Supplementary Material

Biochemical and physiological characterization of *Aedes aegypti* midgut chymotrypsin

Abigail G. Ramirez[1,2,#], Jun Isoe[1,3,#], Mateus Sá Magalhães Serafim[4], Daniel Fong[1], My Anh Le[1], James T. Nguyen[1], Olive E. Burata[5,6], Rachael M. Lucero[5,7], Rebecca K. Spangler[5,8], and Alberto A. Rascón, Jr.[1,5*]

[1]School of Molecular Sciences, Arizona State University, 551 E. University Dr. Tempe, AZ, 85281, USA

[2]Current address: Department of Chemistry, University of Wisconsin-Madison, Madison, WI, 53706, USA

[3]Department of Entomology, the University of Arizona, Tucson, AZ, 85721, USA

[4]Department of Microbiology, Federal University of Minas Gerais, Belo Horizonte, 31270-901, Minas Gerais, Brazil

[5]Former address: Department of Chemistry, San José State University, 1 Washington Square, San José, CA, 95112, USA

[6]Current address: Department of Bioengineering and Therapeutic Sciences, University of California, San Francisco, CA, 94143, USA

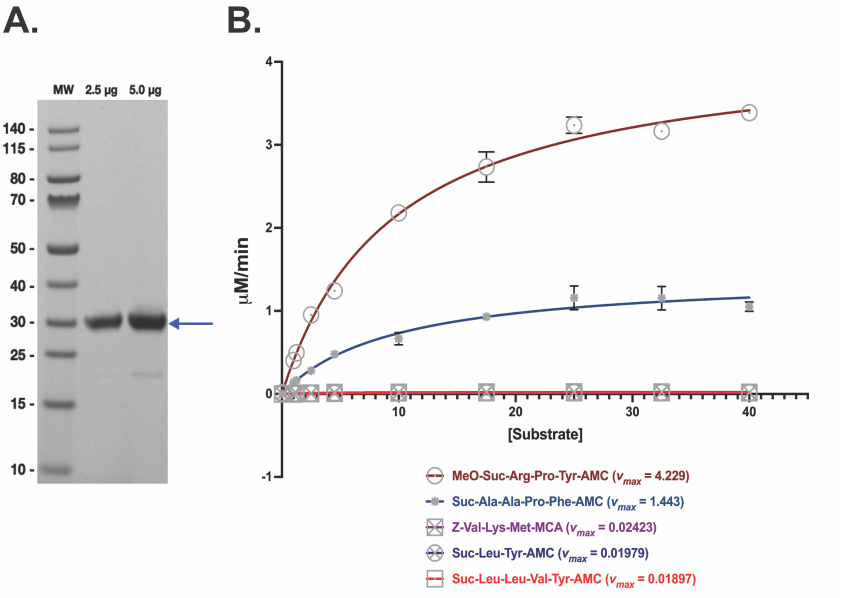
[7]Current address: Revolution Medicines, Redwood City, CA, 94063, USA

[8]Department of Chemistry and Biochemistry, University of California, Santa Cruz, Santa Cruz, CA 95064, USA

[#]First co-authors

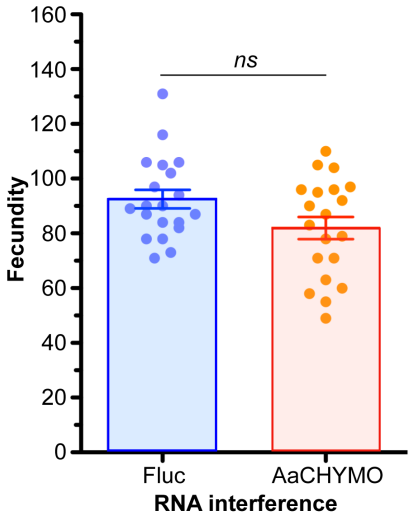
*Corresponding Author:
Alberto A. Rascón, Jr., aarasco1@asu.edu

Figure S1:



Activity assay determination of AaCHYMO under steady state conditions. A. Purified recombinant AaCHYMO (arrow). Different amounts of purified AaCHYMO were loaded onto a NuPAGE 4-12% BIS-TRIS gel (Invitrogen) to assess purity, but for clarity only 2.5 and 5 µg is shown. The protein gel was cropped, and the original unaltered protein gel is presented in **Supplementary File 1**. MW represents the pre-stained PageRuler protein ladder in kilodaltons (kDa) (Thermo Scientific #26616). **B.** Michaelis-Menten plots of active recombinant AaCHYMO obtained from testing different commercially available chymotrypsin substrates (each is listed with their corresponding v_{max}).

