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Heterologous expression, purification and characterisation of the extracellular domain of trypanosome invariant surface glycoprotein ISG75

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ABSTRACT

The invariant surface glycoprotein ISG75 is a transmembrane glycoprotein occurring on the surface of the bloodstream-form *Trypanozoon*. This study describes the expression and purification of the N-terminal extracellular domain of ISG75, a novel target for development of diagnostic tests for trypanosomosis. To facilitate disulfide formation in the cytoplasm, a 1287-bp cDNA fragment encoding ISG75 from *Trypanosoma brucei gambiense* was expressed in a thioredoxin reductase, glutathione oxidoreductase double mutant *Escherichia coli* strain. An accessory plasmid pRIL, providing the *arg*I, *ile*Y, and *leu*W tRNAs, was necessary for efficient heterologous translation of the ISG75 mRNA. The recombinant double-tagged (streptavidine and histidine) ISG75 was purified by two-step affinity chromatography. Addition of L-glutamic acid and L-arginine in the buffer solutions was crucial to stabilise the protein during purification. The purified soluble protein was characterised by circular dichroism spectroscopy, reverse-phase high pressure liquid chromatography and mass spectrometry. It has an alpha-helical folded conformation, is homogeneous and pure (99%). Furthermore, sera of *Trypanosoma brucei*-infected animals specifically recognise this recombinant ISG75; and rabbit antiserum raised against the recombinant ISG75 detects all species of the *Trypanozoon* subgenus in parasite preparations.

1. Introduction

The *Trypanozoon* subgenus causes sleeping sickness in humans and nagana, surra and dourine in animals throughout Africa, Latin America and South-East Asia (Maudlin, 2006). Control measures against trypanosomoses, including diagnosis and vaccination, based on variable surface glycoproteins (VSGs) have faced challenges due to the parasite's ability to switch expression of its VSG coat. All current antibody detection tests for sleeping sickness and surra are based on the predominant VSGs as antigens (Magnus et al., 1978; Verloo et al., 2001). Still these tests have their limitations of misdiagnosis if the parasites expressed other types of VSGs than that used in the tests. Therefore, non-variable surface proteins that are not subjected to antigenic variation, such as ISG75, represent promising alternatives in development of diagnostic tests (Hutchinson et al., 2004).

ISG75 of *Trypanosoma brucei* (*T. b.*) *brucei* was first identified in the membrane extract containing a mixture of invariable surface glycoproteins of 64, 65 and 75 kDa (Ziegelbauer and Overath, 1992). Further investigation by Tran et al. (2006) reveals that ISG75 is encoded by a multicopy gene family, which is conserved in all species and subspecies of the *Trypanozoon* subgenus, including *T. b. brucei*, *T. b. gambiense*, *T. b. rhodesiense*, *T. evansi* and *T. equiperdum*. This gene family consists of two main groups that share 75% and 77% similarities among their cDNA and gDNA sequences, respectively. A putative ISG75, regardless of its origin in group I or group II in the gene family, has a conserved topology: a 28-amino acid N-terminal signal peptide, a 439-amino acid hydrophilic variable region that is predicted to be the extracellular domain, a 23-amino acid single α-helix transmembrane domain, and a 34-amino acid cytoplasmic domain at the C-terminus (Ziegelbauer et al., 1992;

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Fig. 1. Schematic diagram of ISG75 polypeptide and its truncate. The upper bar is the complete ISG75 with amino acid positions of the predicted domains. From the N-terminus, the black box indicates the cleavable signal sequence, the white box and black box depict the variable and conserved region, respectively, of the extracellular domain, the hatched box indicates the transmembrane domain, and the grey box indicates the intracellular domain. The lower bar, with incorporated codons of Strep-tag II and TEV protease site at N-terminus and of His-tag at C-terminus, is the ISG75 truncate inserted to an expression vector (pTbG-ISG75$_{S\text{-}29\text{-}457\text{-}H}$).

Tran et al., 2006). The extracellular domain of ISG75 is exposed to the host environment, and thus carries the most immunogenic determinants that are of interest in immunological studies. Moreover, this large domain bears major folds of the protein structure, of which X-ray crystallographic analysis will offer significant information on the ISG75 three-dimensional structure.

This paper describes the expression of the extracellular domain of ISG75 in a thioredoxin reductase (*trx*B), glutathione oxidoreductase (*gor)* double mutant *Escherichia coli* strain and its purification and characterisation as well as its potential application in diagnosis of trypanosome infections.

2. Materials and methods

2.1. Construct engineering

The ISG75 sequence was derived from a cDNA clone of *T. b. gambiense* LiTat 1.3 belonging to group I in the ISG75 gene family (accession number DQ200239, Tran et al., 2006). The cDNA segment was amplified with a primer set that included the first residue of the mature polypeptide (or amino acid 29 of the full-length polypeptide) and immediately up-stream of the transmembrane domain (amino acid 457) of ISG75 (Fig. 1). A *Nco*I site providing the start codon was incorporated into the forward primer (ISG75-1F). Six histidine codons, a stop codon and a Ba*mH*I site were incorporated into the reverse primer (ISG75-6R) (Table 1). After *Nco*I and *BamH*I digestion (Fermentas), the PCR fragment encoding the His-tagged extracellular domain ISG75$_{29\text{-}457\text{-}H}$ was ligated into *Nco*I–*BamH*I digested pET-15b vector, using Ready-to-go T4 DNA ligase (GE Healthcare). This construct was named pTbG-ISG75$_{29\text{-}457\text{-}H}$ and propagated in *E. coli* K514. To further facilitate protein purification, a Strep-tag II sequence was incorporated at the N-terminus. Two synthetic single-stranded oligonucleotide sequences encoding the Strep-tag II and the Tobacco Etch virus protease site were annealed and ligated into the *Nco*I site of the pTbG-ISG75$_{29\text{-}457\text{-}H}$, resulting in a pTbG-ISG75$_{S\text{-}29\text{-}457\text{-}H}$ construct expressing a double-tagged extracellular domain of ISG75 (Table 1). The correctness of the constructs was confirmed by nucleotide sequencing.

