific allergens from *A. simplex* L3 via a variety of molecular and immunological tools. Thus far, the following 12 protein types have been identified as *A. simplex* allergens: secretory gland protein (Ani s 1) (Moneo et al., 2000), paramyosin (Ani s 2), tropomyosin (Ani s 3) (Asturias et al., 2000; Perez-Perez et al., 2000), protease inhibitor (Ani s 4, 6) (Kobayashi et al., 2007a,b; Rodriguez-Mahillo et al., 2007), SXP/RAL-2 family protein (Ani s 5, 8, 9) (Kobayashi et al., 2007a,b; Rodriguez-Perez et al., 2008), protein with repetitive sequences (Ani s 7, 10, 11, 12) (Caballero et al., 2011; Kobayashi et al., 2011; Rodriguez-Perez et al., 2008). In addition to these identified allergens, many unknown allergens are also thought to exist.

The Di22U protein and its homologs have been previously detected in filarial nematodes (Frank et al., 1999; Sutanto et al., 1985; Vieira et al., 1997). Although the functions of this protein remain poorly understood, they might elicit potent immune responses during the infection period. They are located on the cuticle and hypodermis, and may also be expressed externally via excretory secretory (ES) proteins. Therefore, they may play critical roles in host–parasite interactions during parasitization. Perera et al. evaluated the utility of anti-Di22 antibodies in the diagnosis of human pulmonary dirofilariasis using sera obtained from patients (Perera et al., 1998).

In this study, we have isolated and evaluated the genetic characteristics of 22U homologous (As22U) from *A. simplex* L3, which

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induces anisakidosis in humans (Yu et al., 2007). In an effort to evaluate its potential as an allergen, we constructed recombinant As22U (rAs22U) and evaluated the Th2 chemokine gene expression level of intestinal epithelial cells following rAs22U treatment.

2. Materials and methods

2.1. Parasites

A. simplex L3 were obtained from the viscera, flesh, and body cavities of naturally infected blue whiting (*Micromesistitus poutassou*) and exhaustively washed in PBS (Yu et al., 2007).

2.2. Collection of ES proteins and total extract from A. simplex

The *A. simplex* L3 were incubated in serum-free RPMI 1640 medium containing kanamycin, ampicillin, neomycin, penicillin, and streptomycin. Following incubation, the supernatants were centrifuged for 10 min at 3500 rpm and collected in fresh tubes for ES products. The total extracts from *A. simplex* were acquired using PRO-PREP protein extraction solution (Intron). The ES products and total proteins were desalted with a HiTrapTM desalting column (GE Healthcare, Sweden). All procedures were conducted in accordance with the manufacturer's recommended protocols.

2.3. Identification of a full length sequence of As22U

In a previous study, we isolated a partial clone similar to nematode 22U via *A. simplex* L3 EST analysis (Yu et al., 2007). To determine the full-length sequences of the clone, RACE PCR was conducted as described below. RACE-ready first-strand cDNA was synthesized with 50 ng of *A. simplex* L3 total RNA using a BD SMARTM RACE cDNA amplification kit (BD Biosciences, USA) in accordance with the manufacturer's instructions. The specific 5′ RACE PCRs were carried out using a BD AdvantageTM 2 PCR enzyme system (BD Biosciences, USA). The primer sequence was 5′-CGC AAC ATA CTG AGC TAC TTG ACC GGA GAT T-3′. The amplified PCR products were ligated with pGEM-T easy vector and the sequences were analyzed via Macrogen (Korea). Following sequence analysis, we constructed full-length As22U cDNA clones via PCR using full-length primers (forward: 5′-GGA TCC ATG ATT CGA ATC CTC TTC AC-3′; reverse: 5′-CTC GAG TTA CTG GAT AAT AAT GGT CG-3′). In order to characterize the deduced protein sequences, we analyzed the predicted domain signatures at http://expasy.org/prosite.com.