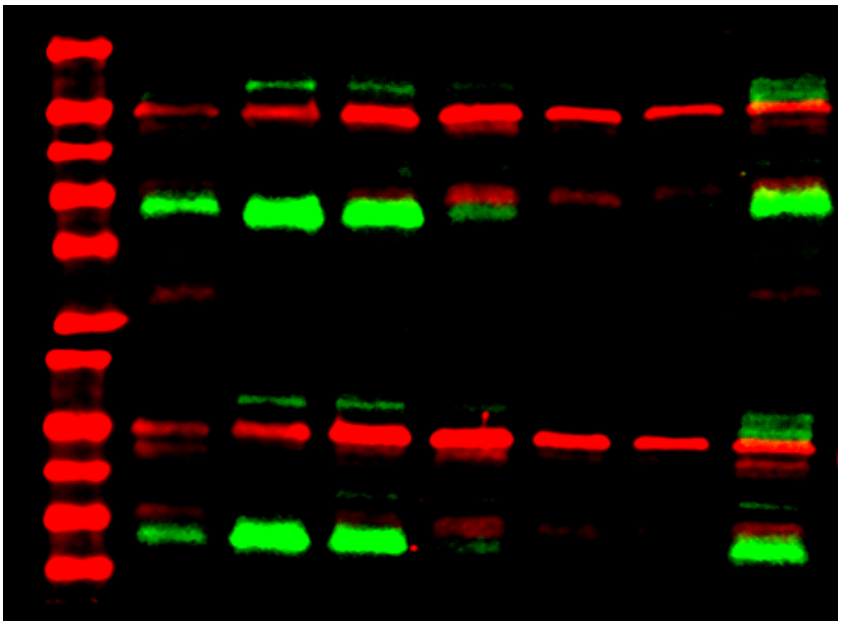
Figure S2:



Effect of AaCHYMO dsRNA injection on fecundity in individual mosquitoes. No significant changes in the number of eggs oviposited are observed between FLUC and RNAi AaCHYMO mosquitoes (ns = no significance, p = 0.052). A total of 3 biological cohorts of 20 individual mosquitoes were chosen for fecundity analysis.

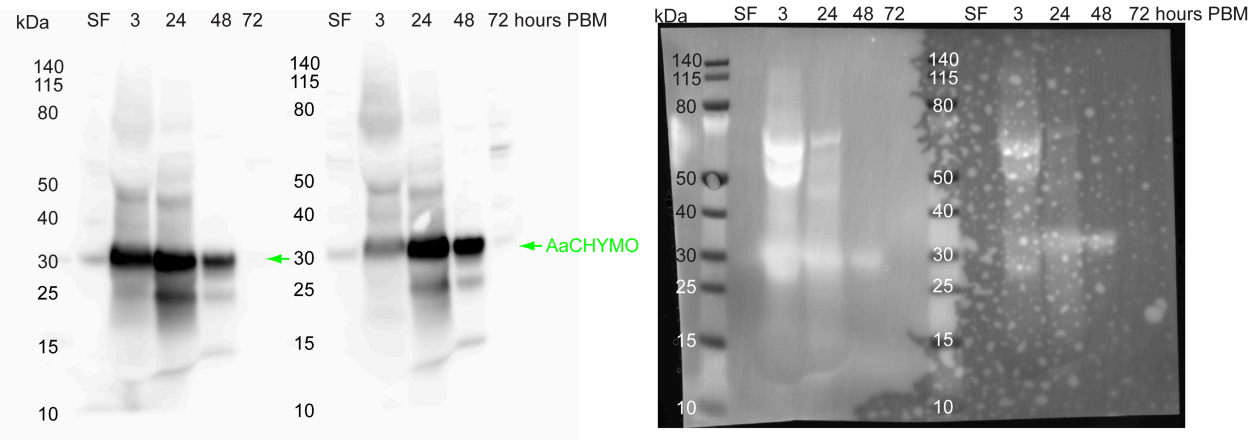
WESTERN BLOT ORIGINAL IMAGES

Fig 1A



Representative fluorescent WB of sugar (SF) and blood fed midgut epithelial cell samples without the food bolus detecting the presence of AaCHYMO at the time points indicated post blood feeding (PBF). α-tubulin was used as an internal control. Uncropped WB original images.

