HHS Public Access

Author manuscript

Biochimie. Author manuscript; available in PMC 2017 March 01.

Published in final edited form as:

Biochimie. 2016 March ; 122: 99–109. doi:10.1016/j.biochi.2015.09.025.

Excretion/secretion products from *Schistosoma mansoni* adults, eggs and schistosomula have unique peptidase specificity profiles

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Abstract

Schistosomiasis is one of a number of chronic helminth diseases of poverty that severely impact personal and societal well-being and productivity. Peptidases (proteases) are vital to successful parasitism, and can modulate host physiology and immunology. Interference of peptidase action by specific drugs or vaccines can be therapeutically beneficial. To date, research on peptidases in the schistosome parasite has focused on either the functional characterization of individual peptidases or their annotation as part of global genome or transcriptome studies. We were interested in functionally characterizing the complexity of peptidase activity operating at the host-parasite interface, therefore the excretory-secretory products of key developmental stages of

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Schistosoma mansoni that parasitize the human were examined. Using class specific peptidase inhibitors in combination with a multiplex substrate profiling assay, a number of unique activities derived from endo- and exo-peptidases were revealed in the excretory-secretory products of schistosomula (larval migratory worms), adults and eggs. The data highlight the complexity of the functional degradome for each developmental stage of this parasite and facilitate further enquiry to establish peptidase identity, physiological and immunological function, and utility as drug or vaccine candidates.

Keywords

Parasite; Fluke; Secretion; Excretion; Protease; Inhibitor

1. INTRODUCTION

Schistosomiasis caused by the *Schistosoma* blood fluke is a chronic disease of poverty infecting more than 200 million people with as many as 800 million people at risk [1, 2]. Schistosome larvae (cercariae), released into freshwater by intermediate snail hosts, penetrate human skin and subsequently develop into adult male or female worms in the host vascular system. Pairs of worms can survive for years, if not decades [3] and release many hundreds of eggs a day [4]. These eggs induce immune-inflammation and tissue damage that contribute to disease morbidity [5]. The disease hampers growth and development of children and severely impacts the ability of families to provide for themselves [6–8].

Proteolysis is a fundamental physiologic process contributing to both health and disease [9, 10]. *Schistosoma* peptidases (a.k.a. proteases or proteolytic enzymes) are vital to successful parasitism, and facilitate invasion of the host, digestion of host proteins, reproduction, modulation of the host's physiology [11–19] and immune response [14, 20]. Interference with these mechanisms by specific vaccines [21, 22] or drugs may provide therapeutic benefits. Indeed, peptidases are excellent druggable targets [23–25] and a large body of literature exists demonstrating the therapeutic benefits of small molecule inhibitors targeting peptidases of schistosomes [26–29] and other infectious organisms [30–36].

Research on parasite peptidases has traditionally been driven by investigator interest in a particular molecule or class of molecules. For example, much of the considerable research focused on schistosome cysteine cathepsin enzymes as drug or vaccine targets has been facilitated by often inexpensive, sensitive and easy-to-use tools that include peptidyl substrates, inhibitors and antibodies. Detailed molecular, structural and immunological characterizations of these enzymes [29, 37, 38] have been greatly aided by their straightforward 'expressability' in heterologous systems such as yeast [38–41]. Over the last 10 years, the increasing availability of accurately annotated genomic [42–45, 47] and transcriptomic data [48–53] has expanded our view of the number and complexity of peptidases (the 'degradome') expressed by the schistosome and how their expression is regulated throughout the parasite's life cycle. In parallel, a number of studies have generated (sub)proteome data for schistosomes (for reviews see [54, 55]), including for the parasite's tegument (surface) [54, 56–58] and excretory/secretory (ES) products [59, 60]. Though

fundamentally informative, these studies do not provide information on which peptidases are functionally active, including at the host-parasite interface.

We performed a global and unbiased analysis of peptidase activity and specificity in the ES products of key *Schistosoma mansoni* developmental stages residing in the human host, namely schistosomula (post-infective migratory larvae), adults and their eggs. We chose to examine ES products as these would contain peptidases more likely to operate at the host-parasite interface rather than extracts which would also include irrelevant somatic activities. The substrate specificity for peptidase activities was detected using Multiplex Substrate Profiling by Mass Spectrometry (MSP-MS). This highly sensitive peptidase assay utilizes tandem mass spectrometry to monitor the degradation of a synthetic peptide library. MSP-MS can simultaneously detect endo- and exo-peptidase activities, and has been successfully employed to profile the proteolytic specificities of human neutrophil extracts [61], ES products from a pathogenic fungi [62] and *S. mansoni* cercariae [63]. In addition, we employed a panel of internally quenched, fluorescent peptidyl substrates in the presence and absence of peptidase-class-specific inhibitors to identify which peptidase classes were contributing to the global activity. Overall, we characterize a number of new proteolytic activities that sets the stage for their formal identification and exploration of their respective biological functions.

2. MATERIALS AND METHODS

2.1 Ethics statement

Maintenance and handling of vertebrate animals were carried out in accordance with a protocol (AN107779) approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California San Francisco.

2.2 Schistosome material

A Puerto Rican isolate of *S. mansoni* is maintained in the laboratory by cycling between Golden Syrian hamsters (*Mesocricetus auratus*) and the freshwater snail, *Biomphalaria glabrata*. Female 3–5 week-old hamsters were subcutaneously injected with 200 cercariae and sacrificed 6–7 weeks post-infection using an intraperitoneal injection of sodium pentobarbital (50 mg/kg). Adults and eggs were isolated as described [64]. Cercariae (infectious larvae) were obtained from infected snails induced to release the parasite under a light stimulus. Cercariae were chilled on ice, collected and mechanically transformed into schistosomula as described previously [65–67].

2.3 Collection of ES products

Fifty pairs of adult worms, 1000 eggs or 1000 schistosomula were washed five times in Basch medium 169 [68] supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 mg/ml streptomycin and 1% Fungizone (Gibco), and allowed to stand for 1 h at 37°C in 5% CO_2. Samples were washed 10 times and then incubated at 37°C in 5% CO_2 in the above medium supplemented with 5% fetal calf serum but in the absence of Fungizone. Adults and eggs were incubated overnight, and schistosomula were incubated for five days to allow for complete transformation from cercariae and remove contaminating cercarial peptidases.

Parasite materials were washed three times in the above medium and then washed 10 times in M-199 medium containing 100 U/ml penicillin and 100 mg/ml streptomycin, but without serum. In 5 ml of the same medium, samples were evenly distributed into a 6-well cultivation dish and incubated for 16 h at 37°C in 5% CO_2.

Medium containing ES products was removed, filtered over an Ultrafree-MC 0.22 mm filter (Millipore), and buffer exchanged into ice-cold Dulbecco's-Phosphate-Buffered Saline (D-PBS). The medium was then concentrated to 2 ml by centrifugation at 4,000 g and 4 °C using an Amicon 10000 Ultra-15 Centrifugal Filter Unit (Millipore). The total volume of PBS used for buffer exchange was 40 ml. Sample materials were quickly frozen in liquid nitrogen and stored at −80°C as 100 μL aliquots. Protein concentration was measured at 280 nm on a NanoDrop 2000c (Thermo Scientific).

