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Functional expression and characterization of *Schistosoma mansoni* cathepsin B and its *trans*-activation by an endogenous asparaginyl endopeptidase☆

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Abstract

Peptidases are essential for the establishment and survival of the medically important parasite, *Schistosoma mansoni*. This helminth expresses a number of gut-associated peptidases that degrade host blood proteins, including hemoglobin, as a means of nutrition. Using irreversible affinity probes, we demonstrate that *S. mansoni* cathepsin B-like endopeptidase 1 (SmCB1) is the most abundant papain family cysteine peptidase in both the parasite gut and somatic extracts. SmCB1 zymogen (SmCB1pm) was functionally expressed in *Pichia pastoris* (4–11 mg l^{-1}). Monospecific and immunoselected antibodies raised against SmCB1pm localized the enzyme exclusively to the gut lumen and surrounding gastrodermis of adult worms. Recombinant SmCB1pm was unable to catalyze its activation, even at low pH. However, recombinant *S. mansoni* asparaginyl endopeptidase (SmAE), another gut-associated cysteine peptidase, processed and activated SmCB1pm *in trans*. Consistent with the known specificity of AEs, processing occurred on the carboxyl side of an asparagine residue, two residues upstream of the start of the mature SmCB1 sequence. The remaining pro-region dipeptide was removed by rat cathepsin C (dipeptidyl-peptidase I)—an action conceivably performed by an endogenous cathepsin C in vivo. The activated recombinant SmCB1 is biochemically identical to the native enzyme with respect to dipeptidyl substrate kinetics and pH profiles. Also, the serum proteins, hemoglobin, serum albumin, IgG, and α-2 macroglobulin were efficiently degraded. Further, a novel application of an assay to measure the peptidyl carboxypeptidase activity of SmCB1 and other cathepsins B was developed using the synthetic substrate benzoyl-glycinyl-histidinyl-leucine (Bz-Gly-His-Leu). This study characterizes the major digestive cysteine peptidase in schistosomes and defines novel *trans*-processing events required to activate the SmCB1 zymogen in vitro which may facilitate the digestive process in vivo.

Keywords: Cathepsin B; Asparaginyl endopeptidase; *trans*-Processing; Peptidyl-dipeptidase; *Schistosoma*

Abbreviations: ACC, 7-amino-4-carbamoylmethyl coumarin; Bz-Gly-His-Leu, benzoyl-glycinyl-histidinyl-leucine; DPP I, dipeptidyl peptidase I (cathepsin C); DTT, dithiothreitol; E-64, L-*trans* epoxysuccinyl-leucylamido-(4-guanidino)-butane; GIC, gastrointestinal contents; GSH, glutathione (reduced); K11777, *N*-methylpiperazine-urea-phenylalanyl-homophenylalanyl-vinylsulfone-benzene; LC-ESI-ToF, liquid chromatography-electrospray ionization-time of flight; NaOAc, sodium acetate; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl flouride; Nle, norleucine; SmCB1, *S. mansoni* cathepsin B1 (a.k.a. Sm31); SmAE, *S. mansoni* asparaginyl endopeptidase (a.k.a. *S. mansoni* legumain; Sm32); Z-Ala-Ala-Asn, benzoyloxycarbanoyl alanyl-alanyl-asparaginyl 4-methyl-7-amido-coumarin; Z-Arg-Arg-AMC, benzoyloxycarbanoyl arginyl-arginyl 4-methyl-7-amido-coumarin; Z-Phe-Arg-AMC, benzoyloxycarbonyl phenylalanyl-arginyl 4-methyl-7-amido-coumarin

☆ *Note:* Both SmCB1.1 (accession no. AJ506157) and SmCB1.2 (accession no. AJ506158) sequences were deposited in the EMBL nucleotide database.

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1. Introduction

Human schistosomiasis causes almost one million deaths per year, affects an estimated 200–250 million people, and is second only to malaria as a global parasitic health problem. Caused by a trematode bloodfluke, adult worms ingest whole erythrocytes using the globin component of hemoglobin as a nutrient source [1]. Though chemotherapy of the disease is available, concerns over possible drug resistance encourage the search for new drug targets. One group of potential targets are those cysteine peptidases that are associated with the parasite gut and digest host proteins to absorbable nutrients [2]. Interruption of their function would limit the parasite's ability to feed and reproduce. Indeed, inhibitors of cysteine peptidases have been shown to decrease worm burden and egg production in mice infected with *S. mansoni* [3]. Characterizing such alimentary peptidases and understanding their biochemical differences compared to orthologous host enzymes is a fundamental requirement to rational and biospecific drug design.

A number of gut-associated cysteine peptidases have been identified in adult *S. mansoni*. These include cathepsin B1 (SmCB1; a.k.a. Sm31) [4,5], cathepsin L1 [6], cathepsin L2 [7], asparaginyl endopeptidase (SmAE; a.k.a Sm32), or schistosome legumain [8] and cathepsin C [9]. In addition, a schistosome cathepsin D-like aspartic endopeptidase has been described [10].

Cathepsin B activity, presumably due to CB1, is the most abundant papain-like, cysteine protease detected in the lumen of the schistosome gut [11]. The gene for SmCB1 encodes a putative signal sequence, pro-region and catalytic domain. The native SmCB1 zymogen is processed to a mature 31 kDa glycosylated protein. SmCB1 is secreted into the gut lumen of adult schistosomes and has also been found in the gastrodermal cells [12,13]. The enzyme has been used as a serodiagnostic marker of schistosomiasis [12]. Though recombinant SmCB1 has been heterologously expressed in insect cells [14], *Saccharomyces cerevisiae* [15] and a cell-free system [16], biochemical analysis has been hampered by poor protein yield or lack of 'correct' processing. As a result, questions as to how pro-SmCB1 is activated, and whether it can efficiently degrade hemoglobin have been raised [10].

For this report, we have overexpressed SmCB1 in *Pichia pastoris* in reagent quantities and characterized the enzyme with respect to zymogen activation, peptidyl- and protein-substrate specificity, tissue localization, and abundance relative to other *S. mansoni* papain-family cysteine peptidases. We also demonstrate for the first time a specific *trans*-processing event necessary for full activation of the SmCB1 zymogen involving a second and biologically relevant clan CD cysteine peptidase, SmAE.