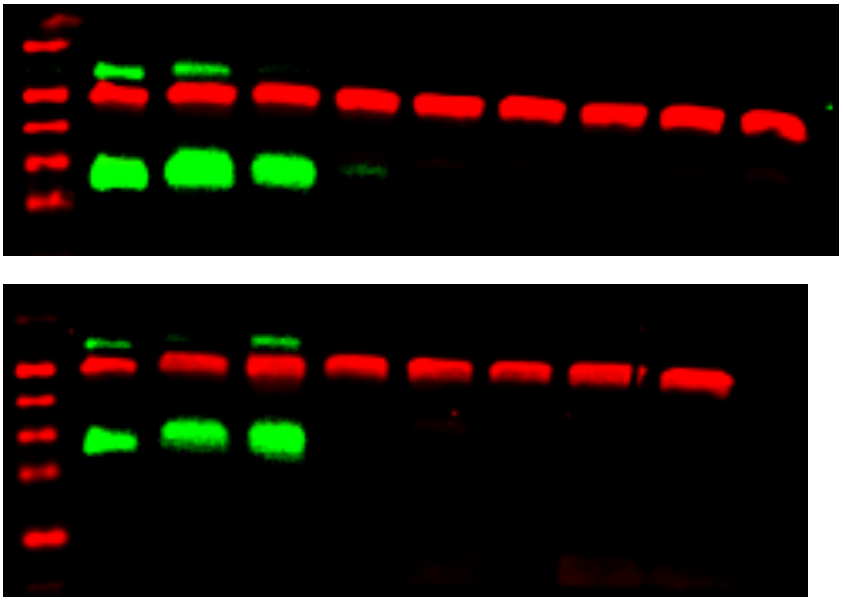
Fig 1C



Representative chemiluminescent WB of sugar- and blood-fed midgut extracts that contain the midgut epithelial cells plus the food bolus (0.5 midgut equivalents were used for analysis). The WB was cropped to separate out each independent set of samples and to allow the addition of the MW ladder. Uncropped WB original images.

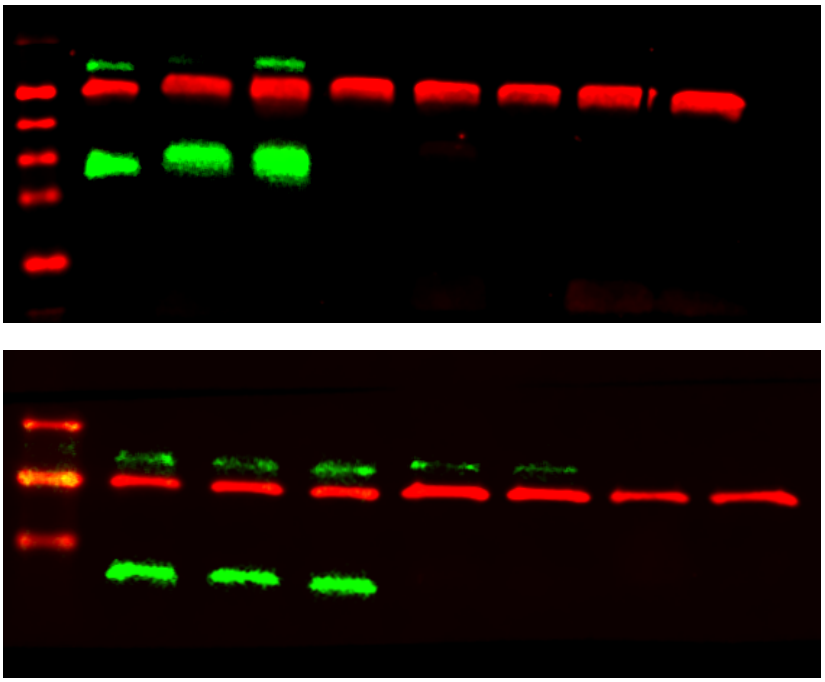
Please note that the two independent samples were run on the same blot. AaCHYMO was detected using chemiluminescence, which in this case only binds to the protein of interest. This is why no MW ladder is visible (picture on the left). Therefore, to properly obtain the MW info, the picture on the right shows the full WB membrane taken under the ethidium bromide settings on the BioRad Chemidoc to illustrate that the protein is present and to show there are no alterations. Additionally, this shows the ladder to be present. These ladders were used to match and align the proper molecular weights on the WB used in the manuscript.