2.4 Multiplex peptide cleavage assay

The Multiplex Substrate Profiling by Mass Spectrometry (MSP-MS) assay was performed as previously described [63] with minor modifications. Briefly, 20 μg/ml of protein from *S. mansoni* ES products of schistosomula, adults or eggs were pre-incubated for 15 min at room temperature with 0.2% DMSO, 1 mM AEBSF (Sigma-Aldrich 76307), 100 μM E-64 (Sigma-Aldrich E-3132) or 2 mM 1,10-Phenanthroline (Sigma-Aldrich 131377) in D-PBS containing 4 mM DTT. Each reaction was split into two tubes containing an equimolar mixture of 62 peptides in D-PBS (124 total). The final assay consisted of 500 nM of each peptide, 10 μg/ml protein, 2 mM DTT, 0.1% DMSO and either 500 μM AEBSF, 50 μM E-64, 1 mM 1,10-Phenanthroline or no inhibitor in D-PBS, in a total volume of 300 μl. Aliquots were removed at defined time intervals, adjusted to <pH 3.0 with formic acid and then desalted using C18 tips (Rainin).

Mass spectrometry was performed on a LTQ FT instrument (Thermo), equipped with a nanoACUITY (Waters) ultra-performance liquid chromatography. Reverse phase LC was performed using an EZ-Spray C18 column (Thermo, ES800, PepMap, 3 μm bead size, 75 μm x 15 cm) at 600 nL/min for loading and 300 nL/min for peptide separation over a linear 65 minute gradient from 2% to 30% acetonitrile in 0.1% formic acid. The mass spectrometer were operated using identical acquisition parameters as reported previously [63]. Substrate specificity profiles were generated using iceLogo software [96].

2.5 Internally quenched peptide assays

All assays were performed at room temperature in D-PBS containing 2 mM DTT and 0.01% Triton X-100. Assays were performed in triplicate in round-bottom 96-well plates in a spectrofluorimeter (Molecular Devices Flex Station) using a λ_{ex} 328 nm and λ_{em} 393 nm. Initial velocities in relative fluorescent units per second were calculated using Softmax Pro. Protein from *S. mansoni* conditioned media was assayed with a set of internally quenched fluorescent substrates (30 μM each). The total protein concentration in the internally quenched (IQ) substrate screen was 1.95 μg/ml, 18.7 μg/ml and 3.3 μg/ml for schistosomula, adults and eggs, respectively. Each IQ substrate consisted of a 7- or 8-mer peptide flanked with 2,4-dinitrophenyl-L-lysine on the carboxyl terminus and either 7-Methoxycoumarin-4-acetic acid or 7-methoxycoumarin-4-yl-acetyl-L-lysine on the amino terminus. IQ substrates

were synthesized using standard Fmoc chemistry and purified to >90% by reverse phase HPLC. Inhibition assays were performed using the same concentration of proteins as above. Assays contained 30 μM of IQ substrate, 1.5% DMSO, 100 μM of E-64, 500 μM of AEBSF or 1 mM of 1,10-Phenanthroline.

3. RESULTS

3.1 Detection of cleavage sites derived from peptidases in S. mansoni ES products

We took a global and unbiased approach to characterizing the proteolytic components of the ES products of three *S. mansoni* life-stages that parasitize the mammalian host, namely schistosomula, adults and eggs. After extensive washing to remove blood and serum components, including contaminant proteases, each developmental stage was placed in serum-free medium overnight. The ES products (conditioned medium) were then concentrated and added to a mixture of 124 physicochemically diverse peptides that are each 14-residues in length. Cleavage of any one of the 1,612 available peptide bonds within these peptides can be readily detected by LC-MS/MS sequencing. This assay is termed Multiplex Substrate Profiling by Mass Spectrometry (MSP-MS) and has been previously used with *S. mansoni* to characterize the specificity of peptidases in conditioned water from parasite-infected snails [63].

Assays on *S. mansoni* ES products were performed at pH 7.4 to mimic physiological pH of the mammalian host. Incubation of schistosomula, adults and eggs ES peptidases with the peptide library for 15 min resulted in 23, 7 and 15 cleavage sites, respectively (Fig. 1A). However, after the longest incubation of 1,200 min, the number of cleavage sites increased to 107, 151 and 301, respectively. In addition, cleavage sites were assessed at the intermediate time intervals of 60 and 240 min (Supplementary File 1).

The complexity of these hydrolytic events is illustrated using a sample peptide, *AYNnWSLYRnIRQE*, from which multiple cleavage sites were detected at various time intervals (Fig. 1B). Peptidases secreted from schistosomula, cleave at the Trp-Ser and Arg-Nle site and products of this hydrolysis were evident after only 15 min. After 60 min, additional cleavage products were detected that corresponded to hydrolysis at the Tyr-Arg bond. Unlike the other two stages, schistosomula ES peptidases could not cleave the Nle-Ile bond. Adult peptidase activity also cleaved the Trp-Ser and Arg-Nle bonds but these products appeared at later time intervals compared to hydrolysis of the Nle-Ile bond. No cleavage of the Tyr-Arg bond was evident in the adult ES products even after 1,200 min incubation, however, products derived from Tyr-Asn hydrolysis were found after 240 min incubation. Finally, ES peptidases from eggs preferentially cleaved at the Arg-Nle site within 15 min and later at the Trp-Ser and Nle-Ile sites. Like adults, egg ES peptidases did not cleave the Tyr-Arg bond. Thus, interrogation of just this single sample peptide from the mixture of 124, indicates that different peptidases are present in the ES products of each of the *S. mansoni* intra-mammalian stages.

The presence of different peptidases in the conditioned media from each of these developmental stages was investigated by directly comparing the cleavage sites. For this, the 145 sites identified after 240 min in the egg ES products was compared to the 107 and 151

sites identified from 1,200 min incubation of schistosomula and adults peptidases, respectively. These assays correspond to the earliest incubation time required to digest at least 5% of the 1612 peptide bonds in the library (≥81 cleavage sites). Peptidases from schistosomula, adults and eggs cleaved at 35, 79 and 62 unique sites, respectively, whereas 45 sites were common to all three (Fig. 1C). This analysis confirms that unique peptidases are present in the ES products from each of the intra-mammalian life stages.

In our experience, exopeptidases that remove mono-, di- or tri-peptides from the amino or carboxy terminus of proteins and oligopeptides are difficult to detect with standard reporter substrates due to the positioning of the fluorescent or colorimetric reporter group. For carboxypeptidases, the reporter group blocks the carboxyl terminus and therefore prevents cleavage. For aminopeptidases, substrates that are too long or too short, will not be hydrolyzed correctly between the canonical P1 residue and the reporter group. Our MSP-MS approach can simultaneously detect exo- and endo-peptidase activity because the peptides employed have free amino and carboxyl termini. The location of each cleavage site within the 14-mer peptides was compared for egg ES peptidases after 240 minutes incubation and adult and schistosomula ES peptidases after 1,200 minutes incubation (Fig. 1D). In general, the majority of peptide bond hydrolysis occurred away from the termini indicating that endopeptidases are most active. However, enzymes in the adult ES products cleaved between position 2 and 3 of the 14-mer peptides at a higher frequency than egg and schistosomula peptidases indicating that an enzyme with di-aminopeptidase activity may be present. Conversely, there is a higher frequency of cleavage by egg ES products between position 12 and 13 which may represent a di-carboxypeptidase activity.