2.2. Protein expression

The pTbG-ISG75$_{S\text{-}29\text{-}457\text{-}H}$ and the accessory plasmid pRIL were co-transformed into Origami B (DE3) (Table 2). All culture media contained 100 μg ml^{-1} ampicillin and 35 μg ml^{-1} chloramphenicol (Duchefa Biochemie). A double transformant was purified and then grown overnight in Lubria Bertani (LB) broth. The overnight culture was diluted 80× in fresh LB medium and incubated at 37 °C with aeration until OD_{600} reached approximately 0.8. Subsequently, expression was induced by 1 mM of isopropyl β-D-thiogalactopyranoside (Fermentas) for 3 h. The ISG75$_{S\text{-}29\text{-}457\text{-}H}$ was detected by SDS-PAGE and immunoblot as described below. The ISG75$_{S\text{-}29\text{-}457\text{-}H}$ was confirmed by N-terminal amino acid sequencing (Alta Biosciences or Alphalyse).

2.3. Double-tagged affinity purification

This purification protocol was achieved after extensive optimisation. *E. coli* were harvested by centrifugation at 6000 rpm for 15 min, and then resuspended in binding buffer containing 0.1 M Tris (pH 8.0), 0.5 M NaCl, 50 mM L-glutamic acid, 50 mM L-arginine, 0.1 mg ml^{-1} 4-(2-aminoethyl)benzensulfonyl fluoride hydroclodride (AEBSF), 1 μg ml^{-1} leupeptine, 50 μg ml^{-1} DNaseI and 10 mM $MgCl_2$. The cell suspension was disrupted at 15,000 kPa in EmulsiFlex-C5 homogenizer (Avestin). Cell debris was removed by centrifugation for 30 min at 18,000 rpm, 4 °C. The clear lysate was loaded into a Strep-tactin Poros 50 (IBA) column (XK 16/20, Pharmacia) equilibrated with the binding buffer. The bound proteins were eluted with 5 mM D-desthiobiotin (IBA) (20 mM Tris (pH 8.0), 1 M NaCl, 50 mM L-glutamic acid, 50 mM L-arginine, 5 mM D-desthiobiotin). The pooled Strep-tactin eluates were batch incubated with Ni-NTA Superflow (Qiagen) for 1 h. The protein–matrix mixture was packed into a column (10/10, GE Healthcare) and washed with 20 mM Tris (pH 8.0), 1 M NaCl, 50 mM L-glutamic acid, 50 mM L-arginine. The bound protein was eluted with a linear gradient over 10 column volumes to 250 mM of imidazole. The peak fractions were dialysed against H_2O prior to lyophilisation for storage at −20 °C. The protein was flash frozen in liquid nitrogen and then applied to a Flexi-Dry MP (FTS systems). All columns were run on an Akta FPLC (GE Healthcare) at room temperature. SDS-PAGE and immunoblot (described below) were employed to monitor the presence of ISG75$_{S\text{-}29\text{-}457\text{-}H}$. The purified protein was confirmed by N-terminal amino acid sequencing and mass fingerprint spectrometry.

2.4. Analysis of the purified ISG75

2.4.1. Native PAGE, SDS-PAGE, and immunoblot

ISG75$_{S\text{-}29\text{-}457\text{-}H}$ was analysed on pre-cast 10% Bis–Tris NuPage SDS-PAGE gels (Invitrogen), with an SDS-2-(N-morpholino) ethane sulfonic acid running buffer. Gels were run at 200 V, 120 mA,

Table 1
Oligonucleotide primers used for constructing the expression vectors

Name	Oligonucleotide sequence (5′–3′)
ISG75-1F	CCGCCATGGAGGAGCTCTCAGTTGCGCAAAAACAGT
ISG75-6R	CCCGGATCCTTAGTGGTGGTGGTGGTGGTGGCTGCCCTCATCATCATGGTCAGCATT
Strep-tag oligo 1	CATGGCTAGCTGGAGCCACCCGCAGTTCGAAAAGAGCGCCGGTGAGAATCTTTATTTTCAGGGCGG
Strep-tag oligo 2	CATGCCGCCCTGAAAATAAAGATTCTCACCGGCGCTCTTTTCGAACTGCGGGTGGCTCCAGCTAGC