2. Material and methods

2.1. Chemicals

Unless stated all chemicals were obtained from Sigma Chemical Co, St. Loius, MO, USA. Restriction endonucleases were purchased from Roche Molecular Biochemicals, Indianapolis, IN. The plasmid vector pPICZαA, the antibiotic Zeocin and X33 strain of *P. pastoris* were from Invitrogen, Carlsbad, CA. *N*-methylpiperazine-urea-phenylalanine-homophenylalanine-vinylsulfone-phenyl (K11777) was synthesized by Dr. Jim Palmer, Celera Genomics, South San Francisco, CA. Benzoyloxycarbonyl phenylalanyl-arginyl 4-methyl-7-amido-coumarin (Z-Phe-Arg-AMC) and benzoyloxycarbanoyl arginyl-arginyl 4-methyl-7-amido-coumarin (Z-Arg-Arg-AMC) were obtained from Bachem, King of Prussia, PA. Recombinant rat cathepsin C (dipeptidyl-peptidase I or DPP I) was a kind gift from Dr. John Pedersen of Unizyme, Denmark. The parasite endopeptidases, cruzain, rhodesain, and SmCB2 are available in this laboratory and falcipain 2 was a gift from Dr. Phil Rosenthal (UCSF).

2.2. Parasite material

Adult *S. mansoni* were obtained as described previously [17]. Prior to the collection of gastrointestinal contents (GIC), parasites were washed in RPMI and then three times with distilled water over a wire mesh to remove any RPMI. GIC were then collected as described [13,18].

2.3. Inhibitor iodination

The E-64 analogue DCG-04 was iodinated as previously described [19].

2.4. Radiolabeling cysteine peptidases in S. mansoni whole worm extract and GIC

Parasite material was incubated with radio-iodinated DCG-04 and processed as described [20]. Prior to radiolabeling, samples were either preheated or labeled in the absence or presence of 10 μM of the cathepsin B-selective inhibitor, MB-074 [21] for 60 min at room temperature. Labeled proteins were visualized by autoradiography using standard methodology.

2.5. pH measurements of GIC

The pH of GIC contents was measured in triplicate at room temperature using either a Micro comb pH probe (Lazar Research Laboratories, Inc., Los Angeles, CA) or ColorpHast indicator strips (pH 6–7; 0.2 unit accuracy; EM Science, Gibbstown, NJ).

2.6. Construction of smcb1 plasmid

Total RNA from mixed-sex adult *S. mansoni* was obtained using the Quickprep Total RNA kit obtained (Ambion, Austin, TX). First strand cDNA was generated using a poly-T oligonucleotide primer and reverse transcriptase (Invitrogen) under standard conditions. This was followed by polymerase chain reaction (PCR)-amplification of the *smcb1* open reading frame encoding the pro- and mature domains (*smcb1pm*).

The start of the pro-region was predicted using the software SigPep at www.exspasy.ch. The forward primer, SmF1, 5′AGCTACTCGAG*AAAAGA*CATAT T T CAGTTAAGAACGAAAAG-3′ contained sequence complementary for the predicted pro-region of SmCB1pm and also included a kexin endopeptidase cleavage site (Lys-Arg; in italic) and an *Xho*I restriction site (underlined) upstream of the gene specific sequence. The reverse primer, SmR1, 5′-GTAATAGCCGGTCGAATAAACT*AA*GCGGCCGCAC T GC-3′-end incorporated the complementary sequence for the coding region of the 3′ of SmCB1 and included the native translation termination codon (italics) and a *Not*I restriction site (underlined). The *smcb1pm* nucleotide sequence was confirmed by bidirectional sequencing at the Biomedical Resource Centre, UCSF using both vector specific and gene specific primers. The pPICZαA-*smcb1pm* plasmid was propagated as recommended by manufacturer's guidelines.

SmCB1pm was also subcloned into the prokaryote expression vector pTrcHis2A by PCR (Invitrogen). The forward primer, pTF1, 5′-TCGGCGGATCCA*CATCATCATCATCATCAT*ATTTCAGTTAAG-3′ contained sequence complementary to the predicted pro-region of *smcb1pm*, a *BamH*I restriction site (underlined) and a sequence encoding a polyhistidine fusion tag immediately upstream of the gene specific sequence. The reverse primer, pTR1, 5′-GCCGGTCGAATAAACT*AA*CTGCAGAATAG-3′ incorporated the complementary sequence for the coding region of the 3′ of *smcb1pm* and included the native stop site (italics) and a *Pst*I restriction site (underlined). The construct was used to transform chemically competent *Escherichia coli* strain DH5α. The nucleotide sequence of the pTrcHis2A-*smcb1pm* plasmid was verified by bidirectional sequencing at the Biomedical Resource Centre.

2.7. Overexpression of smcb1pm in P. pastoris and E. coli

Transfection of X33 strain of *P. pastoris* with SmCB1 was carried out using the methodology previously described [8]. Expression of SmCB1 in *E. coli* was carried out as described by the manufacturer's recommendation (Invitrogen).

2.8. Expression of recombinant SmAE

The expression of SmAE in *P. pastoris* was performed as described previously [8].

2.9. Processing of recombinant Pichia-derived SmCB1pm

The ability of SmCB1pm to undergo inter- or intramolecular processing and activation was studied by incubating the recombinant protein in 100 mM sodium phosphate, 50 mM citrate buffer, 2 mM DTT at 0.5 incremental pH values between pH 3.0 and 8.0. Endopeptidolytic activity was measured using Z-Phe-Arg-AMC and the samples also subjected to SDS–polyacrylamide gel electrophoresis (PAGE) followed by either western blotting or Coomassie Blue staining. The ability of SmAE to *trans*-process the SmCB1pm was also studied. SmAE was activated as described previously [8] and immobilized using *N*-hydroxysuccinamide-activated sepharose (Amersham Biosciences, Piscataway, NJ) using the manufacturer's guidelines. Immobilized SmAE was washed extensively in 50 mM sodium acetate (NaOAc; pH 4.5), until no detectable unbound activity against the SmAE substrate Z-Ala-Ala-Asn-AMC was obtained. The immobilized SmAE was incubated at 37 °C with SmCB1pm (≈15–20:1 mg ml^{-1} protein, SmAE:SmCB1, respectively) in 100 mM sodium phosphate, 50 mM citrate buffer, 2 mM DTT, at 0.5 incremental pH values between pH 3.0 and 8.0 for 3 h at 37 °C on a orbital shaker. Processing and activation of SmCB1pm was monitored using Z-Phe-Arg-AMC and SDS–PAGE followed by Coomassie Blue staining. Processing sites were identified by N-terminal protein sequencing. The activated mature SmCB1 (SmCB1m*) was collected by passing the SmAE/sepharose through an empty chromatography column.