3.2 Comparison of the substrate specificity profiles between life-cycle stages

For each of the cleavage sites generated by the ES peptidases, we obtained prime and non-prime site substrate specificity information (Fig. 2). Using iceLogo software, a P4 to P4' substrate signature was generated for all cleavage sites that occur after a defined incubation time. Peptidases secreted by schistosomula preferentially degraded peptides on the C-terminal side of Arg or Lys residues. This type of substrate specificity is commonly called “trypsin-like”. Gln was most often found in the P2 position and Ser or Arg were frequently found at P1'. In addition, cleavage rarely or never occurred at the C-terminal side of Gly, Pro, Nle or Glu or at the N-terminal side of Pro or Asn (Fig. 2A).

In contrast to schistosomula, much greater cleavage promiscuity was apparent in the ES products of adults suggesting that there are multiple enzymes present (Fig. 2B).Adult peptidases also preferentially cleaved at sites when Arg or Trp were present in the P1 position and Ser or Arg at P1'. The degradation of peptides by the egg ES peptidases had a preference for Arg and Gln at P1, Arg at P1' and bulky hydrophobic residues at P3 (Fig. 2C).

To complete the life cycle, we included the substrate signature generated from our previous study using water conditioned for 1,200 min with *S. mansoni* infected and non-infected *B. glabrata* snails [63]. Non-infected snails secrete at least one peptidase with a trypsin-like specificity (Arg and Lys at P1; Fig. 2D). This is consistent with the previous biochemical characterization of a major tryptase enzyme in snail extracts [69]. The substrate signature generated from conditioned water from *S. mansoni* infected-snails presented a different

substrate specificity profile (Fig. 2E). Infected snails in water release cercariae which contain a number of chymotrypsin-like serine peptidases known collectively as ‘cercarial elastase’. An in-depth characterization of these enzymes uncovered a P1 specificity for Phe and Tyr (Fig 2E). In addition, a preference for Pro at P2 is recorded and this is consistent with the known specificity of purified cercarial elastase using P1-P4 substrate positional scanning [70]. Schistosomula are generated from cercariae by mechanical shearing of tails followed by *in vitro* culture [65]. After 5 days of culture *in vitro*, it is clear that the chymotrypsin-like hydrolysis signature of the cercarial elastase is replaced by one that is trypsin-like.

3.3 Detection of peptidase activity in ES products using IQ substrates

As the majority of activity in ES products was derived from endopeptidases, we utilized a panel of internally quenched (IQ) fluorescent peptides with diverse sequences to quantify this activity. These substrates were previously synthesized by our group to detect aspartic acid and glutamic acid [71], cysteine [72] and serine [73] peptidases from a variety of microbial sources. These peptides were collectively used to detect proteolytic activity in conditioned media from the fungal pathogen, *Pseudogymnoascus destructans* [62]. Each IQ peptide is either seven or eight amino acids long and flanked on the N-terminus by a fluorophore and on the C-terminus by a fluorescent quenching group. Cleavage of any bond results in an increase in fluorescence due to the separation of the quencher from the fluorophore.

Peptidases in the ES products from each life-cycle stage hydrolyzed three IQ substrates containing the sequences QCACSNHE, tQASSRS and GRFGVWKA (Fig. 3). No other peptides were cleaved by schistosomula ES products. Egg-conditioned medium cleaved 5 additional IQ substrates, and in general showed a higher specific activity relative to schistosomula and adult enzymes. This is consistent with egg peptidases cleaving at a greater number of sites in the MSP-MS assay. The conditioned medium from adults cleaved all IQ substrates but generally had low specific activity relative to the schistosomula and egg ES enzymes. tQASSRS was the commonly cleaved IQ substrate, and was therefore deemed to be a useful reporter substrate to quantitatively measure proteolytic activity in the presence and absence of class-specific inhibitors.

3.4 Investigating the contribution of serine, cysteine and metallo-peptidases using class-specific inhibitors

Using the tQASSRS substrate, ES products from each of the *S. mansoni* developmental stages were treated with class-specific inhibitors that target serine, cysteine and metallo-peptidases. All assays were performed at pH 7.4, at which aspartic acid peptidases are unlikely to be active. Therefore, pepstatin A that targets aspartic acid peptidases was not included in the inhibitor screen.

3.4.1 Schistosomula—Cleavage of tQASSRS was unchanged upon treatment with the metallopeptidase inhibitor 1,10-Phenanthroline when compared to the non-inhibited control (DMSO) while the cysteine peptidase inhibitor E-64 actually increased activity. However, in the presence of the serine peptidase inhibitor, AEBSF, turnover of the fluorescent substrate

was abolished (Fig. 4A). These data are consistent with the presence of a predominant serine peptidase activity.

MSP-MS assays were performed with the same inhibitor treated ES products that were used in the IQ studies. Using the sample 14-mer peptide highlighted in Fig. 1B, we show that treatment of medium with E-64 results in a cleavage pattern that is identical to DMSO-treated control (Fig. 4B). In contrast, AEBSF prevented hydrolysis of the Trp-Ser and Tyr-Arg bonds and greatly delayed hydrolysis of the Arg-Nle bond, which was only recorded after 1,200 min. Two new cleavage sites (Tyr-Asn and Nle-Ile) appeared after 1,200 min that were never observed in the DMSO-treated control (Fig. 4B). The appearance of 'new' cleavage sites following treatment with an inhibitor has been observed previously [62]. These sites were not detected in the DMSO-treated control assay because the peptide was rapidly degraded into tri-, tetra- and pepta-peptides by the serine peptidase(s) and therefore any subsequent cleavage sites would not be readily detected by mass spectrometry. Finally, treatment with 1,10-Phenanthroline caused a delay in the processing of the Tyr-Arg bond from 60 to 240 min but otherwise the cleavage sites remained unchanged.

Treatment of schistosomula ES products with AEBSF resulted in the delay in appearance of 91 of the 107 cleavage sites that were detected in the DMSO-treated control. These sites were termed "sensitive to AEBSF" and frequently had Arg and Lys in the P1 position (Fig. 4C). Based on this substrate profile it is reasonable to suggest that a single major peptidase with trypsin-type specificity is present in the ES products of schistosomula.

3.4.2 Adults—Treatment of adult ES products with either E-64 or AEBSF resulted in a 19% and 35% decrease in the overall cleavage of tQASSRS, respectively (Fig. 5A). However, when these compounds were combined, E-64 provided no additional inhibition, indicating that this inhibitor may be targeting the serine peptidase(s). Competitive inhibition of bovine trypsin by E-64 has been previously reported [74]. 1,10-Phenanthroline decreased activity by more than 80%, indicating that metallo-peptidases predominate in adult ES products under the conditions employed. Combining 1,10-Phenanthroline with E-64 and AEBSF completely inhibited cleavage of the tQASSRS substrate (Fig. 5A).