Table 2
List of *E. coli* strains and plasmids used in this study

E. coli strain	Genotype/phenotype	Reference
K514	*sup*E44 *thr*-1 *leu*B6 *lac*Y1 *ton*A21 *hsdR*	Colson et al. (1965)
BL21 (DE3)	F⁻ *ompT [lon] hsdS$_B$* with the Lambda-derived DE3 prophage carrying the *T7RNAP* polymerase gene under the control of the P_{Lac} promoter	Studier et al. (1990)
C43 (DE3)	derivative of BL21 (DE3)	Miroux and Walker (1996)
BL21 AI	F⁻ *ompT hsdS$_B$ gal dcm ara*B::*T7RNAP-tetA*	Invitrogen
OrigamiB (DE3)	*ahpC* Δ*ara–leu7697 araD139* Δ*lacX74 galE galK rpsL* Δ*phoAPvuII phoR malF3 F'*[*lac*$^+$*(lacI^q)*pro] *gor522*::T*n10 trx*B::kan, DE3 prophage	Novagen
Vector	Characteristics	Reference
pET-15b	T7 promoter/lac operator region, ampicillin resistant	Novagen
pRIL	encodes the tRNA genes *arg*U (AGA & AGG), *ile*Y (AUA) and *leu*W (CUA), chloramphenicol resistant	Stratagene
pTbG-ISG75$_{29\text{-}457\text{-}H}$	the ISG75 gene from *T. b. gambiense* encoding the extracellular domain from amino acid 29 till 457, followed by a C-terminal His-tag, cloned in pET-15b	This study
pTbG-ISG75$_{S\text{-}29\text{-}457\text{-}H}$	pET-15b vector carrying the ISG75 gene from *T. b. gambiense* encoding the extracellular domain from amino acid 29 till 457, preceded by the Strep-tag II and the TEV protease cleavage site at the N-terminus and followed by a C-terminal His-tag	This study

for 35 min. Native PAGE analysis was performed on Novex 12% Tris–Glycine gels (Invitrogen) with Tris–Glycine buffer (25 mM Tris, 192 mM Glycine pH 8.3). Gels were run at 125 V, 8 mA for 6 h, at 4 °C. Proteins were stained with Coomassie brilliant blue. Immunoblot was performed according to Sambrook and Russell (2001), using Porablot nitrocelluose membrane (Machery-Nagel). Monoclonal anti-Histidine tag antibody (Serotec) or monoclonal anti-Strep tag antibody (Qiagen). Anti-mouse IgG conjugated with horseradish peroxidase (Sigma) was used for immuno-detection.

2.4.2. Reverse-phase high pressure liquid chromatography (HPLC)

An aliquot of 60 μg of ISG75$_{S\text{-}29\text{-}457\text{-}H}$ in 0.1% trifluoroacetic acid was injected into an analytical C4 column (Vydac) with an acetonitrile gradient of 0–80% in 0.1% trifluoroacetic acid over 35 min. The column was run on 600 S controller and photodiode-array 996 detector (Waters). The elution was continuously monitored at both 214 nm and 280 nm.

2.4.3. Circular dichroism spectroscopy

An aliquot of 0.2 mg ml^{-1} of ISG75$_{S\text{-}29\text{-}457\text{-}H}$ in H_2O was analysed with a J-715 spectropolarimeter (Jasco) in a 1-mm cuvette. The circular dichroism signals were scanned at 180 nm to 255 nm range during four accumulations at constant temperature of 25 °C.

2.4.4. Mass spectrometry

Protein solution was loaded into gold–palladium coated borosilicate nanoelectrospray capillaries (Proxeon). The mass spectrum was acquired on a Q-Tof Ultima mass spectrometer (Waters/Micromass), equipped with a Z-spray nanoelectrospray source and operating in the positive ion mode. The time-of-flight analyser was operated in the W mode. Data acquisition was performed using a MassLynx 4.0 system. The spectrum represents the combination of 1-s scans. Molecular mass of ISG75$_{S\text{-}29\text{-}457\text{-}H}$ was determined after maximum entropy processing of the raw spectrum (MaxEnt1 – Waters/Micromass).

Protein micro-sequencing: protein bands were excised from SDS-PAGE gel, crushed into small pieces, washed with 25 mM NH_4HCO_3 and 50% CH_3CN, 25 mM NH_4HCO_3 and then vacuum-dried. The gel pieces were swollen in an ice-cold bath in 10 μl of a digestion buffer containing 25 mM NH_4HCO_3, and 10 ng μl^{-1} of sequencing grade modified trypsin (Promega). The enzymatic digestion was carried out overnight at 37 °C. The supernatants were collected and the peptides remaining in the gel pieces were extracted sequentially with 0.025 M NH_4HCO_3, 50% CH_3CN 0.025 M NH_4HCO_3 and 50% CH_3CN 5% HCOOH (v/v). The tryptic peptides were analysed by tandem mass spectrometry (MS/MS), data of which were processed by the maximum entropy data enhancement program MaxEnt 3. Amino acid sequences were semi-automatically deduced using the peptide sequencing program PepSeq (Waters/Micromass).

2.4.5. Generation of antiserum against ISG75$_{S\text{-}29\text{-}457\text{-}H}$

Blood was taken from a naive rabbit at day 21 and day 15 before immunisation to prepare pre-immune serum. For immunisation, 1 ml of 230 μg ml^{-1} purified ISG75$_{S\text{-}29\text{-}457\text{-}H}$ was mixed with 1 ml of complete Freund's adjuvant and injected intradermally. At day 24, blood was taken and a boost was given by intradermal injection of 230 μg ISG75$_{S\text{-}29\text{-}457\text{-}H}$ in incomplete Freund's adjuvant. Hyperimmune serum was prepared from the blood taken by heart puncture at day 37.

2.4.6. Enzyme-linked immunosorbent assay

A microplate (Maxisorp, Nunc) was coated with 2 μg ml^{-1} of ISG75$_{S\text{-}29\text{-}457\text{-}H}$. The protocol was based on Lejon et al. (2003) with minor modifications. Goat sera before infection and 30-day post-infected with *T. b. brucei* were tested at 1:1500 dilution in blocking buffer solution of PBS and 1% casein. Anti-goat IgG conjugated with horseradish peroxidase (Jackson) and colorimetric substrate 2,2′-azinobis [3-ethylbenzothiazonline-6-sulfonic acid]-diammonium salt (Roche) were used. Optical densities were read at 415 nm (Multiskan RC Version 6.0, Labsystems).

