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Cloning and molecular characterization of cDNAs encoding three *Ancylostoma ceylanicum* secreted proteins

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Abstract

Ancylostoma ceylanicum belongs to a group of soil-transmitted helminths, which infect almost 576 mln people worldwide and are a major cause of anaemia and malnutrition. Upon contact with a permissive host, third-stage larvae (L3) residing in the environment become activated larvae (ssL3), a process associated with changes in the profile of gene expression. *Ancylostoma* secreted proteins (ASPs) are the major proteins secreted during larvae activation and play a crucial role in hookworm adaptation to parasitism. Here we report the cloning using RACE-PCR technique of three novel ASPs from the hookworm *A. ceylanicum* (*Ace-asp-3*, *Ace-asp-4*, and *Ace-asp-5*) and computational analysis of the protein sequences. All three proteins contain SCP (Sperm Coating Protein) domain characteristic for previously described ASP proteins. Real-time PCR analysis shows significant up-regulation of *Ace-asp-3* and *Ace-asp-5* expression in adult worms and correlated down-regulation in ssL3 larvae. On the other hand, expression of *Ace-asp-4* was increased in ssL3 stages and decreased in adult parasites.

Keywords

Ancylostoma ceylanicum, ASP, *Ancylostoma* secreted proteins

Introduction

Hookworms are soil-transmitted helminths (STH) that infect humans as well as animals. They cause anaemia and malnutrition globally (Hotez *et al*. 2004). Although human infection with STH are most commonly found in tropical and subtropical regions (WHO report 2010), one third of dogs (Bajer *et al*. 2011) and nearly 90% of feral animals (Szafrańska *et al*. 2010) in Poland are infected with hookworms. Among dog hookworms (e.g. *Ancylostoma caninum*, *Ancylostoma braziliense*, *Ancylostoma ceylanicum* and *Uncinaria stenocephala*) only *Ancylostoma ceylanicum* develops in human (Mahdy *et al.* 2012), but it is considered rather as zoonosis whereas the most of human hookworm infections are caused by *Necator americanus* (Bethony *et al*. 2011). There are different strategies to control helminth infection, including deworming with anthelminthics. However, anthelminthic drug therapy is not an efficient way to control hookworm infections. Firstly, in many cases such therapy is not easily accessible to communities. Secondly, rapid re-infection (Albonico *et al*. 1995) and decreasing drug effectiveness with repeated treatments (Albonico *et al*. 2003, Keiser and Utzinger 2008) limit the impact of large scale deworming. Thus there is an urgent need to further study the biology of hookworms in order to develop new methods of control for helminth infections. It seems appealing to search for target genes and proteins that serve as new targets for drug and vaccine development.

During the life cycle of *Ancylostoma* spp. third-stage larvae (L3) residing in the environment become activated upon infection. In this process, the profile of gene expression changes. *Ancylostoma* secreted proteins are major proteins secreted during larvae activation (Datu *et al*. 2008, Hewitson *et al*. 2011) and play a crucial role in hookworm adaptation to parasitism (Hawdon *et al*. 1996, 1999).

ASP, 42kDa protein was the first molecule found by analysis of the protein expression profile during larval activation of *Ancylostoma caninum* (Hawdon *et al*. 1996). Since then at least 16 different ASPs from blood-feeding nematodes have been reported in GeneBank (Benson *et al*. 1998). *Ancylostoma* secreted proteins show several features attributed to this group

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of proteins. The Sperm Coating Protein (SCP) conservative domain is observed in three-dimensional structure of all ASPs (Hawdon *et al*. 1996, 1999; Zhan *et al*. 1999). The same domain is also present in vespid venoms, well described allergens (Lu *et al*. 1993). Vespid venom 5 allergen shows significant similarities to testis-specific protein, which is an autoantigen of the mammalian sperm (Foster and Gerton 1996).

Moreover, the three-dimensional crystal structure of *Ancylostoma* secreted protein 2 from *Necator americanus* (*Na*-ASP-2) shows structural similarities to chemokines. This suggests its role in the immunomodulation of allergic reactions (Asojo *et al*. 2005).