When AEBSF was added to the MSP-MS assay and compared to a DMSO-treated control, 59 of 151 cleavage sites were sensitive to this inhibitor (Fig. 5B). The specificity profile of the AEBSF-sensitive enzymes had a preference for Arg at P1, but a much lower preference for Lys compared to the schistosomula ES peptidase. This indicates that it is unlikely that the same serine peptidases are being measured in ES products of schistosomula and adults.

When the adult ES products were treated with 1,10-Phenanthroline, 107 of the total of 151 cleavage sites in the 14-mer peptide library were sensitive to this agent (Fig. 5C). The substrate specificity profile of the 1,10-Phenanthroline-sensitive enzymes had a preference for bulky residues at P3, Leu at P1 and Arg, Phe and Ser at P1'. In addition, Gln and Lys were most often found at P2' whereas Arg, Ala and Tyr were frequently identified at P3'.

Interestingly, all cleavage sites that occurred between the 2nd and 3rd position of each 14-mer peptide were sensitive to 1,10-Phenanthroline but only two were sensitive to AEBSF

(Fig. 5D). In some cases, AEBSF treatment resulted in an earlier appearance of the cleavage product, when compared to the DMSO control. For example, removal of AY from the sample tetradacapeptide, *AYNnWSLYRnIRQE* occurs after 240 min incubation in the DMSO-treated assay, but the dodecapeptide cleavage product can be detected after only 15 min incubation in the AEBSF treated assay. Thus, in addition to the metallo-endopeptidase activity sensitive to 1,10-Phenanthroline, there is a least one other metallo-peptidase in the ES products with a specificity for amino-terminal dipeptides.

3.4.3 Eggs—Treatment of egg ES products with AEBSF completely inhibited cleavage of tQASSRS indicating that serine peptidase activity is a major proteolytic component (Fig. 6A). Cysteine and metallo-peptidases are also present as E-64 and 1,10-Phenanthroline decreased activity by 61% and 15%, respectively. A substrate specificity profile was generated for both the AEBSF-sensitive (Fig. 6B) and E-64-sensitive (Fig. 6C) cleavage sites. Cleavage sites that were sensitive to AEBSF had a trypsin-like substrate specificity profile using the MSP-MS assay. Interestingly, the cleavage sites that were sensitive to E-64 also had a strong P1 preference for Arg and Gln and generally accepted hydrophobic residues at P2, consistent with that known for cysteine cathepsins [75–77].

The MSP-MS assay on the egg ES products indicated that not all of the cleavage sites were sensitive to either AEBSF or E-64 treatment. Indeed, 61 of the original 145 cleavages identified in the control-treated assay were resistant to both inhibitors suggesting that enzymes other than serine or cysteine peptidases are also present (Fig. 6D). To further probe the egg-conditioned media we utilized the IQ substrate, GRFGVWKA, identified in Fig. 3. This substrate was also cleaved by peptidases in the conditioned media from all developmental stages tested, although with lower activity than the tQASSRS substrate. Using the GRFGVWKA substrate, AEBSF treatment only caused a 35% decrease in overall activity, whereas 1,10-Phenanthroline abolished activity (Fig. 6E). Using these same conditions, the 1,10-Phenanthroline-treated ES products were interrogated with the MSP-MS assay. The resulting sensitive cleavage sites had a preference for Trp, Ile, Ser and Arg at P4, P3, P2 and P1, respectively. In addition, a P1' preference for Arg was evident. Notably, this 1,10-Phenathroline sensitive peptidase differs in specificity from the metallo-peptidases in adults. Taken together, these studies indicate that serine, cysteine and metallo-peptidases are all active in the ES products of *S. mansoni* eggs.

4. DISCUSSION

Understanding the biological function of a peptidase in any organism, including *Schistosoma*, requires orthogonal data inputs, including gene expression, protein localization, post-translational modifications and substrate specificity. Global ‘omic’ analyses such as those have made key contributions in these respects. To date, however, there has been no global *functional* profiling (qualitative nor quantitative) of the peptidases across developmental stages. Our employment of peptidase-agnostic technologies in combination with class-specific peptidase inhibitors reveals previously unrecognized activities that are released by key schistosome developmental stages that parasitize the human host. The technologies applied to schistosomula, adults and eggs have been previously utilized in a number of different contexts [61, 62] including to characterize *S.*

mansoni cercarial secretions [63]. The present report is, therefore, a natural extension of that work. We employed ES products as these are likely to contain peptidases operating at the host parasite interface and that potentially influence host immunology and physiology. Finally, ES products were collected and processed at neutral (physiologic) pH to inactivate or at least mitigate the proteolytic contributions of aspartic and cysteine proteases [39, 78, 79], not least those adult gut proteases that have been extensively characterized [11, 13, 29, 80, 81] and are regurgitated by the worm into culture medium [11, 78, 82, 83]. Thus, our conditions facilitate the identification of novel peptidolytic activities, specifically, metallo- and serine proteases.

We utilized two IQ substrates as reporters of activity and inhibitor sensitivity. The cleavage sites within these substrates were not investigated as the specificity profile was revealed in the subsequent MSP-MS assays. The proteolytic profile of schistosomular ES products is the simplest measured and centers on a single or predominant serine peptidase with a strict P1-P1' specificity for charged amino acids and only minor amino acid engagements at the other prime and non-prime binding sites. The activity may be due to one or more of a number of schistosomular serine peptidases we previously measured by both gene expression profiling and functional activity analyses with small peptidyl substrates [19]. Importantly, the specificity profile of this serine peptidase activity differs markedly from the chymotrypsin-like cercarial elastase that is secreted by the *S. mansoni* cercariae and from which we derive schistosomula [63, 70].

In contrast to schistosomula, the specificity profiles of adults and eggs are much more complex and different from one another with respect to the proteolytic contributions of serine, metallo- and cysteine proteases. Adults produce a serine peptidase that has a strong preference for P1-Arg but not Lys. Generally, trypsin-like serine peptidases cleave peptides with these P1-amino acids equally well. These data support our previous finding that a number of different trypsin peptidases are expressed in adult *S. mansoni* [19]. In addition, a second serine peptidase activity is present that has chymotrypsin-like P1 specificity for Trp. However, this enzyme has little preference for P1-Tyr, -Phe and -Leu which are normally associated with chymotrypsin. Adults also produce at least two metallo-peptidases, one of which is a metallo di-aminopeptidase activity, thus underscoring the utility of the MSP-MS approach to not only detect endopeptidase activity but also exopeptidase activity. Neither of the metallo-peptidase specificity profiles identified are similar to those recorded for a M17 family leucine aminopeptidase activity previously characterized in *S. mansoni* [84, 85].

