On *Blastocystis* secreted cysteine proteases: a legumain-activated cathepsin B increases paracellular permeability of intestinal Caco-2 cell monolayers

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SUMMARY

Blastocystis spp. pathogenic potential remains unclear as these anaerobic parasitic protozoa are frequently isolated from stools of both symptomatic and asymptomatic subjects. *In silico* analysis of the whole genome sequence of *Blastocystis* subtype 7 revealed the presence of numerous proteolytic enzymes including cysteine proteases predicted to be secreted. To assess the potential impact of proteases on intestinal cells and gut function, we focused our study on two cysteine proteases, a legumain and a cathepsin B, which were previously identified in *Blastocystis* subtype 7 culture supernatants. Both cysteine proteases were produced as active recombinant proteins. Activation of the recombinant legumain was shown to be autocatalytic and triggered by acidic pH, whereas proteolytic activity of the recombinant cathepsin B was only recorded after co-incubation with the legumain. We then measured the diffusion of 4-kDa FITC-labelled dextran across Caco-2 cell monolayers following exposition to either *Blastocystis* culture supernatants or each recombinant protease. Both *Blastocystis* culture supernatants and recombinant activated cathepsin B induced an increase of Caco-2 cell monolayer permeability, and this effect was significantly inhibited by E-64, a specific cysteine protease inhibitor. Our results suggest that cathepsin B might play a role in pathogenesis of *Blastocystis* by increasing intestinal cell permeability.

Key words: *Blastocystis*, cysteine proteases, Cathepsin B, Legumain, Caco-2, permeability.

INTRODUCTION

Blastocystis spp. are the most prevalent intestinal parasites recovered from human stool samples. At least 17 subtypes (STs) of these anaerobic unicellular eukaryotes have been described from a wide range of animal hosts, nine of which (ST1-ST9) have been found in human (Tan, 2008; Wawrzyniak *et al*. 2013). Their pathogenic potential is still unclear since these parasites can be isolated from stools of both symptomatic and asymptomatic patients. In symptomatic subjects, blastocystosis may be associated with abdominal pain, diarrhoea, nausea and bloating, a pattern of symptoms similar to that of patients suffering from irritable bowel syndrome (IBS). Interestingly, although it is still debated, a higher prevalence of *Blastocystis* was reported in IBS cohorts (reviewed in Poirier *et al*. 2012; Nourrisson *et al*. 2014). IBS is a chronic functional gastrointestinal disorder of multifactorial origin including cellular abnormalities with a low-grade inflammation (Chadwick *et al*. 2002), dysregulation of the brain-gut axis, alterations of the intestinal microbiota (dysbiosis) and increased intestinal permeability (Mayer *et al*. 2014; Ohman *et al*. 2015). On the basis of the *Blastocystis* ST7 genomic data, we recently proposed a hypothetical model deciphering the mechanisms of pathogenicity and the potential role of secreted proteases in the pathophysiology of *Blastocystis* infections (Poirier *et al*. 2012). Indeed, cysteine proteases are well-known to play key roles in the pathogenesis of several parasite diseases (Que and Reed, 2000; Sajid *et al*. 2003; Mottram *et al*. 2004; McKerrow *et al*. 2006). In *Blastocystis*, high levels of cysteine protease activity from both parasitic live cells and cell lysates were reported to be associated to Interleukin-8 production and modification of gut epithelial permeability (Sio *et al*. 2006; Puthia *et al*. 2008; Mirza *et al*. 2012; Wu *et al*. 2014). Analysis of the *Blastocystis*

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ST7 whole genome sequence revealed the presence of 29 cysteine proteases predicted to be secreted, including 16 members from the C1 family, 11 from the C13 family, 1 that belongs to the C39 family and 1 to the C45 family according to the MEROPS Database (https://merops.sanger.ac.uk). Among these predicted secreted cysteine proteases, a legumain (accession number: CBK21815·2) and a cathepsin B-like proteases (accession number: CBK25506·2) were identified by mass spectrometry from *Blastocystis* ST7 culture supernatant (Bcs) (Wawrzyniak *et al*. 2012). Legumains belong to the C13 protease family and were described in some plants and animals (Rawlings *et al*. 2014). Their activation is triggered by acidic pH and appears to be autocatalytic. Legumains specifically cleave asparaginyl bonds, promoting activation of other proenzymes into their active forms (Dalton *et al*. 2009). In contrast, cathepsin B-like cysteine peptidases belong to the C1 family. They possess both exo- and endopeptidase activities and their structure is based on 2 polypeptide chains linked by disulfide bonds. Cathepsins are synthesized as zymogens and endoproteolytic cleavage of their propeptide is a prerequisite for their activation. In helminth parasites, removal of the cathepsin B prosegment would be done by an asparaginyl endopeptidase (i.e. legumain) (Sajid *et al*. 2003; Beckham *et al*. 2006; Dalton *et al*. 2009). The *Blastocystis* legumain is a protein of 45·2 kDa (397 amino acids) in size containing several asparagine residues at the carboxy-terminal region that may constitute potential cleavage sites for autocatalytic activation. This protease was previously reported to be located at the parasite cell surface and shown to be active in the parasite culture supernatants (Wu *et al*. 2010; Wawrzyniak *et al*. 2012). The cathepsin B, also found in the culture supernatant, is a 35·7 kDa (320 aa) proenzyme with a C-terminal propeptide of 50 aa (Wawrzyniak *et al*. 2012). This propeptide contains a 20 aa conserved sequence corresponding to an occluding loop with terminal asparaginyl residues. Wawrzyniak *et al*. hypothesized that this domain may represent a target for other proteases such as legumains (Wawrzyniak *et al*. 2012). The aim of the present study was (i) to investigate the potential activation of the cathepsin B by the legumain and (ii) to clarify the role of these two secreted cysteine proteases as potential virulence factors for *Blastocystis* ST7. We first characterized specific legumain and cathepsin B proteolytic activities from *Blastocystis* ST7 supernatants (Bcs). Both legumain and cathepsin B were also cloned and produced as active recombinant proteins, and conditions allowing their maximal proteolytic activity were determined (Wawrzyniak *et al*. 2012). Finally, we explored the effects of ST7 Bcs and each recombinant protease on paracellular permeability of Caco-2 cell monolayer.