Even though intensive research is being conducted on the mechanisms of parasite infection in the host organism, the molecular interactions between the parasite and the host are still not sufficiently described. Thus, the main aim of this study was to clone, perform bioinformatics analyses and preliminary molecular studies of three novel *Ancylostoma ceylanicum* secreted proteins (*Ace-asp*-3, *Ace-asp*-4 and *Ace-asp*-5).

Materials and methods

L3 activation

In order to obtain serum stimulated L3 larvae (ssL3), 5000 *Ancylostoma ceylanicum* L3 larvae (hamster adapted strain obtained from Nottingham University cultivated according to the method described by Braisfold and Behnke 1992 were incubated in 1 ml of $RPMI_{1640}$ supplemented with 50% bovine serum for 2 hours (37°C, 5% CO_2) (Chu *et al*. 2004).

Total RNA isolation

Total RNA was isolated from 30 adult *A. ceylanicum* worms using TRIzol reagent (Invitrogen). Worms were homogenised in GLAS-COL homogeniser in 1 ml of TRIzol. 200 µl of chloroform was added, the sample was vigorously shaken for 15 seconds and centrifuged 15000 x g for 10 min, the aqueous phase was collected. 500 µl of ice cold isopropyl alcohol was added and the sample was incubated in –70°C for 30 min. RNA was precipitated by centrifugation (15000 x g for 20 min) and the pellet was washed with 1 ml of 70% ethanol, air dried and dissolved in 30 µl of RNase free water.

cDNA synthesis

First strand synthesis of cDNA was performed using 5 µg of total RNA, 0.5 µg of oligo(dT) primer containing *BamH*I restriction site (pTBam 5'-CCGCCACGCGTGGATCCGTTT TTTTTTTTTTTTTT-3') and M-MuLV Reverse Transcriptase (MBI Fermentas) according to the manufacturer's instructions.

RACE technique (Frochman 1994) was used to clone the full-length cDNAs encoding previously unknown *Ancylostoma* secreted proteins. Primers for 3' RACE-PCR were designed based on multiple sequence alignment (ClustalW, EBI) of known ASPs from *A. ceylanicum* and closely related nematodes (*A. caninum*, *N. americanus*, *A. duodenale*, *Haemonchus contortus*, *Cooperia punctata*). Primers for 5' RACE-PCR were designed based on cloned 3'-end sequence. Primer sequences and PCR conditions are shown in Table I. Full length cDNAs were subcloned into pCR2.1 vector (Invitrogen), their DNA sequences were determined and submitted in GenBank under accession numbers: *Ace-asp-3*,

Table I. Sequences of the primers and reaction conditions used in 5'RACE - PCR, 3'RACE - PCR. If primer was contains sequence recognized by restriction enzyme this sequence is underlined and the name of the enzyme recognizing that sequence is in the bracket.

	Ace-asp-3
3' RACE primer	Asp3L: 5'-CGGGATCCATGGCTGGACCAAGCTGCG-3' (*BamH*I)
3'RACE – PCR conditions	94°C – 4 min; 40 x (92°C – 30 s, 56 °C-1 min, 72 °C–30 s); 72 °C-10 min
5'RACE primer	Asp3R:5'- ATAAGCACAGATGAAGTGGAAGAG -3'
5'RACE –PCR conditions	94°C – 4 min; 40 x (92°C – 30 s, 56°C-1 min, 72°C–30 s); 72 °C-10 min
	Ace-asp-4
3' RACE primer	Asp4L: 5'-CGGGATCCATGGCTGGACCAAGCTGCG-3' (*BamH*I)
3'RACE – PCR conditions	94°C – 2 min; 35 x (92°C – 30 s, 59°C-1 min, 72°C–60 s); 72°C-10 min
5'RACE primer	Asp4R: 5' CCAAGCTTTGGTTTTAGACACACAATC-3' (*Hind*III)
5'RACE – PCR conditions	94°C – 2 min; 35 x (92°C – 30 s, 59°C-1 min, 72°C–60 s); 72°C-10 min
	Ace-asp-5
3' RACE primer	Asp5R: 5'-ACGACATATAGAAAATCGACGTGTGAC-3'
3'RACE – PCR conditions	94°C – 2 min; 35 x (92°C – 30 s, 56°C-1 min, 72°C–60 s); 72°C-10 min
5'RACE primer	Asp5L: 5'-CTCAAGCAATGCAGAGCTTGTCATCG-3'
5'RACE – PCR conditions	94°C – 2 min; 35 x (92°C –30 s, 59°C-30 s, 72°C–60 s) ; 72°C-10 min