2.4.7. Preparation of trypanosome lysates

For immunoblot using rabbit anti-ISG75$_{S\text{-}29\text{-}457\text{-}H}$ antiserum, detergent-soluble extracts of whole trypanosomes were prepared. They included the bloodstream forms of *T. b. brucei* AnTat 2.2, *T. b. gambiense* AnTat 9 1, *T. b. rhodesiense* AnTat 25 1S, *T. evansi* RoTat 1.2, *T. equiperdum* OVI, *T. congolense* TRT17 and the procylic form of *T. b. brucei* AnTat 1. Briefly, a pellet of 10^8 trypanosomes, purified from rat blood over DEAE-cellulose (Lanham and Godfrey, 1970), was suspended in 0.45 ml of 20 mM Na_2HPO4, 2 mM NaH_2PO4 (pH 7.5), 0.1 μg ml^{-1} AEBSF, 50 μg ml^{-1} leupeptin, 50 μg ml^{-1} DNaseI and 10 mM $MgCl_2$ at room temperature for 15 min. The suspension was centrifuged for 15 min at 13,000 rpm, 4 °C. The pellet was then resuspended in 0.45 ml of 20 mM Tris–HCl (pH 7.5), 100 mM NaCl, 0.5% (w/v) sodium desoxycholate, 0.1% (w/v) sodium dodecyl sulfate and 0.5% (w/v) nonidet P-40, 0.1 μg ml^{-1} AEBSF, 50 μg ml^{-1} leupeptin, 50 μg ml^{-1} DNaseI and 10 mM $MgCl_2$ at room temperature for 15 min. The suspension was centrifuged for 1 h at 13,000 rpm, 4 °C. Thirty μl of the supernatant was used for immunoblot, in which the rabbit anti-ISG75$_{S\text{-}29\text{-}457\text{-}H}$ antiserum and horseradish peroxidase

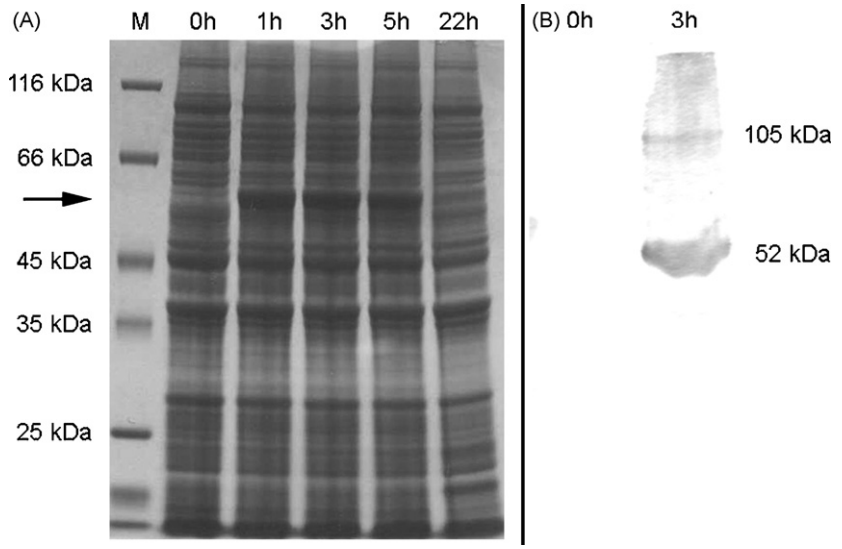

Fig. 2. Expression analysis of ISG75$_{S\text{-}29\text{-}457\text{-}H}$ in Origami B (DE3) strain in the presence of pRIL plasmid. (A) Coomassie-stained SDS-PAGE gel at different induction time intervals. The arrow indicates the position of ISG75$_{S\text{-}29\text{-}457\text{-}H}$; (B) Immunoblot of total cell lysate before induction and 3 h post-induction, using anti-His tag monoclonal antibodies.