MATERIALS AND METHODS

Blastocystis *ST7 culture and preparation of culture supernatants*

Blastocystis subtype 7 (ST7, strain B) used in this study was isolated from the stools of a symptomatic singaporean patient and recently sequenced (Mirza and Tan, 2009; Denoeud *et al*. 2011). Parasites were grown in axenic culture using pre-reduced Iscove's Modified Dulbecco's Medium (IMDM, Gibco), supplemented with 10% horse serum (PAA, Pasching, Austria), 1 $\mu g\,mL^{-1}$ of glutamine (Gibco) and 1 $U\,mL^{-1}$ of Penicillin/Streptomycin (Gibco) (Ho *et al*. 1993). Cultures were incubated at 37 °C under anaerobic conditions using the AnaeroJar system (Oxoid, Thermo Fisher Scientific). *Blastocystis* culture supernatants (Bcs) were collected from 2-days-old cultures by centrifugation at 1000 ***g*** for 10 min. Bcs were filtrated through 0·22 μm-membranes (Millipore) and applied on Amicon Ultra (Millipore) at 4000 ***g*** during 40 min at 4 °C to obtain a 10-fold concentrated solution of proteins with molecular weights higher than 3 kDa. Such concentrated Bcs were stored at −80 °C until used.

Protease assays

Total protease activity of Bcs was measured using azocasein assay as previously described (Wawrzyniak *et al*. 2012). One azocasein unit was defined as the amount of proteolytic enzyme producing an increase of 0·01 optical density units per hour. Specific protease activities of Bcs were carried out at 37 °C using the fluorometric substrates Z-Ala-Ala-Asn-AMC (100 μM) (Sigma-Aldrich) for legumain and Nα-CBZ-Arg-Arg-7-AMC (20 μM) (Bachem) for cathepsin B according to the manufacturer's recommendations. Activities were measured after 5 and 30 min of incubation with each fluorometric substrate and were expressed in relative fluorescence units (RFU). The fluorescent hydrolysis products were quantified with excitation and emission wavelengths of 355 nm and 460 nm, respectively, using a Fluoroskan Ascent FL (Thermo Lab Systems). Protease assays were also performed for recombinant *Blastocystis* cysteine proteases expressed in *Escherichia coli*. Specific enzymatic activity of recombinant cysteine proteases was measured with fluorometric substrates as described above and expressed in U/mg after protein quantification using Bradford assay (Coomassie Plus™ Protein Assay Reagent, Thermo Scientific). Proteolytic activities of both legumain and cathepsin B were measured for pH ranging from 2 to 8 using citrate buffer (50 mM). A follow-up from 0 to 11 h was performed to determine the duration of co-incubation of legumain with citrate buffer providing the highest specific activity. The activated-legumain

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(with the highest specific activity) was then incubated with the recombinant cathepsin B. For protease inhibition assays, the cysteine protease inhibitor trans-epoxy-succinyl-L-leucylamido-(4-guanidino)-butane (E-64, Sigma-Aldrich) was pre-incubated with either Bcs or each recombinant cysteine protease at a 50 μM final concentration during 1 h at 37 °C.

Culture of Caco-2 cells

The human colon adenocarcinoma-derived cell line Caco-2 grows as cell monolayer with spontaneous differentiation and displays morphological and functional characteristics of mature enterocytes: polarized morphology with microvilli at the apical surface, tight junctions between adjacent cells, ability to secrete cytokines (Sambuy *et al*. 2005). Caco-2 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco), containing 10% fetal bovine serum (Gibco), 1 μg mL^{-1} of glutamine (Gibco) and 1 U mL^{-1} of Penicillin/Streptomycin (Gibco). Cells were grown in T-25 flasks in an incubator with 5% CO_2 at 37 °C. For cytotoxicity assays, Caco-2 cells were seeded on standard 24-well culture plates (Millipore) at a density of 1×10^5 cm^{-2} and cultured until they reach confluence. For epithelial cell permeability experiments, Caco-2 cells were grown on Millipore transwell inserts (12-Well Millicell) with Polyethylene terephthalate membranes of 1·0 μm pore size placed in 12-well culture plates and seeded at a density of 1×10^5 cm^{-2}. Transepithelial electrical resistances (TEER) of Caco-2 monolayers were measured with the epithelial volt-ohm meter EVOM2 (WPI, Stevenage, UK). Only cell monolayers with TEER values over 250 Ω cm^{-2} were used.

Cytotoxicity assays

Caco-2 cells were co-incubated with 5, 10 or 20% (v/v) of ST7 Bcs or each recombinant protease in DMEM for 48 h in the presence or absence of 50 μM of the protease inhibitor E-64. Each concentration was tested in triplicate. Lactate deshydrogenase (LDH) assays were performed using the *In Vitro* Toxicology Assay Kit, LDH based (Sigma-Aldrich) and LDH concentrations were measured using a Multiskan™ FC Microplate Photometer (Thermo Scientific) at 490 nm according to the manufacturer's recommendations. A concentration of 20% of Dimethyl Sulfoxyde (DMSO) was used as positive control for cytotoxicity.

Recombinant proteases were also tested for the presence of lipopolysaccharide (LPS) before cellular assays using the Pierce® LAL Chromogenic Endotoxin Quantitation Kit (Thermo Scientific) according to the manufacturer's recommendations. Each condition was tested in triplicate.