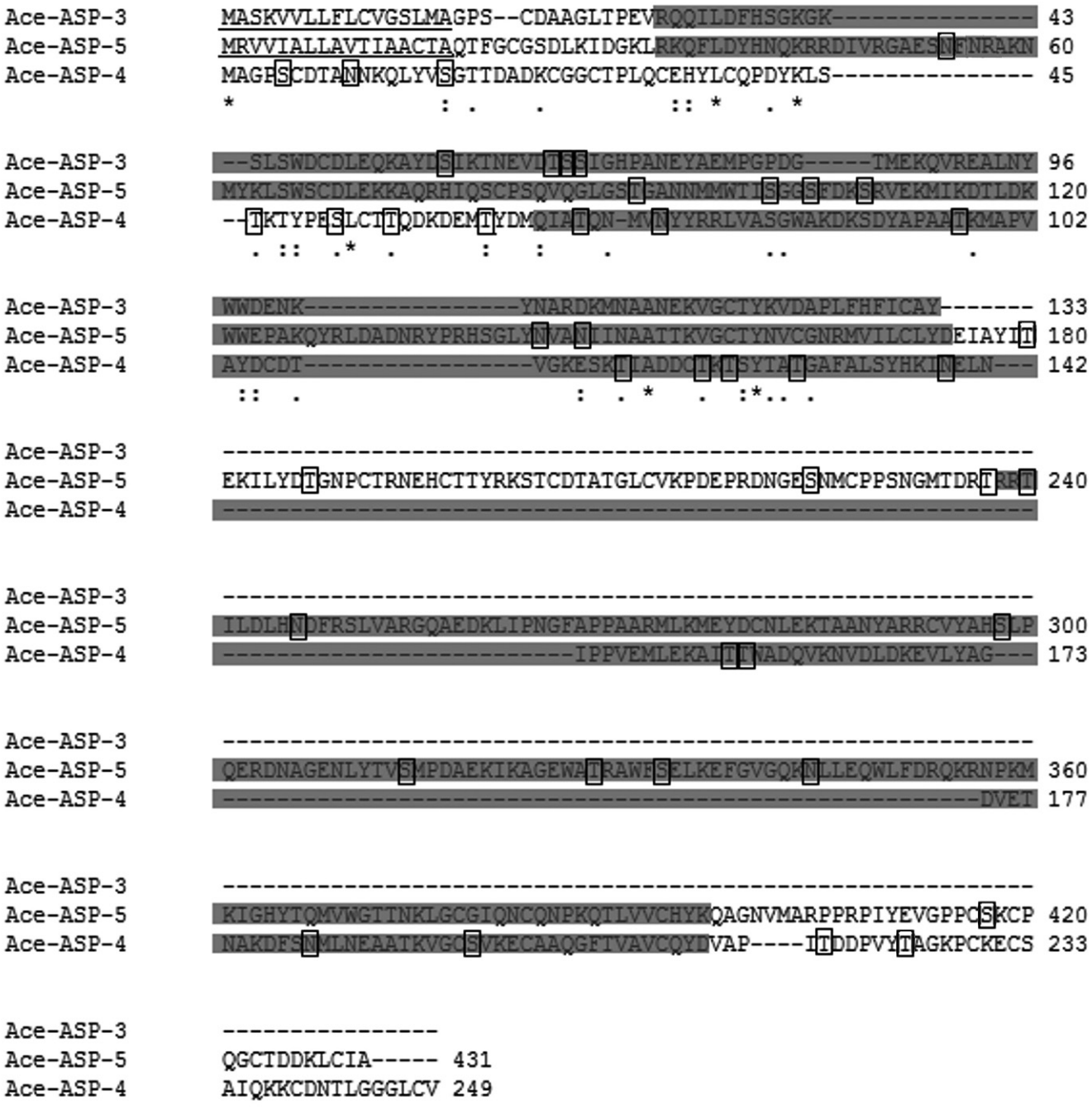

Fig 1. Alignment of deduced amino acid sequences of *Ace*-ASP-3, *Ace*-ASP-4, *Ace*-ASP-5. Signal peptides sequences are underlined, SCP domains are marked in grey, glycosylation sites are marked in frame. Potential glycosylation sites in signal peptide sequence are omitted

Table II. Predicted features and computational analysis of cDNA and amino acid sequences of *Ace*-ASP-3, *Ace*-ASP-4, *Ace*-ASP-5

cDNA	Full length cDNA (bp)	5'UTR (bp)	ORF (bp)	3'UTP (bp)	protein	Mature protein			Signal peptide (AA)	SCP domains	SCP domain (AA)
						AA	p*I*	Mw (Da)			
Ace-asp-3	530	17	405	108	Ace-ASP-3	118	4.96	14990.96	17	1	104
Ace-asp-4	807	6	750	51	Ace-ASP-4	249	4.72	27026.44	0	1	147
Ace-asp-5	1367	19	1296	52	Ace-ASP-5	414	9	48705.68	17	2	142 and 159

Ace-asp-4 and *Ace-asp-5*-AY423765, AY423766 and DQ250680, respectively.

Analysis of Ace-asp-3, *Ace-asp-4 and Ace-asp-5 gene expression of different A. ceylanicum life stages*

Total RNA from 5000 L3, 5000 ssL3 and 50 adult worms was isolated. RT-PCR primers and reaction conditions were as follows: *Ace-asp*-3: ASP3RTL (5'-CAAATCCTTGATTTCCA CAG-3'), ASP3RTR (5'-TCTCATTTGCAGCATTCATC-3') and initial denaturation at 94°C for 3 min, 30 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 30 s, final extension at 72°C for 10 min; *Ace-asp*-4: (ASP4RTL (5'-AGGAGACTCGT TGCATCTGG-3'), ASP4RTR (5'-CAGGAGGAATGT