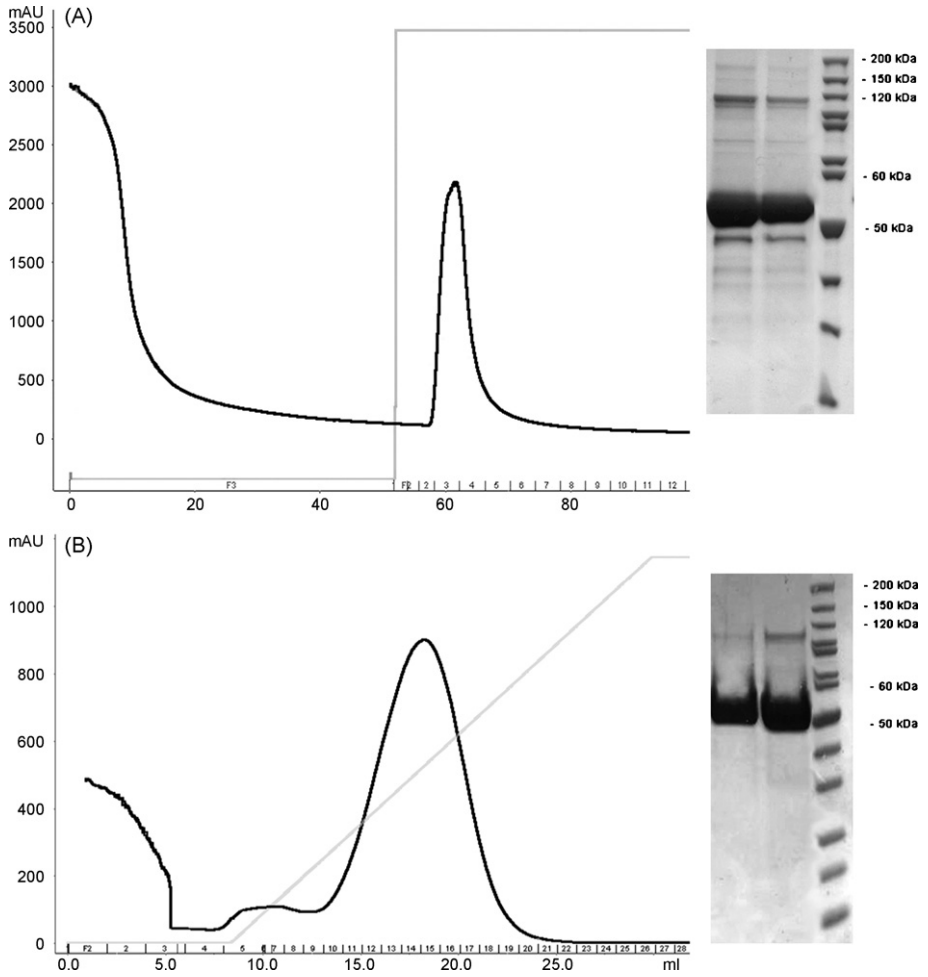

Fig. 3. The elution profiles and Coomassie blue-stained SDS-PAGE gels of the peak fractions of the double-tagged purification of ISG75$_{S\text{-}29\text{-}457\text{-}H}$. (A) Strep-tactin affinity chromatography, the bound proteins were eluted by 5 mM of D-desthiobiotin; (B) Ni-NTA affinity chromatography, the bound protein was eluted by a linear gradient over 10 column volumes to 250 mM imidazole.

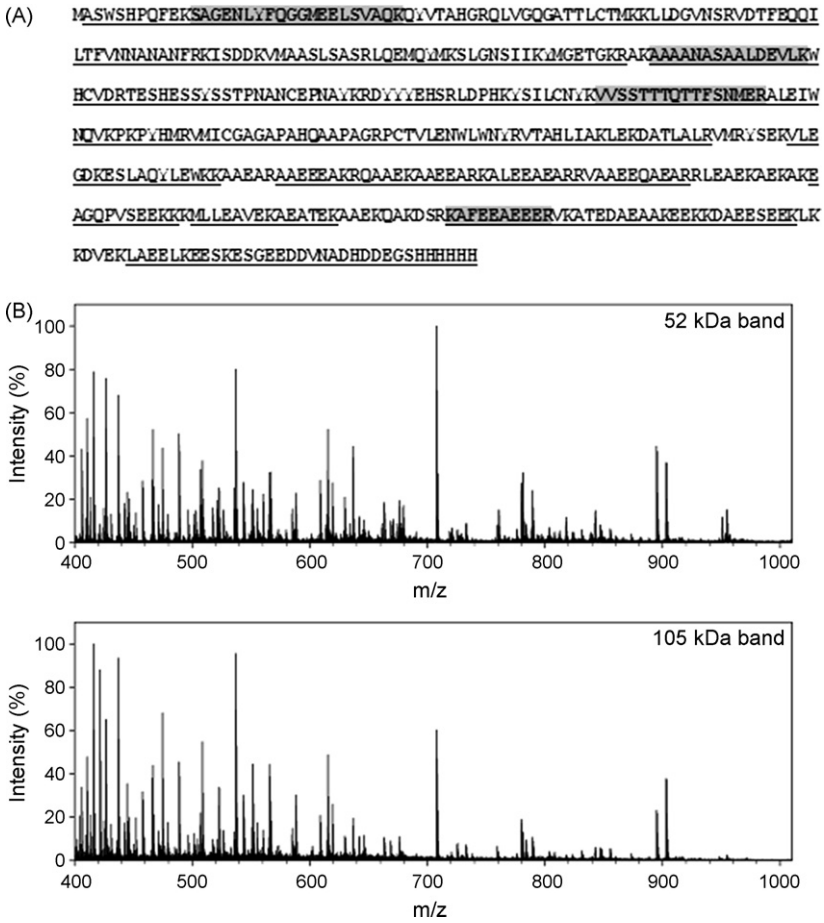

Fig. 4. Analysis by mass spectrometry of the apparent 52 kDa and 105 kDa proteins. (A) Alignment of the peptides that were sequenced (grey boxes) or detected by mass fingerprint (underlined) with the deduced amino acid sequence of the $ISG75_{S\text{-}29\text{-}457\text{-}H}$. (B) Peptide fingerprints of the 52 kDa and 105 kDa bands.

conjugated anti-rabbit IgG (Jackson) were used at 1:1000 dilution.