Paracellular permeability of Caco-2 cell monolayer

Differentiated Caco-2 cells were co-incubated with 10% (v/v in DMEM) of either Bcs or recombinant cysteine proteases (legumain-specific activity: 9·89 ± 0·42 mU mg^{-1}; activated cathepsin B-specific activity: 2·42 ± 0·34 mU mg^{-1}; non-activated cathepsin B-specific activity: 0·15 ± 0·30 mU mg^{-1}) with or without 50 μM of E-64 (Sigma-Aldrich). Each condition was tested in quadruplicate. Diffusion assays were performed by addition of a 0·4 mM final concentration of 4-kDa FITC-conjugated Dextran (FD4) (Sigma-Aldrich) in the apical compartment containing Bcs or recombinant proteases. The diffusion of FD4 across the Caco-2 monolayer was measured in the basolateral compartment after 24, 48 and 72 h of co-incubation. FD4 fluorescence measurement was performed using a Multiskan™ FC Microplate Photometer (Thermo Scientific) at excitation and emission wavelengths of 485 nm and 538 nm, respectively.

Production of recombinant cysteine proteases in E. coli

The *Blastocystis* legumain (accession number: CBK21815·2) and cathepsin B (accession number: CBK25506·2) encoding genes were amplified and cloned in pET23b(+) expression vector (Invitrogen®) for production of these cysteine proteases in *E. coli*. Polymerase chain reaction (PCR) primers were designed to amplify the full sequences of the legumain (LegCBK21815·2-Fw: 5′- CGC GGATCCAGATAACTGGGCCGTGCTTGTT GCC-3′ and LegCBK21815·2-Rv: 5′-CGCCT CGAGATGATGGAAAGCACGAGGG-3′) and the cathepsin B (CatCBK25506·2-Fw: 5′-CGC GGATCCACACCCTCCCAGACTCGTC-3′ and CatCBK25506·2-Rv: 5′-CCGCTCGAGCAGCTC AGGAATTCCAGCAACACC-3′) without the region encoding their N-terminal signal peptide. A truncated form of the cathepsin B lacking the pro-segment (49 first amino acids) was also amplified using the primers CatProCBK25506·2-Fw: 5′- CGCGGATCCAATTGCTATTCGTG GTGAT-3′ and CatCBK25506·2-Rv. In all cases, forward and reverse primers contained one *Bam*HI and one *Xho*I restriction site at their 5′ end, respectively. PCR reactions were performed according to standard conditions (High-Fidelity DNA polymerase) with an annealing step at 54 °C. PCR products were then cloned into the pET23b(+) expression vector (Invitrogen®) and recombinant plasmids were sequenced (MWG, Germany). Each recombinant plasmid was introduced into *E. coli* BL21 Star strain (Invitrogen®) and protein production was induced with 0·5 mM IPTG and bacteria were grown overnight at 16 °C. After centrifugation at 4000 ***g*** for 10 min at 4 °C, cells were disrupted by sonication at 4 °C in a lysis buffer containing 50

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mM NaH_2PO_4 pH 8, 300 mM NaCl, 5 mM EDTA and 1 mg mL^{-1} lysozyme. Protein pellets were washed 3 times in a buffer containing 50 mM NaH_2PO_4 pH 8, 300 mM NaCl, 5 mM EDTA and 3 M. Recombinant proteins were solubilized under denaturing conditions in urea buffer (8 M urea, 50 mM NaH_2PO_4 pH 8, 300 mM NaCl) for 30 min at 4 °C under agitation. Protein refolding was performed by dilution (1/20 v/v) into a renaturation buffer containing glutathione (2 mM GSH/0·4 mM GSSG) during 24 h at 4 °C. Recombinant cysteine proteases were purified using nickel-charged resin (Ni-NTA Agarose, Qiagen) according to the manufacturer's recommendations. Elution was done at 4 °C in 50 mM NaH_2PO_4, 300 mM NaCl pH 8 containing 500 mM imidazole. Imidazole was then removed by centrifugation on Amicon® Ultra 10 K (Millipore) at 4 °C. Protein purity was assessed by 10% SDS-polyacrylamide gel electrophoresis. Bradford protein assay was performed to evaluate the amount of each purified recombinant protein. Purified proteases were tested for endotoxins as described above. Recombinant legumain was activated by incubation in citrate buffer pH 4 at 37 °C during 4 h. Recombinant cathepsin B was incubated in a citrate buffer pH 4 at 37 °C overnight, with or without the pre-activated recombinant legumain.

Production of anti-legumain and anti-cathepsin B polyclonal antibodies

Polyclonal antibodies directed against legumain (CBK21815·2) and cathepsin B (CBK25506·2) were produced in BALB/c mice by intra-peritoneal injection of purified recombinant proteases homogenized with Freund's complete adjuvant or the first injection. Four additional injections were performed at days 14, 21, 28 and 35 with Freund's incomplete adjuvant. Mice were sacrificed 1 week after the last injection and collected sera were stored at -20 °C until used. The animal facility (agreement D631014·5) and the experimental staff (agreement A63-223) were approved by the French Veterinary Service. The experiments were conducted according to ethical rules.

Western-blotting

Recombinant cysteine proteases and total proteins from ST7 Bcs were analysed by 10% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore). The membranes were blocked with 3% (w/v) bovine serum albumin in PBS/0·1% Triton X-100 and incubated overnight at 4 °C with either anti-legumain or anti-cathepsin B polyclonal sera diluted at 1:500 and 1:200, respectively. After 3 washes with PBS/0·1% Triton X-100, blots were probed with HRP-conjugated goat anti-mouse-IgG at 1:7500 (Promega). Revelation was performed using ECL kit (Advanced Bio Design) according to the manufacturer's recommendations.