TGA GTTCG-3') and initial denaturation at 94°C for 2 min, 40 cycles of 94°C for 10 s, 54°C for 5 s, 72°C for 15 s, final extension at 72°C for 10 min; *Ace-asp*-5: ASP5RTL (5'-TG GAATGACAGACAGAACAC-3'), ASP5RTR (5'-CTCAA GCAATGCAGAGCTTGTCATCG-3') and initial denaturation at 94°C for 3 min, 35 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 30 s, final extension at 72°C for 10 min.

Real-time PCR analysis was performed in Roche LightCycler using LightCycler FastStart DNA Master SYBR Green I (Roche) according to the manufacturer's instructions. Relative expression was quantified using 5.8S rRNA as a housekeeping gene. Primers for 5.8S rRNA were PAR168 (5'-CGTATCGATGAAAAACGCAG-3') and PAR169 (5'-ACAACCCTGAACCAGACGTG-3'). Primers for investigated genes were as follows: PAR 162 (5'-TGGAAAG GGTAAATCGTTGA-3') and PAR 163 (5'-TTTGTAGGTG-CAACCAACCT-3') for *Ace*-ASP-3, PAR 164 (5'-TCCT-GTGGCATATGACTGTG-3') PAR165 (5'-GCATTAGTTT CCACGTCACC-3') for *Ace*-ASP-4, PAR 166 (5'-TCAAG-GAGTTTGGTGTTGGT-3') and PAR 167 (5'-TAGATTG-GTCGTGGTGGTCT-3'). Conditions of Real-time PCR were: hot start activation at 94°C for 10 min, 40 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 30 s. Expression level was calculated using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) and presented in relation to gene expression in L3 stage of *A. ceylanicum*. Each expression assay was repeated twice.