2.10. Processing of SmCB1m* by cathepsin C

Rat cathepsin C (DPP I; 0.5 U) was incubated with 10 μg of activated SmCB1m* in 50 mM NaOAc, (pH 5.5), 2 mM DTT at 37 °C for 40 min. To determine the specific activity and processing of SmCB1m*, activity against Z-Phe-Arg-AMC was measured and the *N*-terminal sequence identified, respectively.

2.11. Peptidase assays

Peptidase activities were carried out using the fluorometric substrates Z-Phe-Arg-AMC, Z-Arg-Arg-AMC (both 20 μM final concentration) for SmCB1m* and Z-Ala-Ala-Asn-AMC (20 μM final concentration) for SmAE. Assays were performed in the presence of 2 mM dithiothreitol (DTT) and at 25 °C using an automated microtiter plate spectrofluorimeter (Labsystems Fluoroscan II) in a final volume of 250 μl. The appearance of 7-amino-4-methyl

coumarin (AMC) was measured with excitation and emission wavelengths of 355 and 460 nm, respectively. One unit of activity was defined as that releasing 1 μmol of AMC min^{-1}. All assays were repeated three times.

A novel exopeptidase activity assay of SmCB1m* was developed using a modified method that employed peptidyl-dipeptidase activity [22] to generate a fluorescent adduct. The substrate benzoyl-glycinyl-histidinyl-leucine (Bz-Gly-His-Leu; final 2 mM) is hydrolyzed by a peptidyl-dipeptidase activity which results in the appearance of His-Leu. The free amine-of His-Leu spontaneously reacts with fluorescamine (0.05 mg ml^{-1} from a 0.1 mg ml^{-1} stock in acetone) and the fluorescent adduct formed is excited at 390 nm and measured at 475 nm. SmCB1m* was incubated with substrate in 100 mM NaOAc, 2 mM DTT (pH 5.5), and the reaction allowed to proceed at 37 °C for 90 min. The reaction was terminated by the addition of 1.0 M glycine/NaOH buffer (pH 9.0) to a final concentration of 200 mM.

A 2D-gel analysis approach was used to monitor serum protein degradation by SmCB1m* (data not shown). Protein spots that either appeared or disappeared in the presence of SmCB1 were excised and digested with trypsin using standard methodologies. Peptide fragments analysis was carried out at the Protein and Nucleic Acid Facility, Stanford University.

2.12. Identification of the initial hydrolysis sites of hemoglobin by SmCB1m*

A 4% (w/v) filtered bovine hemoglobin solution in 100 mM ammonium acetate buffer (pH 5.5), 2 mM GSH, was incubated with SmCB1m* (1–2 RFU min^{-1} with Z-Phe-Arg-AMC). The reaction was carried out for 0, 15, 30, and 60 s, after which activity was stopped by addition of 10 μM E-64 and immediate placement in liquid nitrogen. Samples were stored at −70 °C until required and analyzed by liquid chromatography-electrospray ionization-time of flight (LC-ESI-ToF) using the Mariner software (Applied Biosystems, Foster City, CA) by Dr. David Maltby at the Mass Spectrometry Unit, UCSF.

2.13. Production of monospecific anti-SmCB1pm antibodies and immunoelectron microscopy

Pure recombinant SmCB1pm (3.0 mg) expressed in *P. pastoris* was subjected to SDS–PAGE stained with Coomassie Blue and the corresponding protein band excised and supplied to Covance, CA, for generation of polyclonal sera in New Zealand White Rabbits. Serum from the fourth bleed was used in all subsequent experiments and was immunoglobulin purified using a 1 ml HiTrap protein-G column (Amersham Biosciences) using the manufacturer's guidelines.

Immunoselection of the monovalent antibody preparation was carried out using standard methodologies. Briefly, 800 μg of SmCB1pm expressed in *E. coli* (see above) was subjected to SDS–PAGE. Following electrophoresis, proteins were electrotransferred to polyvinylidene difluoride (PVDF) membranes as described below. The transferred proteins were visualized using 0.1% (v/v) Ponceau-Red and SmCB1pm excised and washed briefly with 0.1 M sodium hydroxide to remove the stain. The excised PVDF was blocked with 5% (w/v) BSA in PBS containing 0.05% (w/v) Tween 20 (PBS/T), washed extensively with PBS/T and incubated with the Protein-G purified anti-SmCB1 antibody for 15 h at room temperature. After further washing, the bound antibodies were eluted with 100 mM glycine–HCl (pH 2.7), and the preparation immediately adjusted to pH 7.2 using 1.0 M phosphate buffer. The eluted antibodies were used in western blot analysis to confirm their specificity (data not presented).

Immunoelectron microscopy was carried out as described by Yezzi et al. [23].

2.14. SDS–PAGE and western blotting

Proteins were separated by SDS–PAGE using precast Invitrogen 4–12% NuPage gradient gels. The gels were either stained with Coomassie Blue or electroblotted onto nitrocellulose (Schleicher and Schuell, Keene, NH) or PVDF membranes (Millipore, Bedford, MA). After transfer, the membranes were blocked with 5% (w/v) bovine serum albumin in PBS/T and incubated for 1 h with polyclonal anti-SmCB1pm rabbit serum diluted 1:3000. After washing with PBS/T, membranes were incubated for a further 1 h with goat anti-rabbit antibodies coupled to alkaline phosphatase (1:4000 in PBS/T; Invitrogen). The membranes were washed extensively with PBS/T and stained with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Promega, St Luis Obispo, CA).

2.15. Amino terminal sequencing

Following separation by SDS–PAGE and electroblotting onto PVDF, membranes were stained briefly with Coomassie blue and destained (45% (v/v) methanol, 10% (v/v) glacial acetic acid) and washed extensively in distilled water. Amino-terminal sequencing was carried out by Dr. Ralph Reid at the Protein Sequencing Unit of the Biomolecular Research Centre, UCSF.