Like adult ES products, those from eggs contain serine and metallo-peptidase activity profiles, however, these differ from the adult profiles and from each other suggesting that different enzymes are being measured. It's possible that the serine peptidase activity may be in part due to a PMSF-sensitive fibrinolytic activity previously described in egg extracts [18] although no protein identification for this activity was subsequently carried out. Regarding the metallo-endopeptidase activity, we are not aware of such being described in egg ES products but it's clear that a major 1,10-Phenanthroline-sensitive activity is present. Interestingly, egg conditioned medium also contains a cysteine protease activity that is robustly active under the neutral pH conditions employed. This is in contrast to adult ES products that predominantly contain neutral pH-labile cathepsins that arise from the

gastrointestinal tract [78]. The presence of cysteine protease activity has been described in eggs [86, 87] and miracidia (which eventually emerge from the egg) [88]. Peptidase activity has also been measured at neutral pH in egg ES products [89] and live eggs were shown to degrade the glycoprotein component of an artificial extracellular matrix at neutral pH. This activity was enhanced in the presence of reducing agent suggesting a role for cysteine peptidases [90]. Accordingly, it's possible that eggs release a specialized cathepsin–like activity that facilitates their passage through host tissues and their eventual escape into the environment via the urogenital or digestive tracts.

Our application of peptidase-agnostic technologies in combination with peptidase class-specific inhibitors reveals the heretofore unrecognized complexity of peptidolytic activities released by key schistosome life-stages parasitizing the human host. Further biochemical studies are required to identify the peptidases responsible but there are now well-annotated genomic and transcriptomic data (references cited in the Introduction) that can be brought to bear in such studies. Aided by knowledge of both substrate specificity and peptidase class, we can now develop tools to characterize the individual enzymes in greater detail, *e.g.*, activity-based probes that bind to the peptidase active site. Such probes require knowledge of both the substrate recognition sequence and the nature of the active-site nucleophile, and have been previously engineered to identify and characterize peptidases, and image disease as biomarkers [91–93].

5. CONCLUSION

This study describes the application of an unbiased and global technology to characterize *S. mansoni* excretion-secretion peptidase activity. Each intra-mammalian developmental stage produces a different set of activities and all stages except cercariae secrete a trypsin-type serine peptidase. In addition, a metallo-peptidase with dipeptidase specificity was observed in adult ES products and eggs release a cysteine protease that is active at neutral pH. These studies will facilitate the development of selective active-site directed affinity probes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

6. ACKNOWLEDGMENTS

Research was funded by the University of California, San Francisco Program for Breakthrough Biomedical Research and the Sandler Foundation (AJO and CSC). JD, AL and LU were supported by the grant P302/11/1481 from the Czech Science Foundation. JD was supported by a FP7 Marie Curie International Re-integration Grant 248642. MH, MM, AL and PF are supported by the project InterBioMed LO1302 from the Ministry of Education, Youth and Sports of the Czech Republic and by the institutional project RVO 61388963. CRC is supported in part by NIH-NIAID grants R01AI089896 and R21AI107390. We thank to K.C. Lim his help with *S. mansoni* life-cycle and Chris Franklin for the life-cycle illustration. MS analysis was performed in the Bio-Organic Biomedical Mass Spectrometry Resource at University of California, San Francisco (A. L. Burlingame, Director), supported by the Biomedical Technology Research Centers program, National Institutes of Health, National Institute of General Medical Sciences 8P41GM103481.

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- Unbiased and global peptidase specificity technology to detect novel activities
- Excretion/secretion peptidase activity detected in *Schistosoma mansoni*
- All stages except cercariae secrete a trypsin-type serine peptidase(s)
- Eggs and adults, but not schistosomula, release metallo-peptidases
- Eggs release a cysteine protease that is active at neutral pH