Bioinformatical analysis

Amino acid sequence was determined and Open Reading Frame was identified using Translate (http://web.expasy.org/translate/), signal peptide was predicted using SignalP (http://www.cbs.dtu.dk/services/SignalP/), putative molecular weight and isoelectric point were predicted using Compute pI/Mw tool (http://web.expasy.org/compute_pi/), conservative domains were analysed using Conserved Domain Database from NCBI (http://www.ncbi.nlm.nih.gov/cdd) and SMART (http://smart.embl-heilderberg.de). Glycosylation sites were predicted using GlycoPred (http://comp.chem.nottingham.ac.uk/glyco/). Proteins structures were predicted Local Meta-Threading-Server (LOMETS) (Wu and Zhang 2007) and visualised with PyMOL (The PyMOL Molecular Graphics System, Version 1.1r1 Shrödinger, LLC) to reveal disulphide bonds.

Phylogram was conducted using Phylogeny, (ClastalW2, version 2.1, EBI), neighbour-joining method, based on the alignment of 16 known ASPs sequences (accession numbers): Ace-ASP-1 (AY136548), Ace-ASP-2 (AY288090), Ace-ASP-3 (AY423765), Ace-ASP-4 (AY423766), Ace-ASP-5 (DQ250680), *A. caninum*: Ac-ASP-1 (ACU26187), Ac-ASP-2 (AF089728), Ac-ASP-3 (AY217004), Ac-ASP-4 (AY217005), Ac-ASP-5 (AY217006), Ac-ASP-6 (AY217007), Ac-ASP-7 (JN038056), *N. americanus*: Na-ASP-1 (AF079521), Na-ASP-2 (AY288089), *A. duodenale*: Ad-ASP-1 (AF077402), Ad-ASP-2 (AY288088).

Results

Ace-asp-3, Ace-asp-4 and Ace-asp-5 sequence analysis

Features of newly cloned *Ace-asp-3*, *Ace-asp-4* and *Ace-asp-5* cDNAs are shown in Table II.

Analysis of conserved domains indicate the presence of SCP domains in all deduced amino acid sequences. Interestingly, *Ace*-ASP-5 showed the presence of two SCP domains (Table II, Fig. 1). Analysis by SignalP indicates the presence of a signal peptide in *Ace*-ASP-3 and *Ace*-ASP-5 but not in *Ace*-ASP-4 (Table II, Fig. 1). Computational prediction of glycosylation sites, done by GlycoPred showed multiple glycosylation sites in all sequences (Fig. 1). Protein structure modeling revealed one predicted cysteine disulphide bond in the structure of *Ace*-ASP-3 (Cys119-Cys132), three in *Ace*-ASP-4 (Cys120-Cys200, Cys195-Cys210, Cys232-Cys248) and eight in *Ace*-ASP-5 (Cys22-Cys68, Cys81-Cys163, Cys158-Cys171, Cys191-Cys205, Cys227-Cys280, Cys293-*Cys3*83, Cys378-Cys393, Cys419-Cys429*)*.

The alignment of deduced amino acids sequences showed the highest identities of *Ace*-ASP-5 to *Ace*-ASP-1 (AY13 6548), *Ac*-ASP-1 (ACU26187), *Ad*-ASP-1 (AF077402) and *Na*-ASP-1 (AF079521), which were identical in 42.6%, 42.7%, 42.6%, 42.5% respectively. *Ace*-ASP-3 showed 18.9% identity with *Ace*-ASP-4. *Ace*-ASP-4 showed 21.8% identity with *Ac*-ASP-2. Amino acid sequences were also compared in the aspect of their phylogenetical relation. Results of this analysis are shown in Fig. 2.