Fig 2A



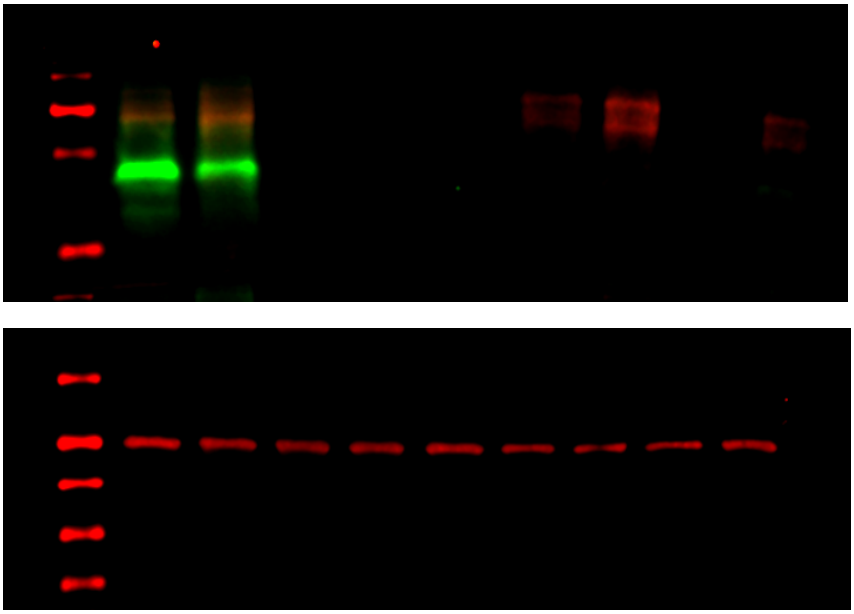
RNAi knockdown efficiency and chymotrypsin-like activity determination in individual *Ae. aegypti* mosquito midgut tissue extracts. Representative western blot (WB) of mosquito midgut extracts at 3 h PBM (**A**). α-tubulin was used as an internal control. Uncropped WB original images.

Fig 2B



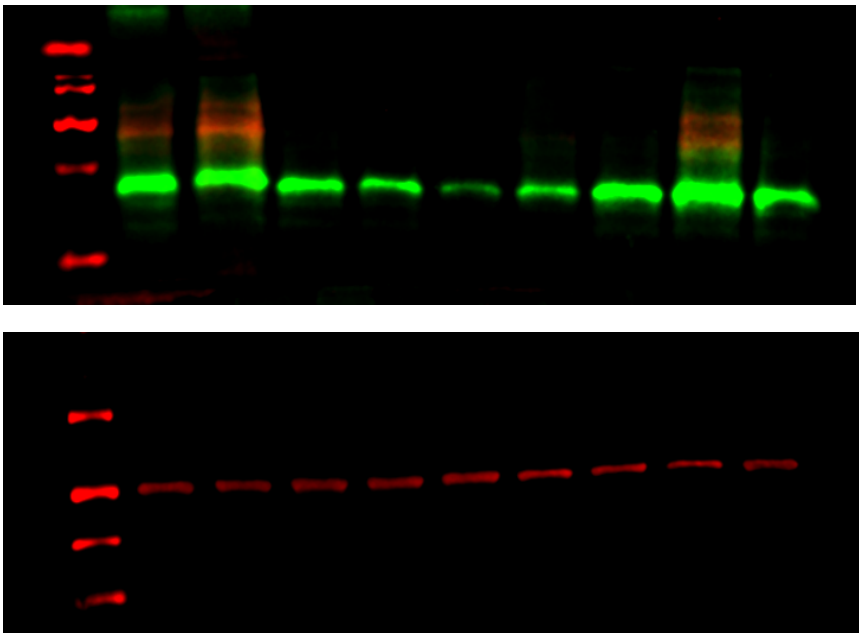
RNAi knockdown efficiency and chymotrypsin-like activity determination in individual *Ae. aegypti* mosquito midgut tissue extracts. Representative western blot (WB) of mosquito midgut extracts at 18 h PBM (**B**). α-tubulin was used as an internal control. Uncropped WB original images.