3. Results

3.1. SmCB1 is the most abundant papain family cysteine peptidase in the parasite gut

Schistosome extracts and GIC incubated with the radiolabeled site-directed cysteine peptidase probe, ^{125}I-DCG-04 [20], identified a number of cysteine peptidases by autoradiography (Fig. 1). As might be expected, there were more

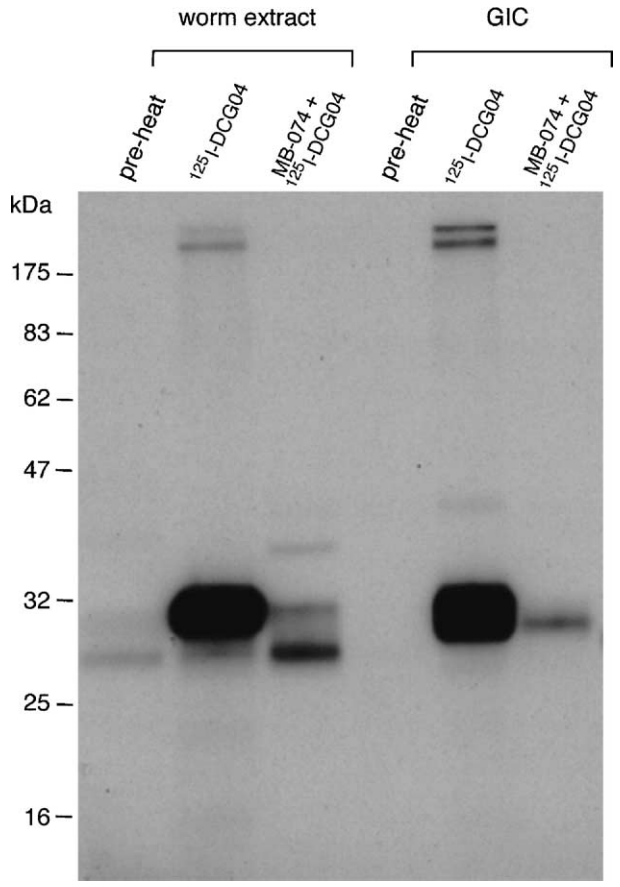

Fig. 1. Cathepsin B activity is the most abundant papain-family cysteine peptidase in *S. mansoni*. Total worm extract or gastrointestinal contents (GIC) were incubated with the irreversible epoxide inhibitor ^{125}I-DCG-04 for 60 min at room temperature. Preincubation of worm extract or GIC with MB-074, a selective cathepsin B inhibitor, prior to labeling with ^{125}I-DCG-04 confirmed the 31 kDa peptidase as a cathepsin B-like enzyme and suggests that the 200 kDa doublet has cathepsin B-like activity.

peptidases resolved in whole worm extract than GIC. In both worm preparations, a major reactive species, presumably SmCB1, was labeled at 31 kDa. This species was identified as a cathepsin B by preincubating extracts or GIC with the cathepsin B-selective inhibitor, MB-074 [20], prior to addition of ^{125}I-DCG-04. This cathepsin B species is the most abundantly labeled peptidase in worm extracts and GIC. A relatively minor peptidase activity 27 kDa was also labeled by ^{125}I-DCG-04 in worm extracts. The lack of sensitivity of this activity to MB-074 and its molecular mass suggests a cathepsin L-like enzyme. This peptidase activity was not observed in the GIC. A high molecular weight cysteine peptidase doublet of approximate 200 kDa, which was also sensitive to MB-074, was present in both worm extracts and GIC.

3.2. There are two isoforms of SmCB1

Using the previously published sequence of SmCB1 [4] a BLAST (tblastn) analysis was carried out at http://www.ncbi.nlm.nih.gov/blast/Blast.cgi using the non-redundant database at the GenBank EST division. Multiple contiguous sequences of adult ESTs revealed the presence of a second isoform of SmCB1. Both SmCB1.1 (accession no. AJ506157) and SmCB1.2 (accession no. AJ506158) isoforms sequences are available on request. Both isotypes differ from the previously described sequence. However, each difference is accounted for in either 1.1 or 1.2. Therefore, it is highly likely that the previously reported SmCB1 sequence was obtained from a mix of SmCB1.1 and SmCB1.2. The present work has been carried out using the SmCB1.1 isoform as this clone was available in the laboratory.

3.3. The pH of the GIC is slightly acidic

Using a micro-pH probe, the GIC content of mixed adults was estimated to be pH 6.84 ± 0.16 ($n = 5$). This pH was reproducible and was distinct from the pH of either RPMI (pH 7.57 ± 0.10) or distilled water (pH 6.01 ± 0.16). The pH of the GIC was also estimated using finely graduated pH indicator strips. This methodology revealed the pH of the GIC to be approximately 6.4 ($n = 2$), and that of distilled water and RPMI to be pH 6.0 and 7.2–7.4, respectively.

3.4. SmCB1 is found exclusively in the parasite gut lumen and gastrodermis

Monospecific and immunoselected antibodies raised to the pro-mature SmCB1 (SmCB1pm) localized the enzyme by histocytochemistry to the gut lumen and gastrodermis (data not presented). Immunoelectron microscopy localized SmCB1 to amorphous structures in the gut lumen and discrete vesicles in the gut gastrodermis (Fig. 2). No localization was seen in the tegument, parenchyma, or reproductive organs (data not presented).

3.5. Functional expression of SmCB1pm in Pichia pastoris

P. pastoris expressing SmCB1pm secreted 4–11 mg l^{-1} recombinant protein into induction media. By SDS–PAGE, a major 41 kDa protein (Fig. 3, lane 1) was identified as SmCB1pm by N-terminal sequencing (His^{18}-Ile-Ser; SmCB1 numbering). Some preparations contained a minor protein species migrating at 38 kDa which had the same N-terminal sequence (Fig. 3, lane 1). Thus, the higher molecular mass protein probably represents SmCB1pm glycosylated at the single potential site (Asn^{183}His-Thr; SmCB1 numbering). SmCB1pm degraded Z-Phe-Arg-AMC albeit at a very low rate (1–3 RFU min^{-1}) and this activity was sensitive to inhibitors of cysteine peptidases (data not shown).