Analysis of Ace-asp-3, *Ace-asp-4 and Ace-asp-5 gene expression among different life stages of A. ceylanicum*

RT-PCR analysis showed presence of *Ace-asp-3*, *Ace-asp-4* and *Ace-asp-5* mRNA in all investigated life stages of *Ancylostoma ceylanicum*, related to expression levels of L3 stages (assigned as 1.0).

Relative expression performed by Real-time PCR showed that the expression of *Ace-asp-3* was nearly 70 fold higher in adult worms than in L3, while it was down-regulated in ssL3. The ex-

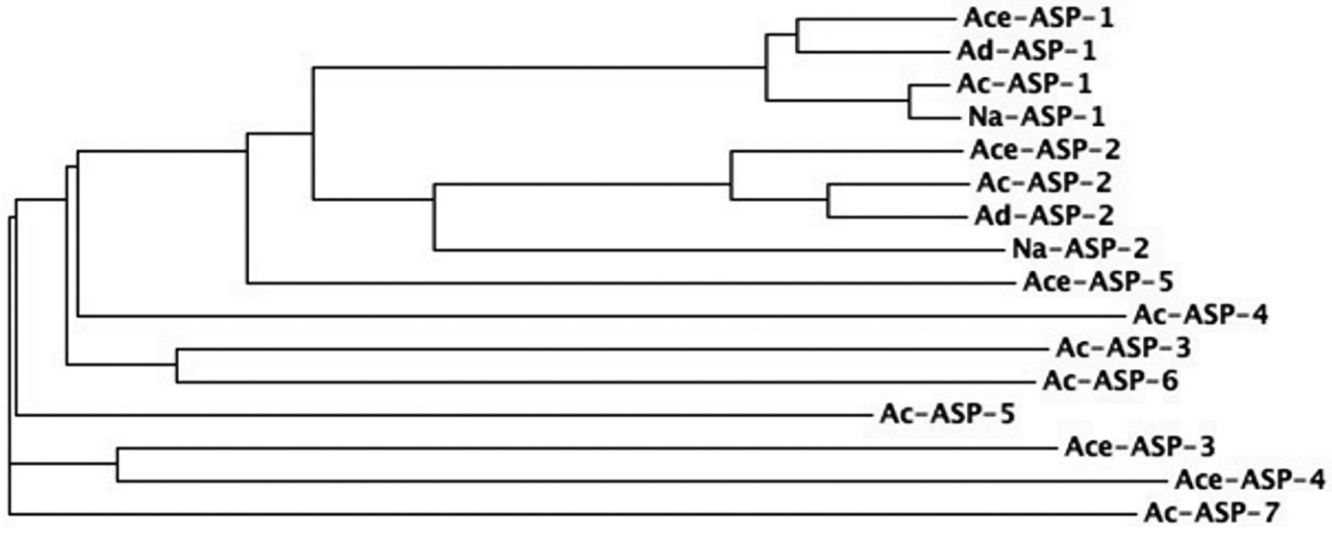

Fig. 2. Phylogenetic relationship of nematode secreted proteins from different species generated with ClustalW