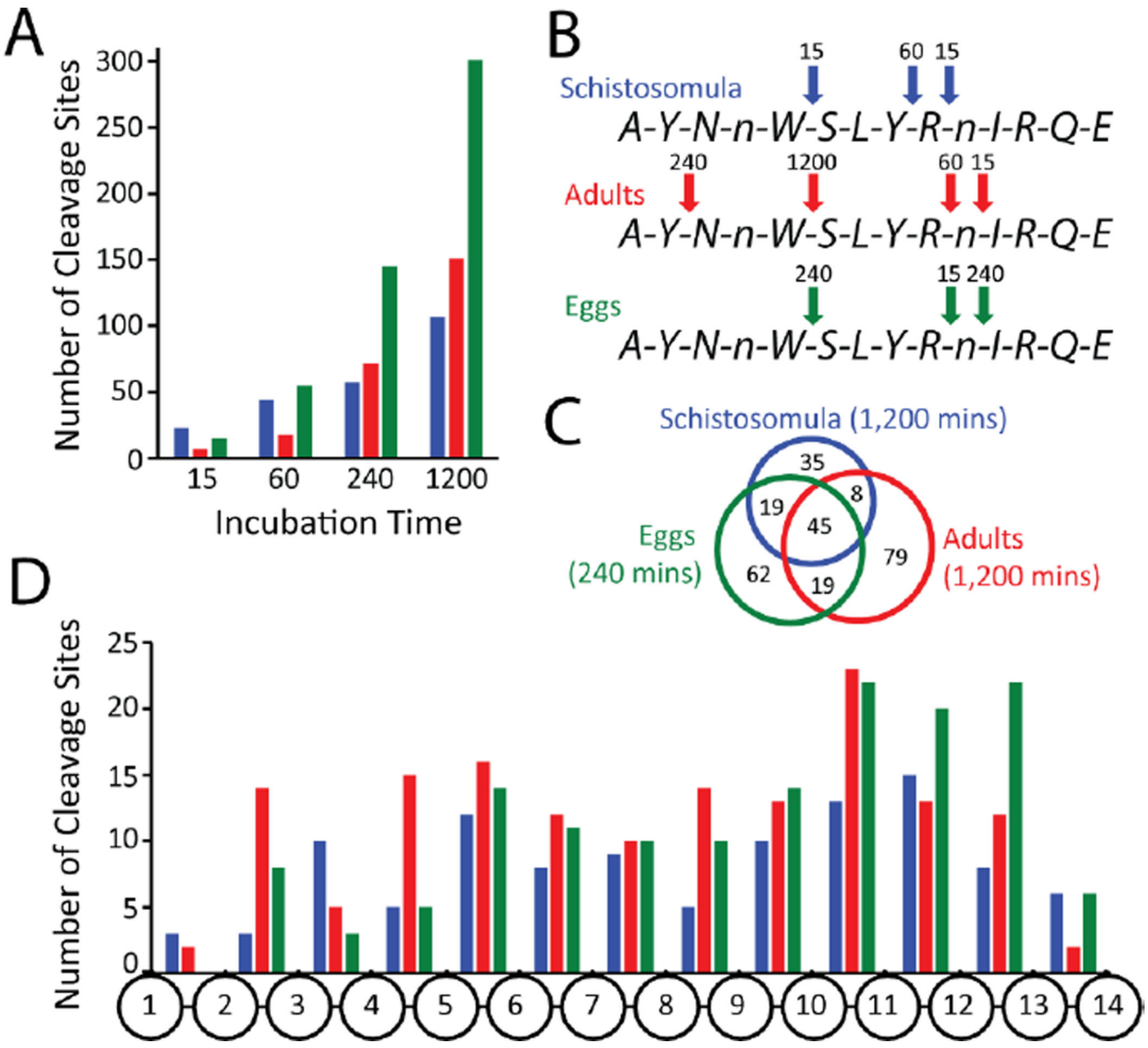

Figure 1. Detection of proteolytic activity in the ES products of *S. mansoni*

A. ES products from schistosomula (blue), adult worms (red) and eggs (green) were incubated for 15 to 1,200 min with a 14-mer peptide library. LC-MS/MS sequencing was used to detect the appearance of peptidase cleavage sites **B**. A sample 14-mer peptide illustrating the complexity of peptidase cleavage. The position and time (minutes) that cleavage products were first detected are indicated. Amino acids are shown in single letter code. Lowercase "n" corresponds to norleucine (Nle). **C**. Venn Diagram showing the number of unique and shared cleavage sites. **D**. Spatial distribution of cleavage sites within the 14-mer peptide scaffold.

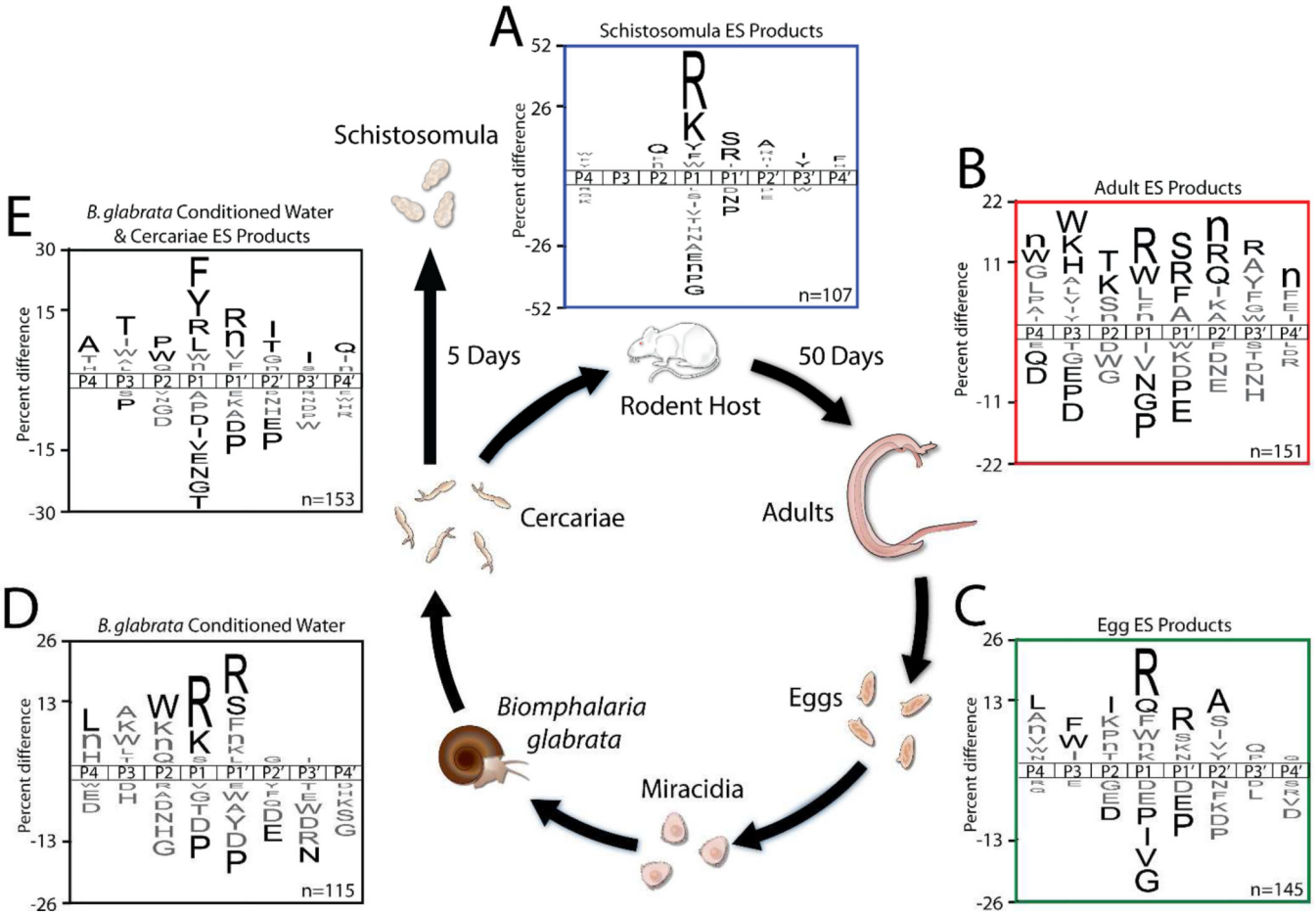

Figure 2. Generation of a proteolytic specificity signature in the ES products of *S. mansoni* at different life cycle stages

Generation of a substrate specificity iceLogo signature for **A**. schistosomule, **B**. adult and **C**. egg secretions following 240 or 1,200 minutes incubation with the 14-mer peptide library. Amino acids colored black are significantly increased ($p<0.05$) in the position relative to a control dataset that corresponds to all possible cleavage sites. Amino acids above the X-axis are found at the given position with higher frequency than the control dataset while amino acids below the axis are rarely or never found at the given position. IceLogo signature showing the substrate specificity profiles of conditioned water from **D**. uninfected and **E**. *S. mansoni* infected *Biomphalaria glabrata*.