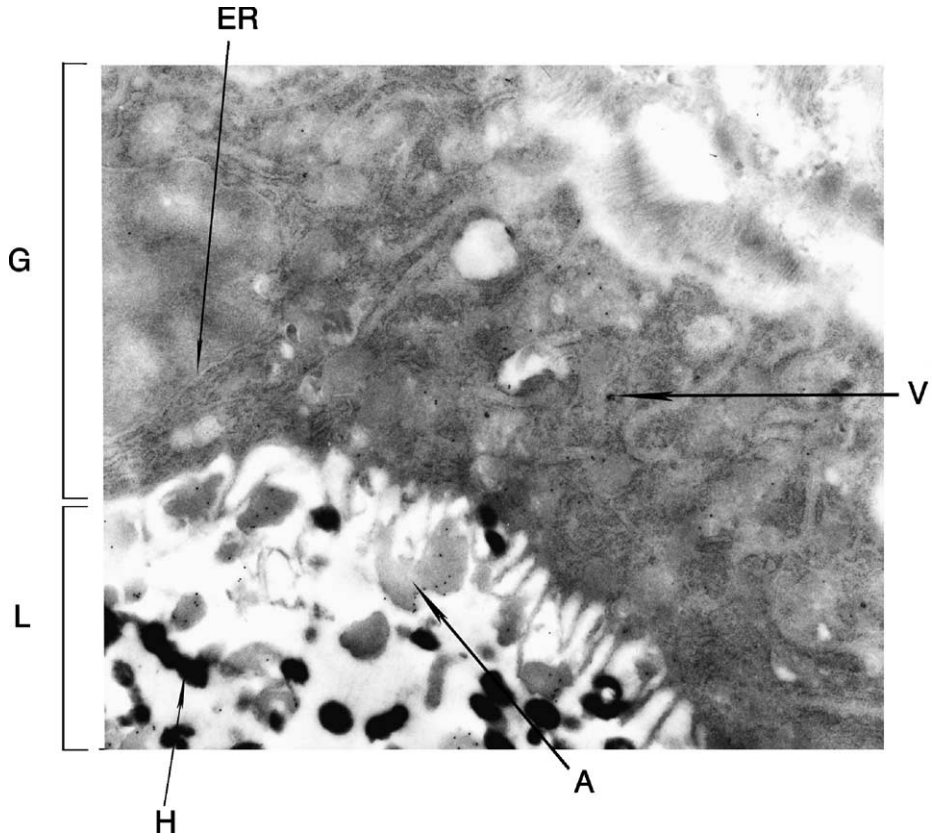

Fig. 2. SmCB1 is exclusively localized to the parasite gut lumen and surrounding gastrodermis. Thin-sections of resin-embedded male and female parasites were incubated with monovalent and immunoselected rabbit anti-SmCB1 antibodies. Indicated are the lumen (L), gastrodermis (G), vesicles (V), hemozoin (H), endoplasmic reticulum (ER), and amorphous structures (A).

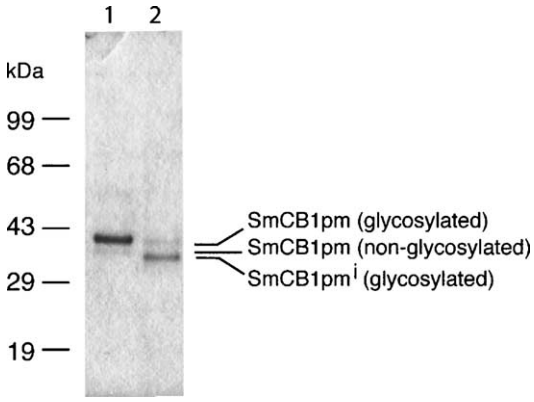

Fig. 3. SmCB1 zymogen, overexpressed in *P. pastoris*, undergoes partial processing without enzymatic activation. Pro-mature SmCB1 (SmCB1pm) is efficiently overexpressed in *P. pastoris* as a secreted major glycoprotein of 41 kDa and minor non-glycosylated protein of 39 kDa (lane 1). Incubation of SmCB1pm at pH 5.5 generated an intermediate form termed SmCB1pm^{i} (lane 2). The N-terminal sequence of SmCB1pm^{i} (Leu^{56}-Asp-Asp-Ala-Arg) revealed that the processing was not at the known N-terminal sequence of the wild-type SmCB1 (^{89}Ile-Pro-Ser). SmCB1pm^{i}, had no increased enzymatic activity compared to the parent protein.

3.6. Partial processing of the pro-domain of SmCB1pm does not result in enzymatic activation

SmCB1pm was incubated in various pH conditions and the presence of DTT to study processing and subsequent activation. However, protein representing mature enzyme (approximately 31 kDa) was not detected by SDS–PAGE or western blotting. Instead, there was a time dependent formation of an intermediate zymogen species (SmCB1pm^{i}) at pH 5.5 (Fig. 3, lane 2). The N-terminal amino acid sequence of SmCB1pm^{i} was Leu^{56}-Asp-Asp. No significant increase in activity against Z-Phe-Arg-AMC compared to the parent SmCB1pm (2–6 RFU min^{-1} and 1–3 RFU min^{-1}, respectively) was determined.

3.7. SmCB1pm is trans-processed and activated by the SmAE

SmCB1pm was incubated in the presence of recombinant activated SmAE and 2 mM DTT at various pH values. SmAE *trans*-processed and activated SmCB1pm in between pH 4.5 and 6.5 as evidenced by a marked increase in enzymatic activity against Z-Phe-Arg-AMC, from 1–3 to 180–260 RFU min^{-1} (Fig. 4A). At pH 5.5, a time

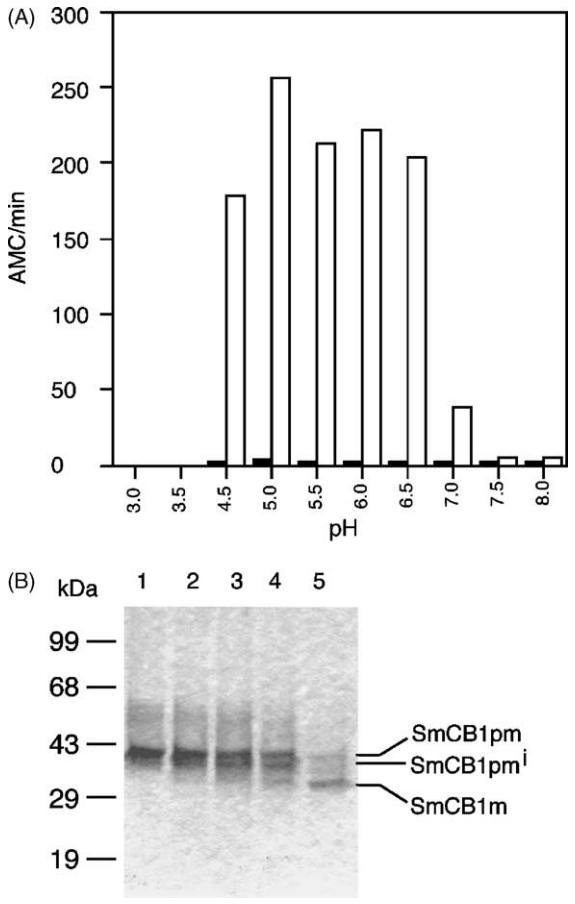

Fig. 4. Recombinant SmCB1pm is *trans*-processed and activated by SmAE; (A) SmCB1 endopeptidolytic activity was measured in the absence (■) and presence (□) of SmAE between pH 3.0 and 8.0 using Z-Phe-Arg-AMC as substrate (see experimental procedures). Activation was concomitant with the appearance of SmCB1m* (Fig. 4B) and occurred over a broad pH range between 4.5 and 6.5 ($n = 3$, S.E.M. < 10%); (B) SmCB1 was co-incubated with the SmAE (lane 1) over a time course of 10 (lane 2), 30 (lane 3), 60 (lane 4), and 120 min (lane 5) at 37 °C in 50 mM NaOAc, pH 5.5, 2 mM DTT buffer.