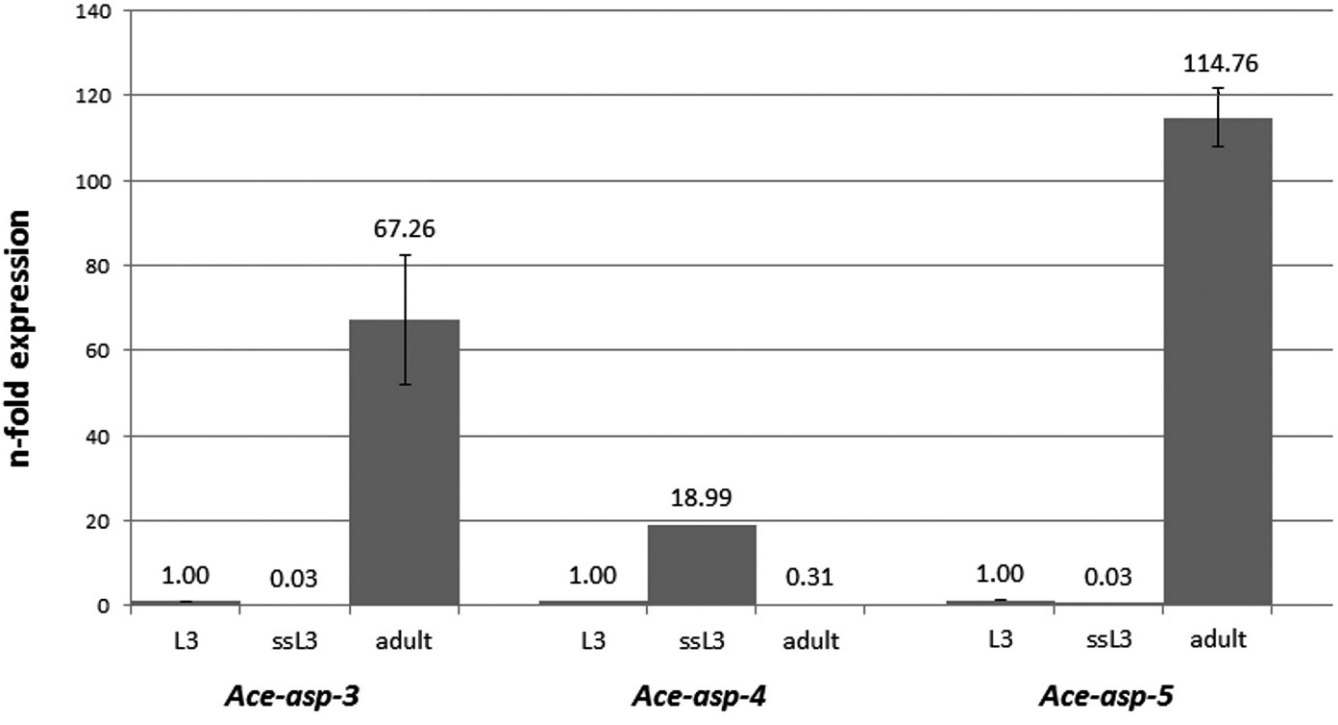

Fig. 3. Relative expression of cDNAs encoding *Ace-asp*-3, *Ace-asp*-4, *Ace-asp*-5 among L3 larvae, ssL3 (serum activated L3 larvae) and adult life stages of *Ancylostoma ceylanicum*, related to expression levels of L3 stages (assigned as 1.0)

pression of *Ace-asp-4* was 19 fold higher in ssL3 and down-regulated in adult worms. The expression of *Ace-asp-5* was decreased in ssL3 and more than 100 times increased in adult worms. Gene expression levels were calculated in the relation to expression levels of L3 stages, which was assigned as 1.0 (Fig. 3**)**.

Discussion

In this study we report the molecular cloning of three novel *Ancylostoma* secreted proteins from the hookworm *A. ceylanicum*: *Ace-asp-3*, *Ace-asp-4* and *Ace-asp-5*. Phylogenetic

analysis of 16 related sequences showed significant homology between *Ace*-ASP-5 and ASP-1, as well as between ASP-2 from *A. caninum*, *A. duodenale* and *N. americanus*. Moreover, the presence of SCP domains in three novel ASPs provides evidence of belonging to the ASP family. Even though that *Ace*-ASP-3 and *Ace*-ASP-4 are not highly homologous to other ASPs, they still may belong to the same protein family, along with *Ac*-ASP-7, which also shows less identity to other ASPs (Fig. 2).

Computational analysis of the deduced amino acid sequences of three novel ASPs revealed differences in numerous features. Firstly, the isoelectric point, which is similar for *Ace*-ASP-3 and *Ace*-ASP-4 but different for *Ace*-ASP-5. The p*I* correlates with the charge of amino acids present in the protein. Since *Ace*-ASP-5 is the largest protein and it shows the most differences in amino acid sequence therefore its p*I* is different from other two investigated proteins. Secondly, the alignment of obtained sequences showed 18.9% identity between *Ace*-ASP-3 and *Ace*-ASP-4, but only 10.3% and 15.1% between pairs: *Ace*-ASP-5 - *Ace*-ASP-3 and *Ace*-ASP-5-*Ace*-ASP-4, respectively.