3. Results

3.1. Protein expression

The expression and purification of the extracellular domain of ISG75 underwent several optimisation steps. Firstly, cytoplasmic expression of pTbG-$ISG75_{29\text{-}457\text{-}H}$ was tested in different strains of *E. coli*, including BL21 (DE3), C43 (DE3) and BL21 AI (Table 2). However, the $ISG75_{29\text{-}457\text{-}H}$ protein level was too low to be detected by Coomassie-stained SDS-PAGE. Secondly, attempts to express TbG-$ISG75_{29\text{-}457\text{-}H}$ in the periplasmic space, using the signal sequences from DsbA or PelB, were unsuccessful. Since $ISG75_{29\text{-}457\text{-}H}$ contained six cysteines that could engage in disulfide bonds, expression of pTbG-$ISG75_{29\text{-}457\text{-}H}$ in a reducing environment of the cytoplasm was not ideal. Therefore, in the third approach, a *trx*B *gor* double mutant strain of *E. coli*, Origami B (DE3), was selected as expression host. In addition, as the TbG-$ISG75_{29\text{-}457\text{-}H}$ gene contained several codons that are reported to be translated with low frequencies in *E. coli*, pRIL plasmid supplying the *arg*I, *ile*Y, and *leu*W tRNAs was co-transformed into Origami B (DE3). The $ISG75_{29\text{-}457\text{-}H}$ was well expressed in Origami B (DE3) in the presence of pRIL, since a protein band of the expected molecular weight of the soluble protein fractions of IPTG-induced cells was observed in Coomassie-stained SDS-PAGE and in immunoblot using anti-His tag monoclonal antibodies. The correctness of this protein band was subsequently confirmed by N-terminal amino acid sequencing. Purification of $ISG75_{29\text{-}457\text{-}H}$ however exhibited difficulties in obtaining the desired purity. We therefore incorporate an N-terminal Strep-tag to improve the purification (construct pTbG-$ISG75_{S\text{-}29\text{-}457\text{-}H}$). Origami B (DE3) containing pRIL displayed good expression of $ISG75_{S\text{-}29\text{-}457\text{-}H}$. Fig. 2 shows the cytoplasmic expression of soluble $ISG75_{S\text{-}29\text{-}457\text{-}H}$ in 1–5 h post-induction. Overnight incubation resulted in loss of the $ISG75_{S\text{-}29\text{-}457\text{-}H}$ due to degradation.

3.2. Double-tagged affinity purification

A reliable affinity purification protocol consisting of Strep-tactin affinity chromatography followed by Ni-NTA affinity chromatography was obtained (Fig. 3). Early purification of the $ISG75_{S\text{-}29\text{-}457\text{-}H}$ exhibited problems of purity, stability and solubility. Several buffer solution conditions were tested, such as buffer solutions under native or reducing condition (β-mercaptoethanol, 1,4-dithiothreitol (DTT), or containing detergents (Triton X-100, Tween 80, 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate). The addition of 50 mM L-glutamic acid and 50 mM L-arginine in the buffer solutions helped stabilise and solubilise the protein (Golovanov et al., 2004). Concentrating the purified $ISG75_{S\text{-}29\text{-}457\text{-}H}$ by centrifugation in a Vivaspin 10 K concentrator (Vivascience AG) resulted in protein aggregation. Interestingly, aggregation during concentration was avoided by lyophilisation. Approximately 0.5 mg of pure soluble $ISG75_{S\text{-}29\text{-}457\text{-}H}$ per liter of bacterial culture was obtained after purification.

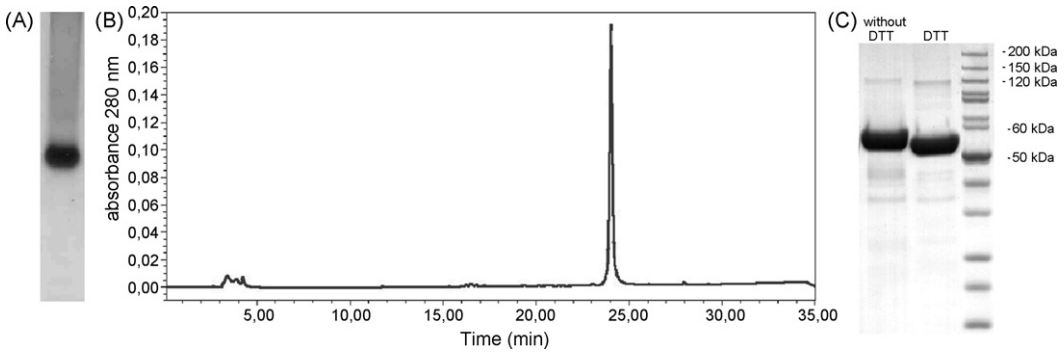

Fig. 5. Analysis of protein homogeneity and purity. (A) Native PAGE gel stained by Coomassie blue; (B) reverse-phase HPLC profile of ISG75$_{S\text{-}29\text{-}457\text{-}H}$ with absorbance of 280 nm over 35 min; (C) disulfide bonds analysis, the protein was incubated with 100 mM DTT for 1 h at room temperature before being applied to SDS-PAGE and Coomassie blue staining.

3.3. *Characterisation of the purified ISG75$_{S\text{-}29\text{-}457\text{-}H}$*

The identity of ISG75$_{S\text{-}29\text{-}457\text{-}H}$ of apparent 52 kDa was confirmed by N-terminal amino acid sequencing and micro-sequencing by mass spectrometry (Fig. 4A). A band at 105 kDa was consistently observed in the eluates of both the affinity chromatography steps. This band was therefore analysed by N-terminal amino acid sequencing and mass spectrometry. The N-terminal amino acid sequence and the peptide mass fingerprints of the 52 kDa and 105 kDa were identical, indicating that the 105 kDa protein band was the dimeric form of the ISG75$_{S\text{-}29\text{-}457\text{-}H}$ (Fig. 4B).