dependent appearance of a 31 kDa protein species was detected by SDS–PAGE (Fig. 4B) and western blotting. The appearance of this 'mature' SmCB1 (SmCB1m*) was unaffected by pretreatment and subsequent inactivation of SmCB1pm by 1 μM E-64. The N-terminal sequence of SmCB1m* was consistent with a *trans*-processing event by SmAE at Asn^{86} to yield ^{87}Val-Glu-Ile, i.e. two residues upstream of the N-terminus of the wild-type mature enzyme. SmCB1m* had a pH optimum of 4.5–6.5 (data not presented) and was sensitive to cysteine peptidase inhibitors 1 μM E-64, 10 μM iodoacetamide, and 1 μM K11777, but not 10 mM EDTA, 10 mM 1–10 *bis*-phenanthroline, 20 μM pepstatin A, 1 mM phenylmethylsulfonyl flouride (PMSF), and 10 μM puromycin. SmCB1m* had apparent K_m values of 38 μM (S.E.M. ± 14, $n = 3$) and 46 μM (S.E.M. ± 19, $n = 3$) for Z-Phe-Arg-AMC and Z-Arg-Arg-AMC, respectively.

3.8. SmCB1m* can be further processed by cathepsin C

Rat cathepsin C (DPP I) hydrolyzed the remaining dipeptide (Val-Glu) of SmCB1m* to yield the N-terminal sequence observed in the wild-type protein, ^{89}Ile-Pro-Ser. This final mature product (SmCB1m) had indistinguishable pH and inhibitor profiles from SmCB1m (data not presented). Further, the K_m value of 42 μM for SmCB1m* (S.E.M. ± 6, $n = 3$) using Z-Arg-Arg-AMC was similar to that recorded for SmCB1m (46 μM; see above).

3.9. SmCB1m* degrades hemoglobin α and β chains

SmCB1m* degraded native hemoglobin in vitro, consistent with the enzyme's putative in vivo activity. Hemoglobinolysis was completely inhibited by 1 μM E-64. Early cleavage within the α-chain of hemoglobin was determined by mass spectrometry; the β-chain was degraded too rapidly to assign unambiguous peptides. Indeed, the only monoisotopic unambiguous masses that were contiguous at a mass tolerance of 0.5 Da were, 3402.710 and 11659.603 with in the α-chain. These values demonstrate initial cleavage within the α-chain at Glu-Arg-Met-Phe^{33}-↓-Leu-Ser-Phe-Pro. All other monoisotopic masses, derived from either the α or β chain of hemoglobin, were ignored as they were either ambiguous or not part of a complete contiguous

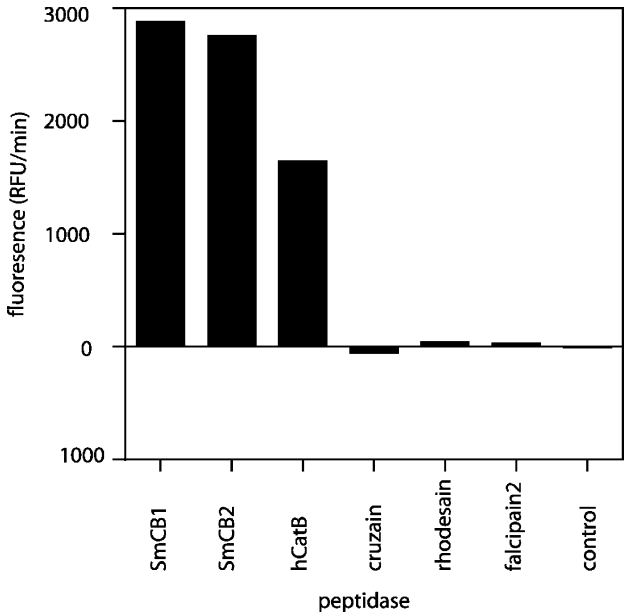

Fig. 5. SmCB1 has exopeptidase activity. Dipeptidyl-peptidase action by SmCB1m* on the substrate Bz-Gly-His-Leu facilitates the quantifiable reaction of fluorescamine with the exposed amino group of His. For comparison, *S. mansoni* cathepsin B2 (SmCB2), human cathepsin B (hCatB), and the cathepsin L-like enzymes, cruzain, rhodesain, and falcipain-2 from *Trypanosoma cruzi*, *T. brucei rhodesiense*, and *Plasmodium falciparum*, respectively, were also tested in the assay. The parasite cathepsin L-like endopeptidases had no exopeptidase activity and fluorescence obtained was similar to that of the control reaction lacking enzyme ($n = 3$; S.E.M. < 10%).

polypeptide sequence. *m*/*z* values of peptide fragments were assigned to cleavage sites using the software Findpept at http://ca.expasy.org/tools/findpept.html.

3.10. SmCB1m* degrades additional macromolecular proteins in the blood meal

In addition to hemoglobin, SmCB1m* degraded the serum proteins, serum albumin, IgG, and α-2 macroglobulin (monoisotopic peptide masses available on request).

3.11. SmCB1m* is also an exopeptidase

A novel application for a peptidyl-dipeptidase assay was used to verify SmCB1m* as an exopeptidase. SmCB1m* released the dipeptide His-Leu from the peptidyl-dipeptidase substrate Bz-Gly-His-Leu (Fig. 5). The appearance of the His-Leu was monitored by reaction with fluorescamine. Other cathepsin B-like enzymes, namely SmCB2 and human cathepsin B also possess peptidyl-dipeptidase activity. The cathepsin L-like endopeptidases, cruzain, rhodesain, and falcipain 2, had no peptidyl-dipeptidase activity.

4. Discussion

Several cysteine peptidases and an aspartic endopeptidase are secreted into the gut of *S. mansoni* and each of these has been proposed to facilitate degradation of ingested host proteins including hemoglobin [2,4,13,18,24]. Whether a single peptidase is the major digestive enzyme or a number act in concert is unknown. Previous work using peptidyl substrates and inhibitors suggested, the most abundant cysteine peptidase activity in the GIC to be a cathepsin B-like activity, most likely associated with SmCB1 [11]. This finding is verified here with the use of irreversible active-site probes that target papain-family peptidases. In addition to labeling the major cathepsin B-like activity at 31 kDa in whole worm extract and GIC, the inhibitor ^{125}I-DCG-04 [20,21] also labeled a less abundant putative cathepsin L-like activity at 27 kDa and a cysteine peptidase doublet of high molecular mass (200 kDa). Use of the cathepsin B-selective inhibitor, MB-074 [21], in a competition assay with ^{125}I-DCG-04, confirmed the identity of the 31 kDa enzyme as a cathepsin B and suggests that the 200 kDa doublet is also a cathepsin B-like peptidase(s). The cathepsin L-like activity was not detected in the GIC, suggesting that this enzyme is unlikely to be a major digestive enzyme.