Statistical analyses

P-values were determined by Student's *t*-test. *P*-values of 0·05 or below were considered as significant (bilateral).

RESULTS

Cysteine protease activities in the ST7 Blastocystis *culture supernatants (Bcs)*

The whole proteolytic activity of ST7 Bcs was measured using the azocasein assay as previously described (Wawrzyniak *et al*. 2012). The activity reached 57·9 ± 14·1 azocasein units at pH 6 after incubation for 1 h at 37 °C. Addition of the cysteine protease inhibitor E-64 significantly decreased this proteolytic activity to 15·3 ± 6·0 azocasein units (73·6% decreased; $P = 0·013$) and no proteolytic activity could be detected when Bcs were boiled (Fig. 1A). Specific activities of both legumain and cathepsin B were also measured in Bcs using the fluorometric substrates Z-Ala-Ala-Asn-AMC and Nα-CBZ-Arg-Arg-7-AMC, respectively. Results were expressed in RFU. Bcs exhibited a legumain activity of 181·8 ± 6·1 RFU (Fig. 1B) and a cathepsin B activity of 183·0 ± 31·6 RFU (Fig. 1C). Cysteine protease inhibitor E-64 significantly decreased specific activity of cathepsin B (1·9 ± 0·2 RFU; $P = 0·001$) (Fig. 1C) whereas legumain activity remained unchanged (208·0 ± 5·0 RFU; $P = 0·236$) (Fig. 1B). As expected, specific activities of both proteases were inhibited when Bcs were boiled. All assays were performed in quadruplicate.

Proteolytic activities of recombinant cysteine proteases expressed in E. coli

Recombinant legumain and cathepsin B proteases produced in *E. coli* formed inclusion bodies preventing their solubilization under native conditions. Protein aggregates were thus solubilized in denaturing conditions with 8 M urea and purified recombinant proteases were then refolded. SDS-PAGE analysis revealed bands of the expected sizes at ~40 kDa for the recombinant legumain ~33 kDa for the recombinant cathepsin B and ~28 kDa for the recombinant cathepsin B without its pro-segment (data not shown). The maximal specific activity of the purified recombinant legumain (30·1 ± 0·4 mU mg^{-1}) was obtained at pH 4 (Fig. 2A) and this activity was significantly decreased by E-64 ($P < 0·001$; Fig. 2B). We then determined the effects of incubation time on proteolytic activity. As shown in Fig. 2C, the maximal activity of the

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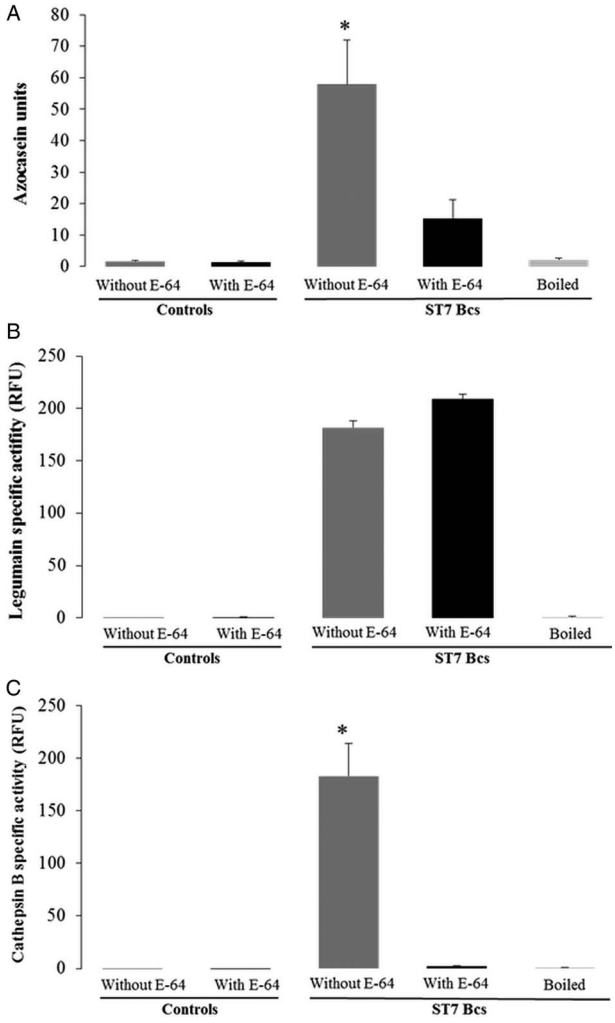

Fig. 1. Protease activity assays from *Blastocystis* ST7 culture supernatants (Bcs). (A) Azocasein assay represents total proteolytic activity of ST7 Bcs. Controls correspond to measurement of protease activities in IMDM medium. (B, C) Proteolytic activity assays were performed using fluorometric substrates specific of legumain (100 μM Z-Ala-Ala-Asn-AMC, B) or cathepsin B (20 μM Nα-CBZ-Arg-Arg-7-AMC, C). Specific protease activities were measured after 5 min of incubation with each substrate and were expressed in RFU because the amount of each cysteine protease secreted in Bcs was undetermined. Inhibition assays were performed using E-64 (50 μM final concentration), which is a specific inhibitor of cysteine proteases but known to have low effect on legumain activity. Protease activity of boiled Bcs was also measured. Bars represent standard errors of the mean (all assays were performed in quadruplicate), $*P < 0{\cdot}05$. IMDM, Iscove's Modified Dulbecco's Medium; RFU, relative fluorescence units.