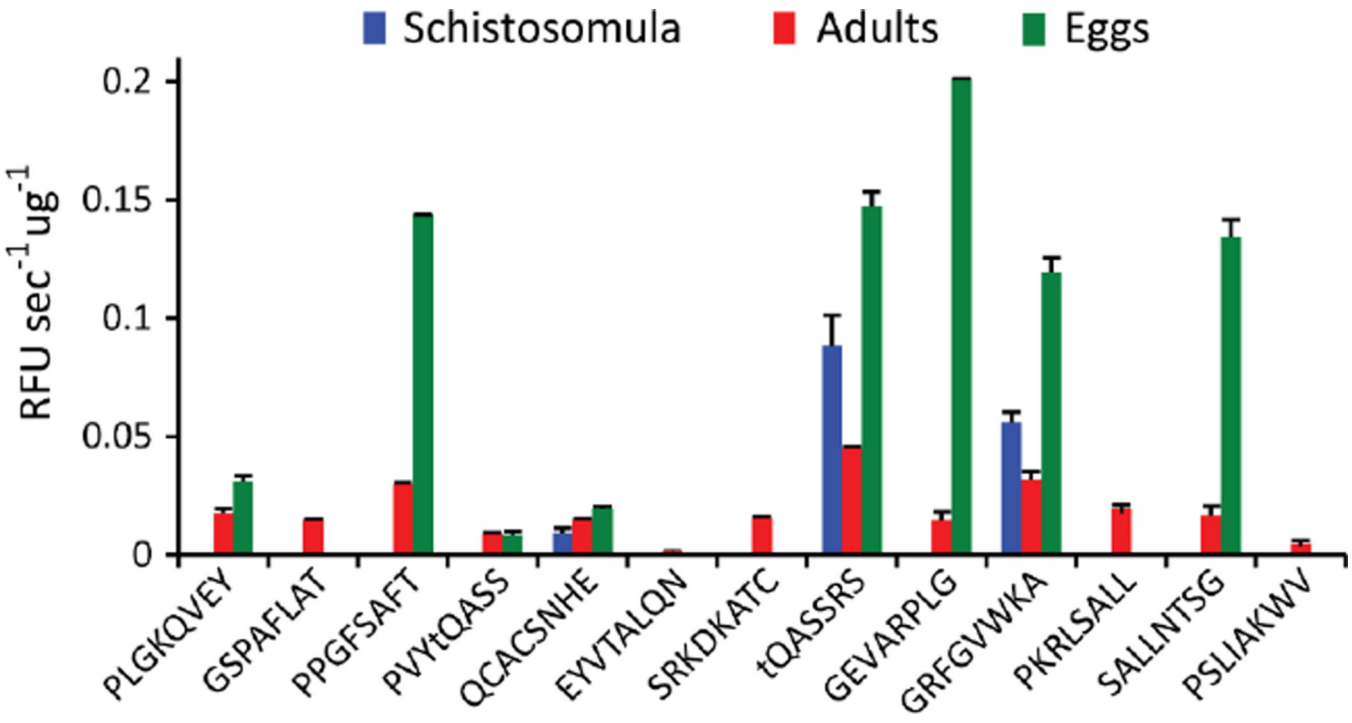

Figure 3. Quantitation of proteolytic activity in ES products of *S. mansoni* at different life cycle stages

Proteolytic activity in the conditioned media was detected using internally quenched fluorescent substrates. Amino acids are described in single letter code and lowercase ‘t’ corresponds to *tert*-butyl glycine.

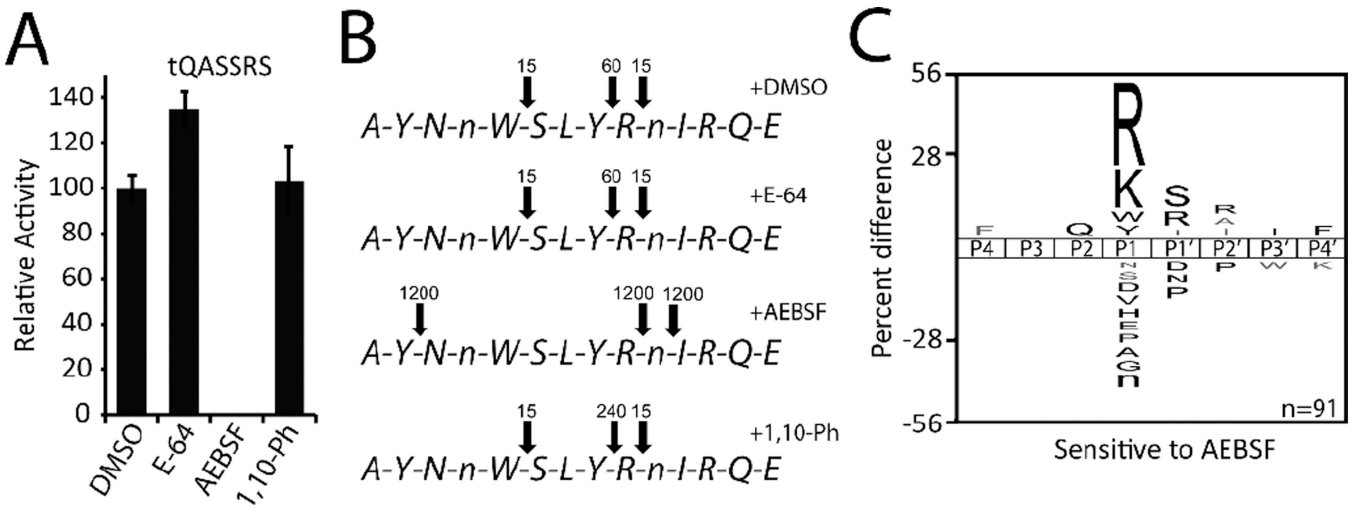

Figure 4. Use of class-specific inhibitors to characterize peptidase activity and specificity in schistosomula secretion

A. ES products were assayed with the internally quenched tQASSRS substrate in the presence of class-specific inhibitors. **B**. Use of a sample 14-mer peptide to illustrate the time dependent changes in cleavage site accumulation in the presence of class-specific inhibitors compared to a non-inhibited DMSO control. **C**. An iceLogo substrate profile generated from all cleavage sites in the 14-mer peptide library that were sensitive to AEBSF.