Here, SmCB1 has been exclusively localized in the gut lumen and surrounding gastrodermis of adult worms. A previous study suggested localization to several other structures in addition to the gut but the anti-sera use had a high background due to non-specific cross reactivity [25]. To overcome this problem, anti-SmCB1 antibodies that were both monovalent and immunoselected were used to conclusively localize this endopeptidase to the gut alone. No immunoreactivity in other tissues including the tegument, parenchyma, and reproductive organs was seen. SmCB1 is present in discrete organelles in the gastrodermis and associated with amorphous material in the gut lumen. SmCB1 is, therefore, ideally placed to hydrolyze blood proteins in the gut lumen and continues catabolism of polypeptides in vesicles within the gastrodermis.

Previous attempts at recombinant expression of SmCB1 using heterologous expression in insect cells [14] and in vitro transcription and translation [16] failed to yield sufficient active SmCB1 for thorough biochemical analysis. *P. pastoris* has now been successfully employed as a convenient and efficient system to heterologously express the SmCB1 in reagent quantities. Expression of SmCB1 results in secretion via the *P. pastoris* secretory pathway does not require further purification following buffer exchange with a PD10 column.

SmCB1pm zymogen expressed in *P. pastoris* was unable to autocatalytically remove the complete pro-domain in a fashion consistent with the processing reported with other cysteine peptidases (Fig. 6) [26]. A similar lack of activation was observed when SmCB1pm was expressed in *S. cerevisiae* [15]. Although some autoprocessing was observed with that SmCB1 preparation, the site of processing has not been demonstrated in vivo and may be an in vitro artefact of SmCB1 expressed in yeast. In our preparation, it was not possible to determine whether the processing was in *cis* or in *trans*. There was no significant concomitant increase in endopeptidolytic activity against Z-Phe-Arg-AMC between the parent SmCB1pm and the intermediate SmCB1pm^{i}, suggesting that the active site remained occluded by the remainder of the N-terminus of the pro-region. There are precedents for processing intermediates of papain-family peptidases, including vertebrate procathepsins B, L, S, and K [27–29].

We demonstrate here for the first time that active SmAE can *trans*-process and activate the SmCB1pm in vitro. Processing was unaffected by pretreatment of SmCB1pm with E-64, thus confirming that SmAE (which is not inhibited by E-64) and not SmCB1 itself was responsible for hydrolysis. Processing occurred on the carboxyl side of Asn^{86} of the pro-peptide which is consistent with the known selectivity of asparaginyl endopeptidases for Asn at P1 [30]. The ability of SmAE to process SmCB1pm seems to be unique as, of a number of peptidases tested previously [15], only pepsin yielded a processed product. However, this product had unusual kinetic constants with dipeptidyl substrates, preferring Arg rather than Phe at P2 and having a poor ability to degrade globin, both findings contrary to our data presented here. The *trans*-activation of SmCB1pm by SmAE detailed here confirms the original hypothesis put forward by Dalton and Brindley who noted potential asparaginyl-endopeptidase cleavage sites in a number of *Schistosoma* and human pro-cathepsins [31]. Asparaginyl endopeptidases are also reported to process pro-proteins in plants [32] and mammals [33], and human MHC class II antigen [34]. The presence of both SmCB1 and SmAE in