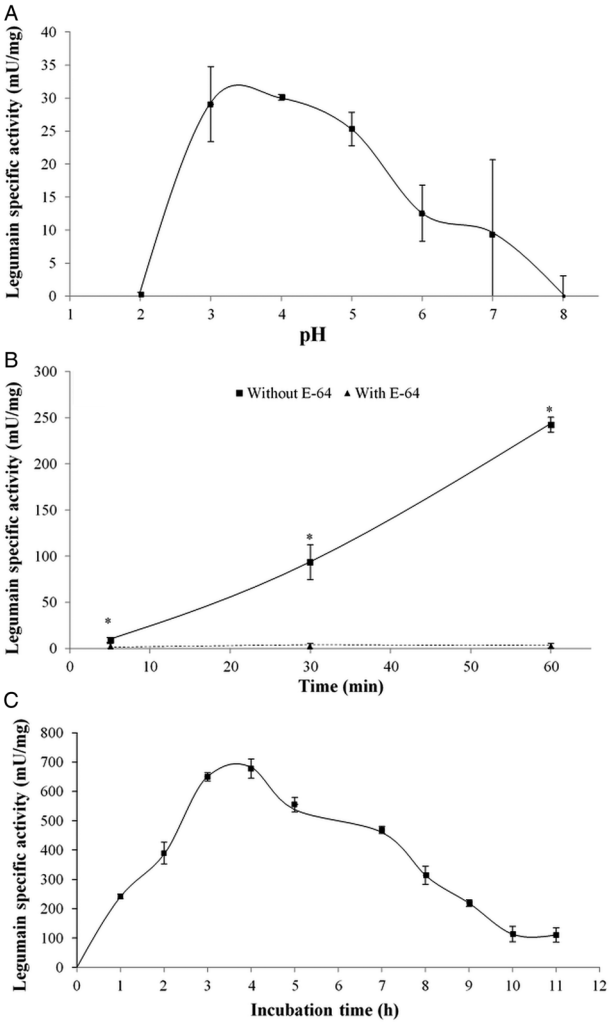

Fig. 2. Optimal pH and time of incubation for proteolytic activity of the recombinant legumain expressed in *E. coli*. (A) Legumain was screened for its proteolytic activity at 37 °C after incubation for 5 min with 50% (v/v) citrate buffers adjusted at different pH (pH 2 to pH 8) using substrate Z-Ala-Ala-Asn-AMC (100 μM). (B) Inhibitory effect of E-64 (50 μM) on specific legumain activity at pH 4 was evaluated at 5–30–60 min after co-incubation with the inhibitor during 1 h at 37 °C. (C) Legumain was incubated at 37 °C pH 4 for 11 h and its proteolytic activity was measured every hour. Bars represent standard errors of the mean (each condition was performed in triplicate), $*P < 0{\cdot}05$.

legumain was achieved after 4 h of incubation at 37 °C ($678{\cdot}0 \pm 32{\cdot}4$ mU mg^{-1}). Specific activity of the recombinant cathepsin B was also measured in the pH range 3–8 and from 1 h to overnight incubation at 37 °C. However, a low activity was only recorded ($1{\cdot}00 \pm 0{\cdot}07$ mU mg^{-1}) after an overnight incubation at pH 4 (Fig. 3). The recombinant cathepsin B was then co-incubated overnight with the pre-activated recombinant legumain (the pre-activation of the legumain was done by incubation for 4 h at pH 4). A significant increase of the specific cathepsin B activity ($5{\cdot}0 \pm 0{\cdot}4$ mU mg^{-1}; $P = 0{\cdot}005$) was observed whereas controls using only pre-activated legumain remained negative (Fig. 3). Cathepsin B activity was completely inhibited by E-64 ($0{\cdot}01 \pm 0{\cdot}07$ mU mg^{-1}; $P = 0{\cdot}005$) (Fig. 3). All assays were performed in triplicate. *In silico* analyses of the *Blastocystis* ST7 genome predicted the secretion of the cathepsin B as inactive zymogen.

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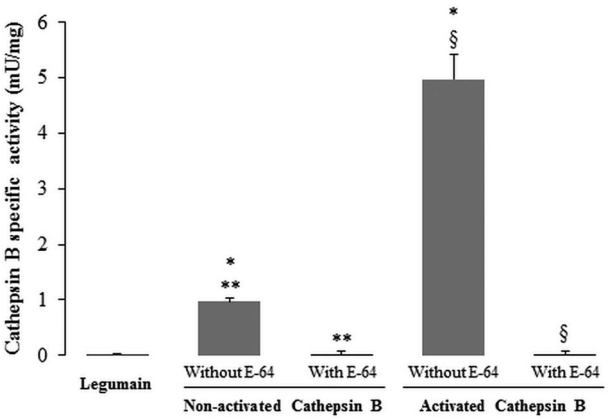

Fig. 3. Proteolytic activity of the recombinant cathepsin B before and after co-incubation with the pre-activated recombinant legumain. Cathepsin B was tested for specific activity in acidic environment (pH 4) using substrate Nα-CBZ-Arg-Arg-7-AMC (20 μM). Activated cathepsin B corresponded to the proteolytic activity when this protease was co-incubated overnight with the activated recombinant legumain. Proteolytic activity was inhibited by addition of E-64 (50 μM). Bars represent standard errors of the mean (all assays were performed in triplicate), *, **, §$P < 0{\cdot}05$.

We thus hypothesized that cathepsin B could be activated by legumain through proteolytic cleavage of the ~7 kDa predicted pro-segment. Cleavage of this pro-segment should induce a mobility shift on SDS-PAGE when compared with the full-length form of the recombinant cathepsin B. However, any difference in size of the recombinant cathepsin B was detected on Western blot before and after co-incubation with the activated legumain (data not shown). A recombinant cathepsin B was also produced without its predicted pro-segment (aa 1–49), but no specific cathepsin B activity was recorded with or without co-incubation with the activated legumain (data not shown).