A molecular weight of 51377 Da was determined by mass spectrometry for the purified ISG75$_{S\text{-}29\text{-}457\text{-}H}$, while the predicted molecular weight of the truncate ISG75$_{S\text{-}29\text{-}457\text{-}H}$ is 51384 Da (ProtParam, Expasy Tools). SDS-PAGE analysis of the protein in the presence and absence of DTT revealed a shift in migration distance of the corresponding bands between the two samples, suggesting a change in compactness due to intramolecular disulfide bonds (Fig. 5C). The discrepancy of 7 Da between the experimental molecular mass and the theoretical mass of ISG75$_{S\text{-}29\text{-}457\text{-}H}$ further supported the occurrence of three disulfide bonds.

For downstream applications, the recombinant protein has to retain its integrity. Therefore, the purified ISG75$_{S\text{-}29\text{-}457\text{-}H}$, after being lyophilised, was analysed by native PAGE, reverse-phase HPLC and circular dichroism spectroscopy. Protein homogeneity was evident as a single band in Coomassie blue-stained native PAGE gel (Fig. 5A). Furthermore, a single elution peak in the reverse-phase HPLC profile indicated a high degree (99%) of purity (Fig. 5B). To assess the secondary structure conformation, the protein was analysed by circular dichroism spectroscopy. The ISG75$_{S\text{-}29\text{-}457\text{-}H}$ had a typical circular dichroism spectrum of an α-helical protein (Yang et al., 1986). It showed a double minimum at 224 nm and 208 nm, and a maximum at 193 nm (Fig. 6). Hence, the recombinant protein preserved its folded conformation at least at the secondary structure.

3.4. *ISG75$_{S\text{-}29\text{-}457\text{-}H}$ as a potential diagnostic tool*

A preliminary assessment of the diagnostic potential of the ISG75$_{S\text{-}29\text{-}457\text{-}H}$ was performed in ELISA on sera from goats infected with *T. b. brucei*. The protein was recognised by antibodies in sera from animals at 30 days post-infection, while reactions with sera from the animals before infection remained minimal (Fig. 7A). On the other hand, rabbit antiserum generated against the ISG75$_{S\text{-}29\text{-}457\text{-}H}$ specifically reacted with the detergent-soluble extracts and not with the water-soluble extracts of different antigenic type parasites, *T. b. brucei* AnTat 2.2, *T. b. gambiense* AnTat 9 1, *T. b. rhodesiense* AnTat 25 1S, *T. evansi* RoTat 1.2 and *T. equiperdum* OVI, as seen in immnoblot (Fig. 7B). Moreover, the anti-ISG75$_{S\text{-}29\text{-}457\text{-}H}$ rabbit antiserum displayed no cross-reactivity with *T. congolense* TRT17, procylic *T. b. brucei* AnTat 1 and VSG LiTat 1.3. These observations showed that the anti-ISG75$_{S\text{-}29\text{-}457\text{-}H}$ rabbit antiserum recognised the ISG75 of the bloodstream-form *Trypanozoon*.

4. Discussion

Sleeping sickness and animal trypanosomosis caused by the *Trypanozoon* subgenus are important diseases, especially in Africa. However, very limited knowledge about the trypanosome's invariant surface glycoproteins has been reported. Native ISG75 in a mixture with ISG64 and ISG65 were first isolated from the plasma membrane extracts of bloodstream-form *T. b. brucei* (Ziegelbauer and Overath, 1992). There are an estimated 5×10^4 ISG75 molecules on the surface of a bloodstream-form trypanosome. They seem to be hidden under the VSG coat and are not subjected to antigenic variation (Overath et al., 1994). Furthermore, the ISG75 gene family is conserved among the *Trypanozoon* (Tran et al., 2006). In this study, we express in *E. coli* the extracellular domain of *T. b. gambiense* ISG75 derived from group I of the gene family.

Due to its inexpensiveness and easy manipulation, *E. coli* is often the host of choice for heterologous protein expression. A number of *Kinetoplastida* proteins have been produced in *E. coli* for several purposes. Examples include functional characterisation of hypoxanthine-guanine phosphoribosyltransferase enzyme of *T. b. brucei* (Allen and Ullman, 1993), X-ray crystallographic analysis of oligopeptidase B from *T. b. brucei* (Rea et al., 2006), or application

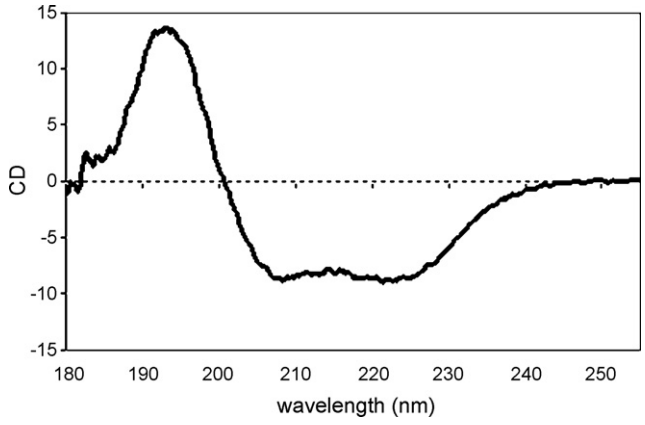

Fig. 6. The far-ultraviolet circular dichroism spectrum of ISG75$_{S\text{-}29\text{-}457\text{-}H}$. This profile with a double minimum at 224 nm and 208 nm, and a maximum at 193 nm is typical of an α-helical protein.