Fig 3A



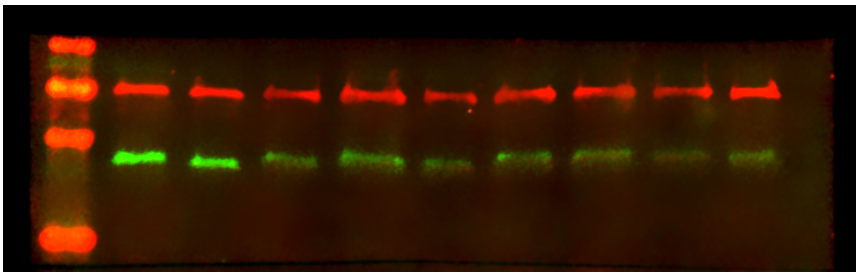
Western blot (WB) analysis showing that the double knockdown led to an effect on AaCHYMO expression (seven individual mosquitoes) and compared to the FLUC control (two individual mosquitoes). α-tubulin was used as an internal control. Uncropped WB original images.

Fig 3C



Western blot analysis showing that knockdown of AaLT has no effect on AaCHYMO expression. α-tubulin was used as an internal control. Uncropped WB original images.

Fig 7A



Western blot analysis of the EcR knockdown effect on AaCHYMO protein expression using an anti-AaCHYMO primary antibody. Midgut protein extracts were prepared from individual mosquitoes microinjected with dsRNA (RNAi-FLUC and -AaCHYMO). Each lane represents the set number of mosquito samples used for analysis. Each lane contains 0.5 midgut tissue equivalent. The Western blot is representative of 3 independent biological replicates. α-tubulin was used as an internal control. Uncropped WB original images.

PROTEIN GEL ORIGINAL IMAGES

Figs 4A-4C. Protein gel electrophoresis analysis of AaCHYMO, human and bovine chymotrypsin digestion assays with the three major human blood proteins: human serum albumin (HSA) (A), hemoglobin (Hb) (B), and immunoglobulin G (IgG) (C), incubated for a total of 60 min post-protease addition. Time = 0 min is the no protease control in each reaction.

Fig 4A – Human Serum Albumin (HSA) Digestion Assays

Bovine Chymotrypsin left samples. AaCHYMO right.