Effects of cysteine proteases on paracellular permeability of Caco-2 cell monolayer

Cytotoxic assays on Caco-2 cell line were first performed with different amounts of ST7 Bcs from 5 to 20% (v/v in Caco-2 cell culture medium) and activated recombinant cysteine proteases (legumain-specific activity: $9{\cdot}89 \pm 0{\cdot}42$ mU mg^{-1}; activated cathepsin B-specific activity: $2{\cdot}42 \pm 0{\cdot}34$ mU mg^{-1}; non-activated cathepsin B-specific activity: $0{\cdot}15 \pm 0{\cdot}30$ mU mg^{-1}). As shown in Fig. 4, a significant increase of 4-kDa FITC-dextran (FD4) diffusion from the apical to the basal compartment ($10{\cdot}31 \pm 1{\cdot}14$ μg mL^{-1}) was observed after incubation with 10% ST7 Bcs when compared with the control ($4{\cdot}61 \pm 1{\cdot}43$ μg mL^{-1}; $P = 0{\cdot}030$). This effect was significantly disrupted when Bcs was pre-incubated with E-64 ($6{\cdot}25 \pm 0{\cdot}42$ μg mL^{-1}; $P = 0{\cdot}043$) (Fig. 4), the diffusion of FD4 being comparable to that of the control ($P = 0{\cdot}230$). The effect of each recombinant cysteine protease was also tested on epithelial permeability. Non-activated recombinant cathepsin B did not significantly increased FD4 diffusion across Caco-2 cell monolayer ($4{\cdot}79 \pm 0{\cdot}17$ μg mL^{-1}; $P = 0{\cdot}301$) contrary to the legumain pre-activated cathepsin B ($12{\cdot}67 \pm 0{\cdot}40$; $P = 0{\cdot}003$) (Fig. 4). E-64 significantly decreased FD4 diffusion induced by the pre-activated cathepsin B ($9{\cdot}26 \pm 0{\cdot}36$ μg mL^{-1}; $P = 0{\cdot}016$) but remained significantly higher than the control ($P = 0{\cdot}011$). In contrast, incubation of Caco-2 cells with the active recombinant legumain alone did not alter epithelial permeability (FD4 diffusion: $5{\cdot}92 \pm 0{\cdot}78$ μg mL^{-1}; $P = 0{\cdot}197$) (Fig. 4).

DISCUSSION

Proteases are well-known to play key roles in various fundamental biological processes and particularly in host–pathogen interactions. These proteolytic enzymes may facilitate penetration and spread of pathogens in host tissues, degradation of host proteins for nutrition and may be involved in the immunomodulation of the host immune system (McKerrow *et al*. 2006). Thus, proteases were proposed as virulence factors for some parasitic protozoa. For example, in *Entamoeba* spp., cysteine proteases play an important role in virulence as their production and extracellular release are 10- to 1000-fold more elevated from *Entamoeba histolytica* than from the non-pathogenic *Entamoeba dispar* (Que and Reed, 2000). Cysteine proteases are also considered as potential therapeutic or vaccine targets. Inhibition of falcipain (another member of the C1 cysteine protease family) was shown to provide antimalarial effect whereas intra-nasal immunization of mice with cysteine proteases of *Trichomonas vaginalis* conferred protection against the parasite (Hernandez *et al*. 2005; Rosenthal, 2011). Pathogenicity of *Blastocystis* spp. is still unclear, however accumulation of evidences suggests virulence capacities (Poirier *et al*. 2012). *In silico* analysis of the *Blastocystis* ST7 genome revealed the presence of a large number of secreted proteases, including 29 cysteine proteases (Denoeud *et al*. 2011; Wawrzyniak *et al*. 2012). Cysteine proteases are the most represented proteolytic enzymes among proteases identified in both *Blastocystis* ST4 and ST7 genomes (Denoeud *et al*. 2011; Wawrzyniak *et al*. 2015). They were suggested to be implicated in IgAs degradation, inflammatory process, and disruption of Caco2 cell monolayer (Puthia *et al*. 2005, 2008; Mirza *et al*. 2012; Wu *et al*. 2014). The secretion of active cysteine proteases by *Blastocystis* ST7 was demonstrated through the identification of a legumain and a cathepsin B in parasite culture supernatant (Bcs) (Wawrzyniak *et al*. 2012). This legumain was

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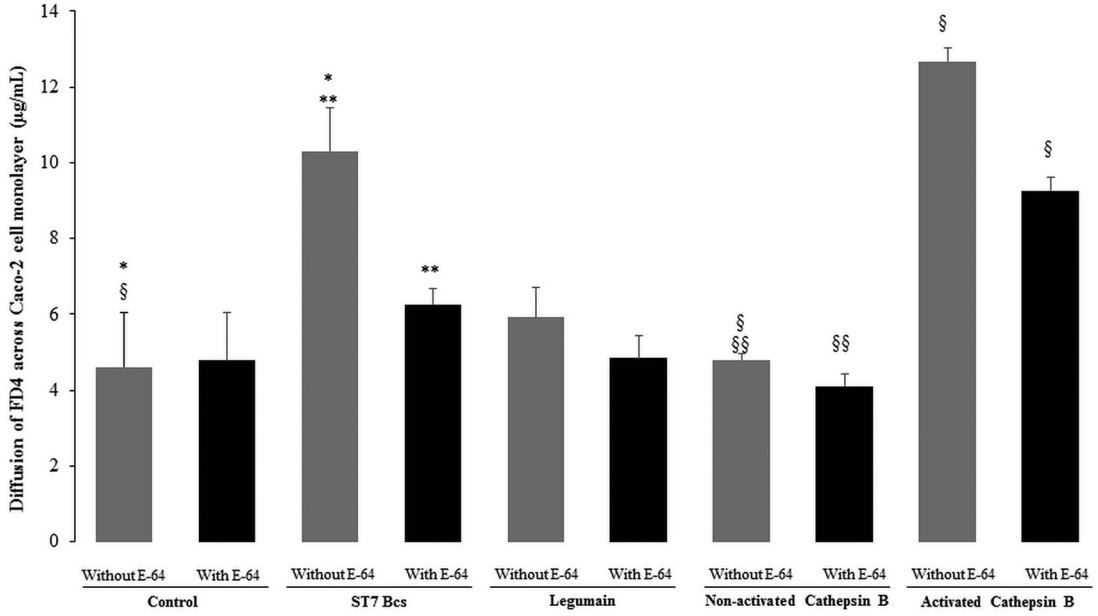