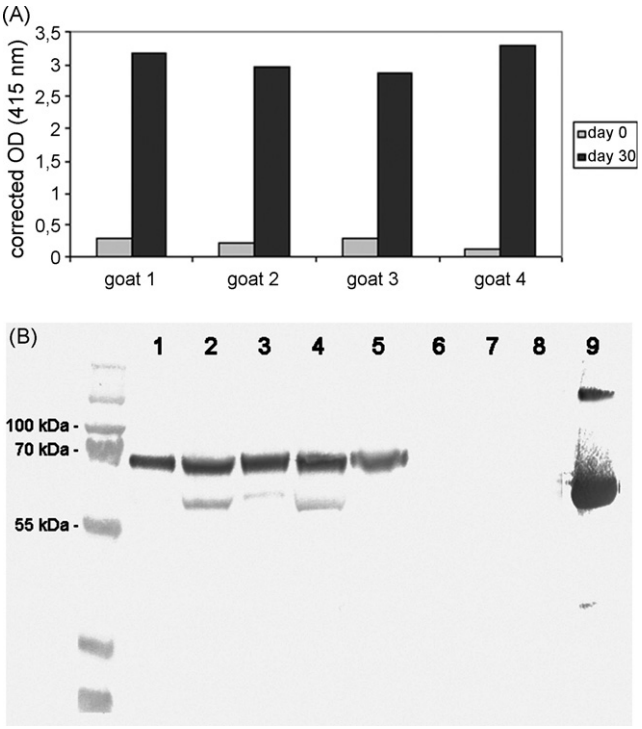

Fig. 7. Immunological properties of ISG75$_{S\text{-}29\text{-}457\text{-}H}$. (A) ELISA of ISG75$_{S\text{-}29\text{-}457\text{-}H}$ with sera from goats experimentally infected with *T. b. brucei*; (B) immunoblot of the detergent extracts of trypanosomes using anti-ISG75$_{S\text{-}29\text{-}457\text{-}H}$ rabbit antiserum. Lanes 1–5, bloodstream forms of *T. b. brucei* AnTat 2.2, *T. b. gambiense* AnTat 9 1, *T. b. rhodesiense* AnTat 25 1S, *T. evansi* RoTat 1.2 and *T. equiperdum* OVI, respectively; lane 6, procyclic *T. b. brucei* AnTat 1; lane 7, bloodstream form of *T. congolense* TRT17; lane 8: VSG LiTat 1.3; lane 9, ISG75$_{S\text{-}29\text{-}457\text{-}H}$.

of calflagin from *Leismania major* in diagnosis of *Trypanosoma cruzi* and *Leishmania* sp. infections (Marcipar et al., 2005).

Several fine-tuning measures have been considered during the protein purification. The construct initially contained only a C-terminal His-tag (pTbG-ISG75$_{29\text{-}457\text{-}H}$). Purification of ISG75$_{29\text{-}457\text{-}H}$ was performed using Ni-NTA affinity chromatography in combination with various methods, including anion exchange, cation exchange, hydrophobic interaction and size-exclusion chromatography. However, it was difficult to obtain the ISG75$_{29\text{-}457\text{-}H}$ at desirable purity and quantity. Therefore, an N-terminal Strep-tag was introduced to improve purification. With this double-tagged construct, we obtained highly pure soluble ISG75$_{S\text{-}29\text{-}457\text{-}H}$. It is essential that the aggregation suppressors, L-glutamic acid (Glu) and L-arginine (Arg), are included in purification buffer solutions. Glu and Arg appear to help reduce protein aggregation to some extent, possibly because of the interactions between the charged amino acids and the protein, and hence to stabilise the protein (Golovanov et al., 2004). It may be assumed that protein aggregation during concentrating by centrifugation is caused by surface polarisation with protein destabilisation as a consequence. On the other hand, lyophilisation can be used as a means to concentrate the protein. The lyophilised powder is easily stored and dissolved in any type of buffer solution.

Disulfide bonds play a crucial role in protein folding and stability, as successful expression of this recombinant protein requires an oxidative environment that is supplied by the cytoplasm of the Origami B (DE3) strain. The addition of a reducing agent DTT and mass spectrometry analyses confirm the existence of three disulfide bonds in the extracellular domain of ISG75. Since the positions of all six cysteines are well conserved in all sequences of the ISG75 gene family (Tran et al., 2006), it suggests that all three disulfide bonds will also be formed on the native ISG75 proteins. The consistent presence of a small proportion of dimer, even at low protein concentrations (such as 0.2 mg ml^{-1}) and in the presence of DTT, indicates that other types of interactions are responsible for dimerisation. It is tempting to speculate that ISG75 might occur as a dimer on the surface of trypanosomes.

It is crucial for downstream applications that the purified ISG75$_{S\text{-}29\text{-}457\text{-}H}$ retains its structural and immunological integrity. The analyses by native PAGE and reverse-phase HPLC show a high degree of protein homogeneity and purity. The protein maintains its folded conformation at least at secondary structure as assessed by circular dichroism spectroscopy. The production of homogeneous soluble folded extracellular domain of ISG75 is a requirement for study of three-dimensional structure, which will offer better understanding of the parasite's surface that has direct interactions with the host environment. Furthermore, we have shown that the ISG75$_{S\text{-}29\text{-}457\text{-}H}$ derived from one *Trypanozoon* subspecies specifically react with sera from goats infected with another *Trypanozoon* subspecies member. In addition, antibodies raised against this recombinant protein readily recognise the native ISG75 from parasite preparations of the *Trypanozoon* subgenus. This recombinant ISG75 and ISG75-binding molecules are thus of relevance for immunological studies as well as for development of novel antigen, parasite and antibody detection tests for all species and subspecies of the *Trypanozoon* subgenus.

In conclusion, we describe an optimised and standardised procedure for the expression and purification of the extracellular domain of ISG75 in Origami B (DE3). The protein was characterised and showed that it is readily applicable in diagnosis and immunological studies.

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