2.4. Construction of recombinant As22U proteins

Following confirmation of the PCR product sequences, As22U was cloned as a his-fusion protein. Thereafter, ligates were transformed into *Escherichia coli* strain BL21. Induction of fusion protein expression was followed by the addition of IPTG at a final concentration of 0.1 mM. The recombinant As22U (rAs22U) protein was purified with 300 mM imidazole using a HisTrap™ HP column (Amersham, Biosciences, UK) and dissolved in PBS using a HisTrap™ desalting column (Amersham, Biosciences, UK). LPS was depleted (endotoxin levels <0.01 μg/ml) from the rAs22U using Detoxi-Gel Affinity Pak prepacked columns (Pierce), in accordance with the manufacturer's instructions. Polyclonal anti-rAs22U Ab was produced in rat which was boosted twice every two weeks with 500 μg of rAs22U, CFA, and IFA (Sigma–Aldrich).

2.5. Western blot analysis

Western blot analysis has been performed according to prescribed method. Briefly, 55 μg of ES products and the total extract of *A. simplex* L3 were separated via SDS–PAGE. Following this, the proteins were transferred from the gel to nitrocellulose membranes (Amersham, Biosciences, UK), and after blocking with skim milk, the membrane was soaked in a primary antibody solution (polyclonal anti-rAs22U antibody) for 2 h at RT. The membranes were then incubated in HRP-labeled secondary goat anti-rat IgG antibodies solution for 1 h at RT. In detecting positive reactions, ECL western blotting detection reagent (Amersham, Biosciences, UK) was used, and the resultant complexes were processed for the detection system using RAS-3000 (Fuzi, Japan).

2.6. Inflammatory-related cytokine gene expression after rAs22U treatment

CT-26 mouse intestinal epithelial cells purchased from the Korean cell line bank (Korea, Seoul) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Hyclone, Logan, USA) supplemented with 10% heat-inactivated FBS (Hyclone, Road Logan, Utah, USA), 2 mM L-glutamine (Sigma, Korea), 100 units/ml of penicillin, and 100 μg/ml of streptomycin (Hyclone, Logan, Utah, USA). The cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2. For rAs22U treatment (final concentration 1 μg/ml), the cells were plated in 10 cm plates at 1×10^6 cells/well and incubated for the indicated time periods. After rAs22U treatment, the quantitative expression of thymus and activation-regulated chemokine (TARC, CCL17), thymic stromal lymphopoietin (TSLP), IL-25, and CXCL1 (Gro-α) mRNAs levels (these genes have been known as initiators of the Th2 allergic response) in CT-26 cells was evaluated via real-time PCR in accordance with the manufacturer's recommended protocols and previous reports (Cho et al., 2011; Yu et al., 2010).

3. Results and discussion

3.1. Identification of a 22U coding gene from A. simplex L3 cDNA library

The partial cDNA (486 bp, As22U), which exhibits a high degree of similarity to the previously-characterized 22U protein-coding genes, was located in an *A. simplex* L3 cDNA library. Following 5′ RACE, we obtained the additional 114 bp nucleotide sequence (included 61 bp untranslated sequence) of the 5′ terminal region (GenBank No. JN241677). The open reading frame of As22U was 636 bp long, coding for 212 amino acid residues with a calculated mass of 23.5 KDa and a PI of 9.06. Based on results from the SignalIP 3.0 program, used to predict the length of the signal peptide, As22U harbored a region encoding for a hydrophobic sequence of 20 amino acids, which may function as a signal peptide.rAs22U was overexpressed in the *E. coli* strain BL21 using a pET28a vector and was found to migrate on SDS–PAGE as a 26 kDa fusion protein with a polyhistidine tag and thus, the molecular weight of rAs22U was calculated to be 24 kDa (Fig. 1A). To determine whether As22U could be secreted by *A. simplex*, we conducted western blot analysis using an anti-rAs22U antibody. The As22U protein was identified in both the total extracts and ES proteins of *A. simplex;* therefore, As22U might be a secretory and structural protein (Fig. 1B).

Twenty-two U molecules have been reported only in a few parasitic nematodes. The first, Di22U was identified in *Dirofilaria immitis*, a dog heartworm, along with other two similar molecular weight proteins (Di20, Di22L); Di20 and Di22L were larval-specific, whereas Di22U was detected in both larval and adult worms