Fig. 4. Effect of cysteine proteases on the paracellular permeability of Caco-2 cell monolayer. Caco-2 cells were grown on transwell inserts and co-incubated with 10% (v/v in DMEM) of *Blastocystis* ST7 supernatant (ST7 Bcs) or with 10% (v/v in DMEM) of recombinant cysteine proteases (active legumain, non-activated cathepsin B and legumain-activated cathepsin B). Effects of Bcs and recombinant proteases were evaluated with or without pretreatment with E-64 (50 μM). Negative controls were made with DMEM. Variations in the paracellular permeability of Caco-2 cell monolayer were measured through the diffusion of 4-kDa FITC-dextran (FD4, 0·4 mM) across Caco-2 cell monolayer (i.e. from the apical to the basolateral compartment). Results present FD4 concentration (μg mL^{-1}) in the basolateral compartment after 24 h of co-incubation. Bars represent standard errors of the mean (all assays were performed in triplicate), *; **;§; §§$P < 0{\cdot}05$. DMEM, Dulbecco's Modified Eagle Medium.

previously identified by Wu *et al*. at the parasite cell surface and would play a pro-survival role in *Blastocystis* (Wu *et al*. 2010). All these studies have been performed from live parasite cells and/or using parasite cell lysates. However, no experiment was performed using *Blastocystis* cell culture supernatant, i.e. focusing on proteins released by the parasite that could be of importance during colonization of the gut. In our study, we analysed the proteolytic activity in Bcs and its impact on Caco-2 cell permeability. It remains difficult to evaluate the local concentration of secreted proteases in intestinal lumen as (i) *Blastocystis* probably creates a local microenvironment, and (ii) parasite load probably varies along the intestinal tract (Fayer *et al*. 2014). Bcs were initially filtrated and 10-fold concentrated to remove proteins with molecular weight lower than 3 kDa. For this reason, cell permeability experiments were done using 10% of concentrated Bcs. As incubation of Caco-2 cells with 10% of either Bcs or recombinant proteases did not show any toxicity (data not shown), we selected this concentration to evaluate their potential effect on the paracellular permeability of Caco-2 cell monolayer.

We measured proteolytic activity of Bcs and observed that it was highly inhibited by E-64, with a decreased activity of 73·6% (Fig. 1A), suggesting the release of many secreted cysteine proteases by the parasite. Indeed, E-64 is a potent inhibitor of many cysteine proteases, which acts through the formation of a thioether linkage with the thiol group of these proteases, but it fails to inhibit most of legumains (Chen *et al*. 2000). We demonstrated that Bcs had legumain and cathepsin B activities by using specific substrates, and that E-64 only inhibited cathepsin B specific activity (Fig. 1B and C). Both legumain and cathepsin B were then produced as recombinant proteins (Wawrzyniak *et al*. 2012). High level expression of these recombinant proteases resulted in their accumulation within inclusion bodies avoiding native extraction. This phenomenon could be linked to disulfide bond formation (Singh and Panda, 2005). Recombinant proteins were thus extracted in denaturing conditions using high concentration of urea (8 M) followed by refolding procedure. Most cysteine proteases are produced *in vivo* as inactive forms (zymogens) and their activation depends on particular conditions. For example, reducing agents such as L-cysteine and dithiothreitol, are able to increase their proteolytic activity (North, 1982). Cysteine proteases are mainly activated at acidic pH so that legumains auto-catalytic activation occurs more rapidly at low pH values (Chen *et al*. 2000). In our study, the highest specific activity for the recombinant legumain was observed at pH 4 as previously shown by Wu *et al*. (2010) and comparable to other recombinant cysteine proteases studied in the literature (Chen

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et al. 1997; Wu *et al*. 2010). As pH in the colon is much more elevated (around 6·5 for distal colon), we hypothesized that *Blastocystis* could create a local acidic microenvironment favourable for legumain activation. Activation of proteases synthesized as zymogens requires the proteolytic cleavage of a propeptide. Interestingly, we showed that the recombinant cathepsin B required to be co-incubated with the pre-activated recombinant legumain to become active. This legumain-dependent activation was previously described for helminth parasites: a two-step mechanism was proposed for *Schistosoma mansoni* for which legumain trans-activates a small number of cathepsin L zymogens which in turn are able to activate other cathepsins L (Dalton *et al*. 2009). This phenomenon was confirmed by a study in legumain-deficient mice that reported an abolishment of cathepsins processing (Shirahama-Noda *et al*. 2003). However, it is the first time that such mechanism is observed among protozoa. We hypothesized that processing and activation of the *Blastocystis* cathepsin B could be performed by the activated recombinant legumain. However, we were not able to highlight the mechanism of this processing. Our results suggest that it is not associated to an enzymatic cleavage of cathepsin B as we did not observe any shift in size in SDS-PAGE for the cathepsin B after incubation with the legumain. In order to verify whether cleavage of the propeptide was a prerequisite for cathepsin B activation, a recombinant cathepsin B lacking the propeptide was also produced in *E. coli* (not shown). This truncated form of the protease had a very low activity even though when co-incubated with legumain (data not shown), suggesting that (i) either the presence of the propeptide is required for the activation by the legumain (ii) or its absence is not sufficient to have active form (Wawrzyniak *et al*. 2012). The low activity obtained could refer to auto-activation at acidic pH. Consequently, other experiments are required to elucidate mechanisms involved in processing and activation of cathepsin B by the legumain. Proteolytic activity of both recombinant cathepsin B and legumain was inhibited by the protease inhibitor E-64 (Figs 2B and 3). In contrast, E-64 had no effect on the legumain specific activity measured in Bcs (Fig. 1B). Several reasons could explain this discordance. (i) We hypothesized that denaturation and refolding steps of the recombinant legumain may have induced modifications in the structural conformation of the protease when compared with the native legumain secreted by the parasite in the culture supernatant. Such modifications may have facilitated binding of E-64 to the protease active site. (ii) Genes coding for other legumain-like proteases predicted to be secreted have been annotated from the *Blastocystis* ST7 genome (Denoeud *et al*. 2011; Wawrzyniak *et al*. 2012) and unpublished data). So, the legumain-specific activity measured in Bcs could correspond to the proteolytic activity of multiple legumains and some of them could not be sensitive to E-64. This would explain the inability of the inhibitor to significantly reduce the total Bcs legumain activity.