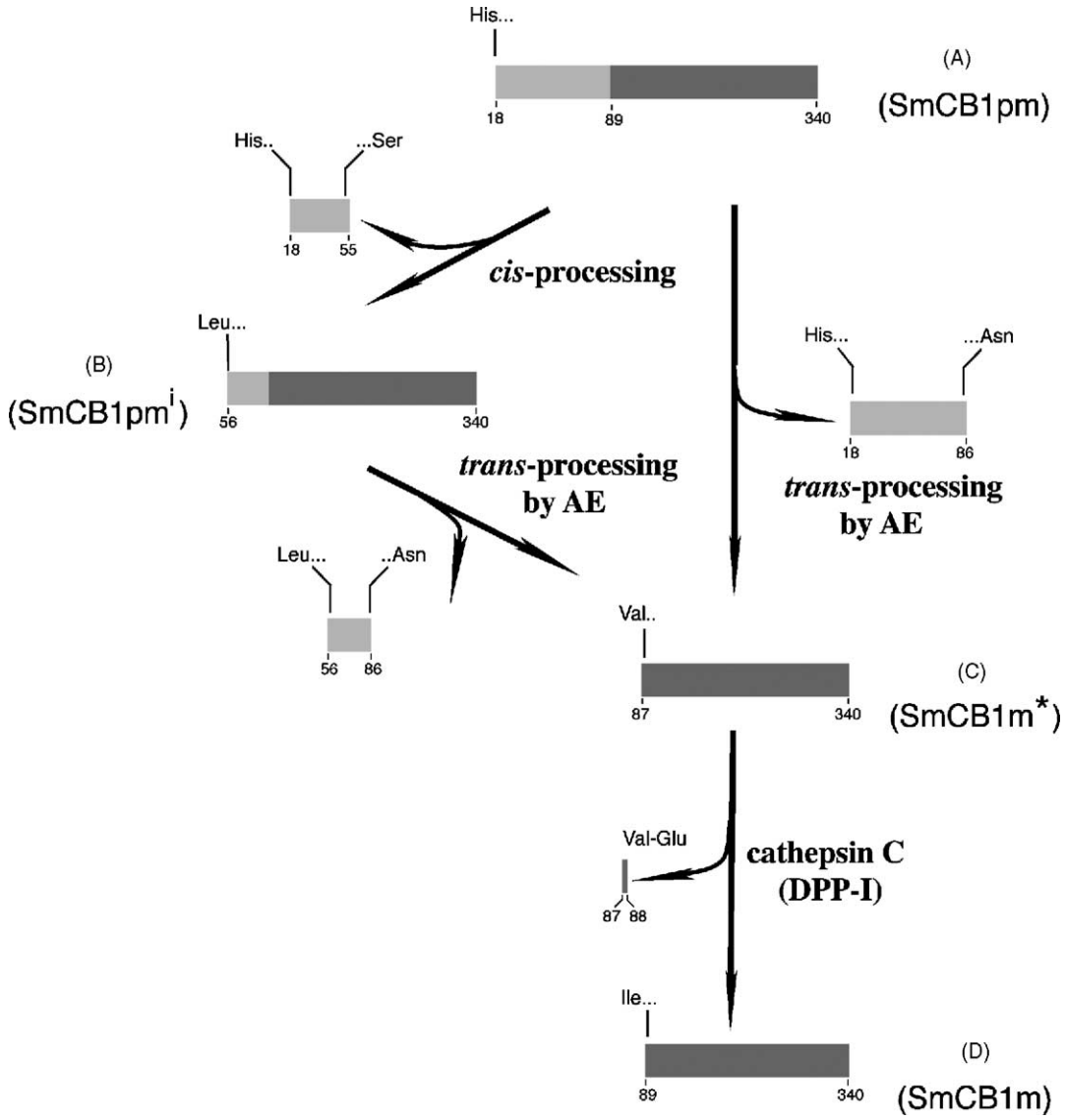

Fig. 6. Schematic of processing and activation of SmCB1pm. (A) SmCB1pm undergoes *cis*-processing in the pro-region at Phe-His-Ser^{55}-↓-Leu-Asp-Asp to yield SmCB1pm^{i}; (B) SmCB1pm^{i} or SmCB1pm is *trans*-processed at Asp-Trp-Asn^{86}-↓-Val-Glu-Ile by the ScistsSmAE to yield activated SmCB1m*; (C) SmCB1m* undergoes further maturation by a cathepsin C-like activity (a.k.a. dipeptidyl peptidase I (DPP I)) that removes the N-terminal dipeptide, Val-Glu to yield SmCB1m; and (D) both SmCB1m* and SmCB1m are catalytically indistinguishable. Numbers refer to the amino acid residue position with respect to the start methionine (no. 1).

the schistosome gut [12,25] is of biological relevance and suggests that *trans*-activation of SmCB1 may occur in vivo.

N-terminal sequencing of the *trans*-activated SmCB1 (SmCB1m*) indicated that two propeptidyl amino acid residues remained (*Val-Glu*-Ile^{89}-Pro-Ser for SmCB1m*; Fig. 6). Using rat cathepsin C we could remove this dipeptide to yield SmCB1m which has the same N-terminus as native SmCB1. *S. mansoni* is known to express a cathepsin C which is localized in the gut [35] and it is conceivable that this exopeptidase may perform the same function in vivo. We found no biochemical differences between the SmCB1m* and SmCB1m which had a similar pH profile and K_m values for Z-Arg-Arg-AMC, and therefore used SmCB1m* in the biochemical characterization.

The pH of the GIC was consistently 6.8 using a pH microelectrode and approximately 6.4 using pH indicator strips. Both readings suggesting an acidification of the blood meal (around pH 7.3) upon ingestion. The discrepancy between the GIC pH values obtained using the different protocols is unclear, however, the result with the pH indicator strips compares reasonably well with pH values previously recorded (6.2 [13] and 5.0–6.0 [36]). As noted in [13], tegumental components sloughed into the regurgitant may contribute to the pH measured. Also, it is possible that any GIC pH value represents an aggregate value not accurately accounting for the possibility of low pH microenvironments in the gut. Such acidic pockets could facilitate the action of proteases with lower pH optima such as cathepsin D (SmCD) [10]. In any

case, both the *trans*-activation of SmCB1pm by SmAE and the activity of SmCB1 against ingested proteins would proceed efficiently given the pH data presented herein or by others.

Sequence comparison with human cathepsin B revealed that SmCB1 has the predicted occluding loop, which has been shown to confer exopeptidase (specifically peptidyl dipeptidase) activity in vertebrate cathepsin Bs [37]. A novel assay for such activity was developed and confirmed that SmCB1 acts as an exopeptidase in addition to its endopeptidolytic activity described herein and elsewhere. The exopeptidase activity may be important in the downstream catabolism of ingested blood proteins. The assay also confirmed SmCB2 and human cathepsin B as exopeptidases. In contrast, none of the cathepsin L-like endopeptidases studied namely, cruzain, rhodesain and falcipain 2, could act as peptidyl dipeptidases.

Activated recombinant SmCB1 has very similar biochemical properties to the native parasite enzyme [5]. These include a similar pH optimum with Z-Phe-Arg-AMC as a substrate (pH 5.5–6.0) and similar K_m values for Z-Phe-Arg-AMC (wild-type, 21 μM; recombinant, 38 μM) and Z-Arg-Arg-AMC (wild-type 41 μM; recombinant, 46 μM).

A major protein in the schistosome blood meal is hemoglobin. Accordingly, it was pertinent to attempt to map the initial cleavage sites produced by recombinant SmCB1m* in the α and β chains of hemoglobin. Such experiments were carried out using a dilute enzyme preparation with reaction samples taken at 0, 15, 30, and 60 s. Mass spectrometry revealed the appearance of peptide fragments in a time dependent manner. However, due to the extremely rapid degradation of substrate by SmCB1, it was difficult to assign unambiguous monoisotopic masses to unique linear regions within the α or β chains of hemoglobin. The only complete contiguous sequence occurred with two polypeptides (3402.7 and 11659.6 Da) in the α-chain, consistent with initial hydrolysis at Arg-Met-Phe^{33}-↓-Leu-Ser-Phe-. Interestingly, the same cleavage site is also targeted as the initial point of hemoglobin hydrolysis by other peptidases implicated in parasite nutrition. These include, *S. mansoni* cathepsin D [10] the hookworm cathepsin Ds, Ac-APR-1 and Na-APR-1 [38], and a preparation of *Plasmodium falciparum* digestive vacuoles [39]. This may represent an evolutionary convergence across parasites and peptidase classes for the most appropriate site of initial hydrolysis of hemoglobin. This area within hemoglobin is referred to as the 'hinge region' and is involved in maintaining stability of the quaternary tetrameric structure. Hydrolysis at this point would relax the molecule and facilitate subsequent catabolism of hemoglobin. In addition to hemoglobin, SmCB1m* also degraded a number of other macromolecular proteins in the blood meal. These included, serum albumin, IgG, and α-2 macroglobulin. Hydrolysis of immunoglobulins may facilitate immune evasion by the parasite. In addition, effective degradation of macromolecular inhibitors such as α-2 macroglobulin, would be conceivably important in maintaining a full repertoire of active peptidases, thus aiding parasite survival.

In conclusion, we have expressed SmCB1 in quantities sufficient for detailed enzymic characterization and have shown that recombinant SmCB1 zymogen requires SmAE for *trans*-processing and activation. SmCB1 efficiently degrades hemoglobin and, as the major cysteine peptidase in the parasite gut, is a potential target for novel chemotherapies of schistosomiasis.

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