Two out of the three ASPs were encoded with a signal peptide. Ace-ASP-4 was the only investigated protein without a predicted signal peptide. It is common that excretory-secretory products (ES) do not contain a signal peptide (Cass *et al*. 2007, Hewitson *et al*. 2008, Moreno and Geary 2008). Several glycosylation sites, typical for eukaryotic posttranslational modifications, were revealed by computational analysis of *Ace*-ASP-3, *Ace*-ASP-4 and *Ace*-ASP-5 as it was described for other ASPs (Zhan *et al*. 1999, Hawdon *et al*. 1999).

A common attribute of all ASPs is the presence of a SCP domain (Zhan *et al*. 1999, 2003), which is found in many phylogenetically diverse proteins including vespid venom allergens (King and Sprangford 2000), mammalian sperm proteins (Giese *et al*. 2002), free-living *Ceanohabditis elegans* proteins (Hawdon *et al*. 1999) and plant pathogenesis proteins (Szyperski *et al*. 1998). It is known that single–SCP–domain ASPs are more widespread among nematodes than double-domain forms (Zhan *et al*. 1999, Hawdon *et al*. 1999). Our results also support this statement as the bioinfomatic analysis of *Ace*-ASP-3, *Ace*-ASP-4 and *Ace*-ASP-5 using Conserved Domain Database from NCBI showed that only *Ace*-ASP-5 is a double-domain form of ASP. Moreover, similarity demonstrated by ClustalW, both in the amino acid and in the phylogenetic tree, indicates that *Ace*-ASP-5 is related to ASPs with two SCP domains. As expected, single-domain *Ace*-ASP-3 and *Ace*-ASP-4 are more closely related to the single-domain ASPs. Although all ASPs comprise at least one SCP domain, they still show too low similarities to speculate about the biological role of ASPs based only on their amino acid sequence homology (Zhan *et al*. 1999).

ASPs show certain differences in their expression patterns of different life stages of the parasite. Zhan and the colleagues reported that RT-PCR analysis of six ASPs from *A. caninum* indicated an exclusive expression of *Ac-asp-1* and *Ac-asp-2* by the L3 stage, in contrast to *Ac-asp-5* and *Ac-asp-6*, which were expressed in adult worms only. On the other hand, *Ac-asp-3* and *Ac-asp-4* were expressed in both life stages of *A. caninum* (Zhan *et al*. 2003). Our analysis of mRNA expression of novel *A. ceylanicum* ASPs showed that *Ace-asp-3*, *Ace-asp-4* and *Ace-asp-5* were present in all three stages (L3, ssL3 and adult). Furthermore, real-time PCR analysis showed clear differences in relative expression of ASPs. It was evident that *Ace-asp-3* and *Ace-asp-5* were up-regulated in adult worms. This suggests that *Ace*-ASP-3 and *Ace*-ASP-5 may play an important role in the biology of adult *A. ceylanicum*. On the other hand, *Ace-asp-4* expression was increased in ssL3 larvae, which may indicate a role in adaptation to parasitism and molecular interaction with the host. Those data were consistent with Western-blot analysis performed with polyclonal antisera obtained in mouse with r*Ace*-ASP-4 and r*Ace*-ASP-5 (data unpublished). Moreover, an interesting pattern in the change of expression of investigated genes was observed. When the gene was up-regulated in adult stage of the parasite, at the same time it was down-regulated in ssL3 stage. In case of *Ace-asp-4* the pattern was conversed.

In conclusion, we report the cloning and characterisation of three novel cDNAs belonging to *Ancylostoma* secreted proteins. It is of primary interest to further express those genes as recombinant proteins and test for their pharmaceutical and vaccine potential.

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