Physiologic activity of *Blastocystis* secreted proteases on host cells has never been explored. Previous studies have suggested an increase of intestinal permeability associated with *Blastocystis* parasites cells and/or lysates, which could be due to proteases (Dagci *et al*. 2002; Puthia *et al*. 2006; Mirza *et al*. 2012). Modulation of intestinal epithelial barrier function is often a step in pathophysiology of many enteric pathogens (O'Hara and Buret, 2008). The regulation of epithelium paracellular permeability implicates the tight junctions (TJs) protein complex, which is essential for intestinal homeostasis. Therefore, disruption of TJ structure by enteric pathogens may have significant detrimental effects on intestinal function. Disruption of the TJs results in a water and electrolytes leak into the lumen. This may contribute to the diarrhea commonly associated with acute gastro-intestinal infections. In addition, loss of intestinal barrier function may allow luminal antigens diffusion to the submucosa leading to an activation of the host's immune system or enteric neurons. Increasing evidence also suggests that this may contribute to the development of chronic intestinal disorders like inflammatory bowel disease (IBD) and IBS. Interestingly, although it is still debated, several epidemiological surveys highlight a higher prevalence of *Blastocystis* spp. among patients suffering from IBS (reviewed in Nourrisson *et al*. 2014). In order to evaluate the potential impact of *Blastocystis* spp. on digestive epithelium, we incubated Bcs with intestinal Caco-2 cells which form monolayers mimicking an epithelial barrier with TJs. Addition of 10% Bcs induced an increase of 4-kDa FITC-dextran (FD4) diffusion through cell monolayer, without any cytotoxic effect, suggesting an increase in paracellular permeability. This effect was significantly decreased in presence of E-64 (diffusion decrease of 71·1%, see Fig. 4) suggesting that it was mostly due to cysteine proteases present in Bcs. We observed a significant increase of Caco-2 cell monolayer permeability with the recombinant cathepsin B but not with the recombinant legumain. E-64 significantly reduced the effect of the cathepsin B on Caco-2 cell monolayer confirming the role of this protease in paracellular permeability modifications. Although the direct impact of legumain on permeability seems to be limited, this protease would activate the cathepsin B as increase of cell permeability was higher and more significant when both recombinant cysteine proteases were combined. As indicated above, legumains have previously been shown to be implicated in virulence of *Blastocystis* spp. (Puthia *et al*. 2005, 2008; Wu *et al*. 2010). In

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contrast, there is no data concerning the role of cathepsin B in *Blastocystis* pathogenesis.

As amounts of Bcs used in our study were not cytotoxic, we hypothesized that the modification of Caco-2 cell permeability would involve disruption of the TJs complex. It is well described that ZO-1 has a central role in TJ complex and reorganizations of TJs complex implicate rearrangement of ZO-1. On a Caco-2 model, Mirza *et al*. showed that the rearrangement of ZO-1 induced by live *Blastocystis* ST7 was inhibited by E-64, suggesting the role of cysteine proteases (Mirza *et al*. 2012). In our study we identified two of the cysteine proteases involved in the mechanism of modification of epithelial permeability by *Blastocystis*. To our knowledge there is no data showing the specific impact of secreted cysteine proteases on cellular cohesion of an *in vitro* model. It would be of interest to search for cellular receptor(s), which could be activated by these proteases and which pathways could be implicated. It has been suggested that cysteine proteases of *Blastocystis* induce rho kinase (ROCK)-dependent disruption of intestinal epithelial barrier function with reorganization of cytoskeletal F-actin and tight junctional ZO-1 (Mirza *et al*. 2012). Caspase 3- and 9-dependant enterocyte apoptosis could also play a role in *Blastocystis*-mediated epithelial barrier compromise (Wu *et al*. 2014).

To conclude, we described for the first time a work in tandem for legumain and cathepsin B of *Blastocystis* ST7, although the accurate mechanism of cathepsin B activation still remains to be elucidated. Cysteine proteases would be able to alter integrity of gut epithelia, which in turn could induce an imbalance of the luminal environment and favour implantation of the parasite. This work supports the evidence that secreted cysteine proteases could be important virulence factors for *Blastocystis*, particularly by increasing epithelial intestinal permeability. This context could promote gut inflammation by luminal antigens diffusion to submucosa. Orthologs of both legumain and cathepsin B are also present in the recently sequenced *Blastocystis* ST4 (WR1 strain), which could suggest that other STs possess these virulence factors (Wawrzyniak *et al*. 2015). However, the reasons why *Blastocystis* do not induce intestinal symptoms among all infected subjects remain to be explained. Wu *et al*. showed that a protease activity varies between ST but especially between strains within the same ST (Wu *et al*. 2014). Then, levels of cysteine proteases expression and production remain key factors and their potential variability between isolates could explain differences in clinical presentation.

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