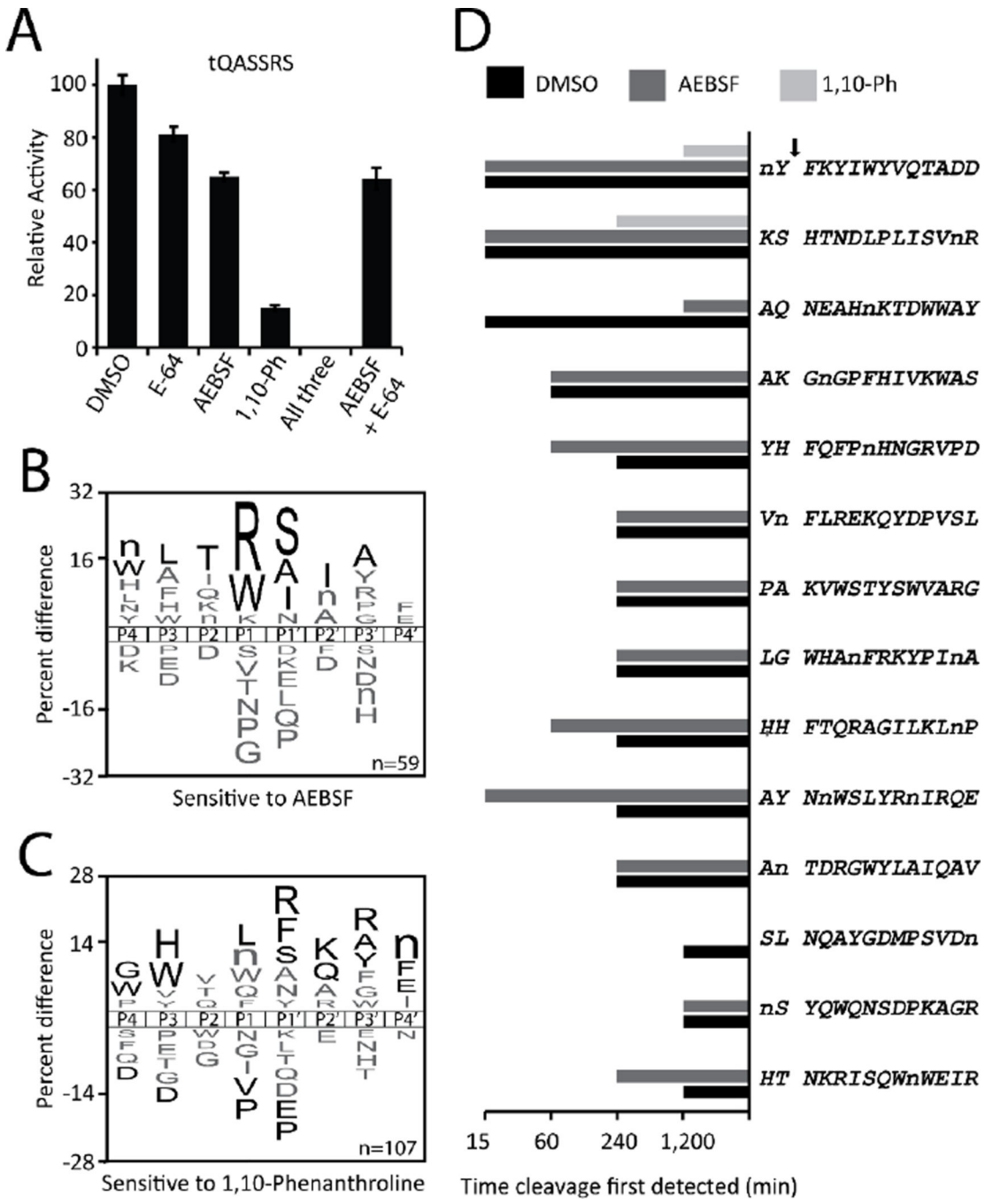

Figure 5. Use of class-specific inhibitors to characterize peptidase activity and specificity in adult secretion

A. ES products were assayed with the internally quenched tQASSRS substrate in the presence of class-specific inhibitors. **B**. An iceLogo substrate profile consisting of all cleavage sites in 14-mer peptides that were sensitive to AEBSF treatment. **C**. An iceLogo substrate profile consisting of all cleavage sites in 14-mer peptides that were sensitive to 1,10-Phenanthroline treatment. **D**. Analysis of the di-aminopeptidase specificity detected in

adult ES products. The bar graph represents the time interval at which each the cleavage product was first detected.

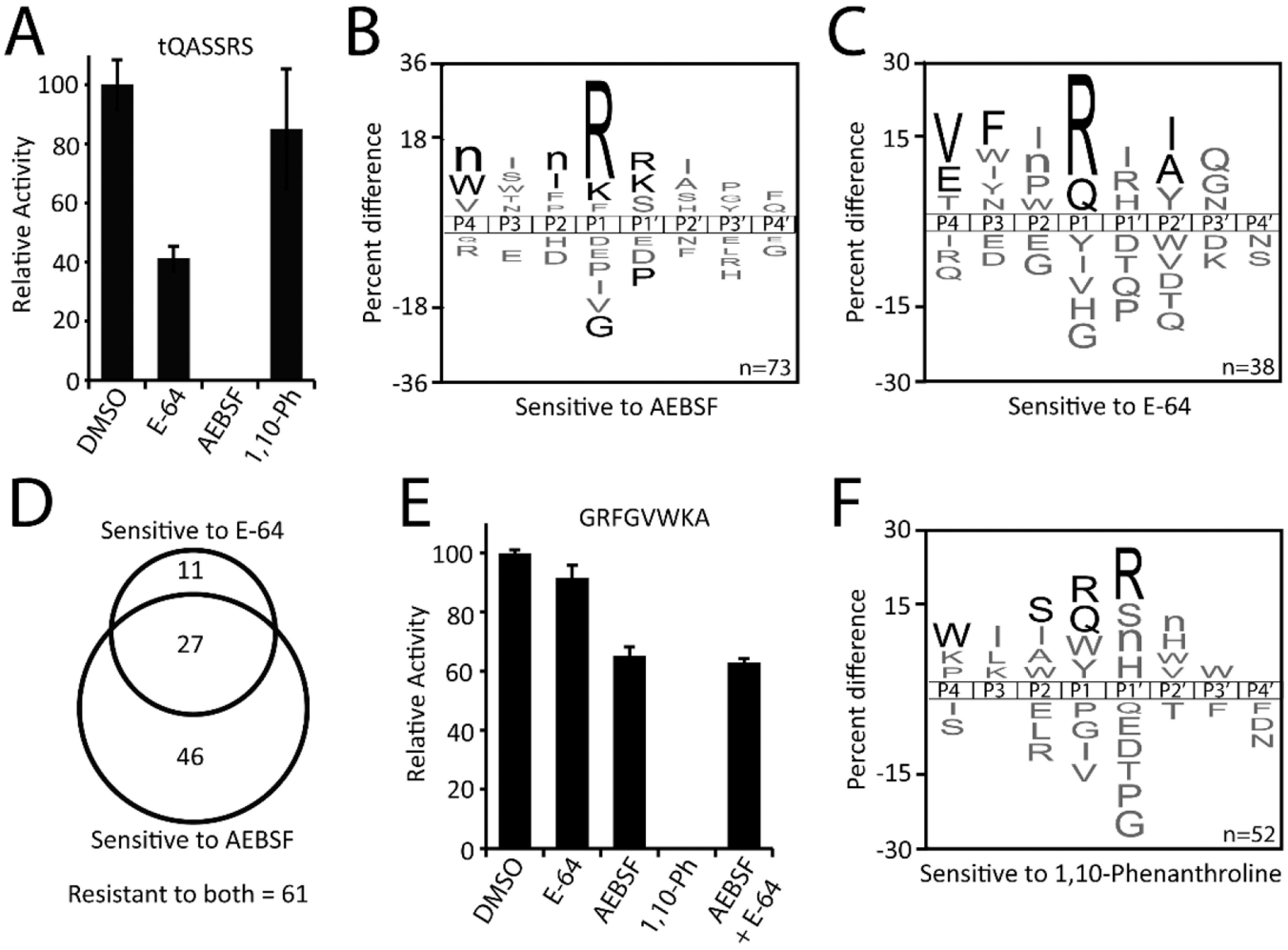

Figure 6. Use of class-specific inhibitors to characterize peptidase activity and specificity in egg secretion

A. ES products were assayed with the internally quenched tQASSRS substrate in the presence of class-specific inhibitors. **B**. An iceLogo substrate profile consisting of all cleavage sites in the 14-mer peptides that were sensitive to AEBSF treatment. **C**. An iceLogo substrate profile consisting of all cleavage sites in the 14-mer peptides that were sensitive to E-64 treatment. **D**. A Venn diagram showing that many of the cleavage sites are resistant to both AEBSF and E-64. **E**. ES products were assayed with the internally quenched GRFGVWKA substrate in the presence of class-specific inhibitors. **F**. An iceLogo substrate profile consisting of all cleavage sites in the 14-mer peptides that were sensitive to 1,10-Phenanthroline treatment.