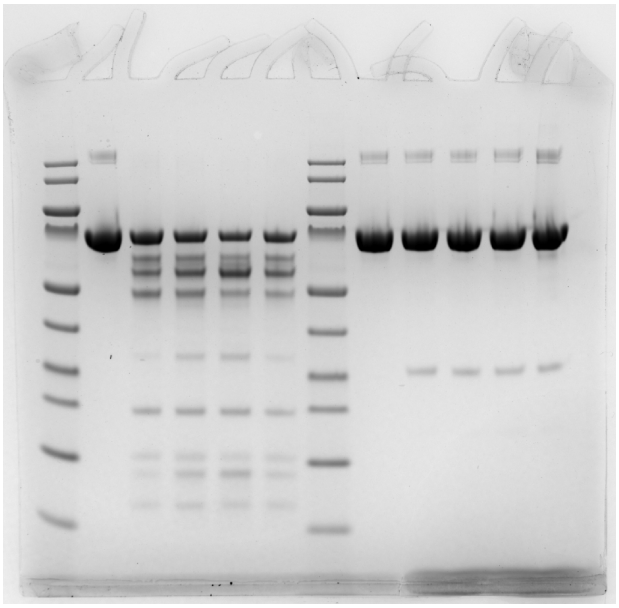


Fig 4A – Human Serum Albumin (HSA) Digestion Assay: Human Chymotrypsin Only

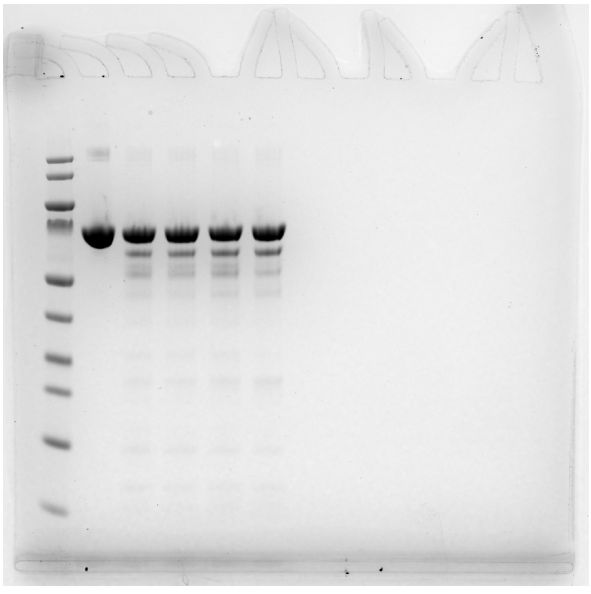


Fig 4B – Hemoglobin (Hb) Digestion Assays

AaCHYMO left samples. Human Chymotrypsin right.

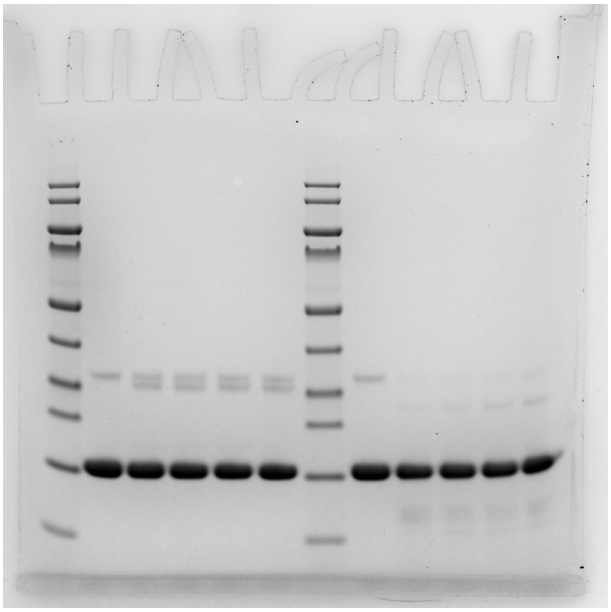


Fig 4C – Immunoglobulin G (IgG) Digestion Assay Left samples for Bovine Chymotrypsin. **Fig 4B**. Hb Digestion Assay Right samples for Bovine Chymotrypsin

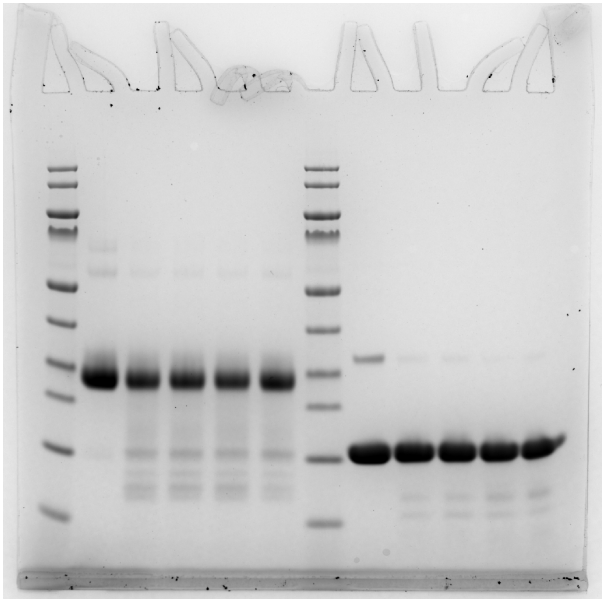


Fig 4C Immunoglobulin G (IgG) Digestion Assays

Human Chymotrypsin left samples. AaCHYMO right.

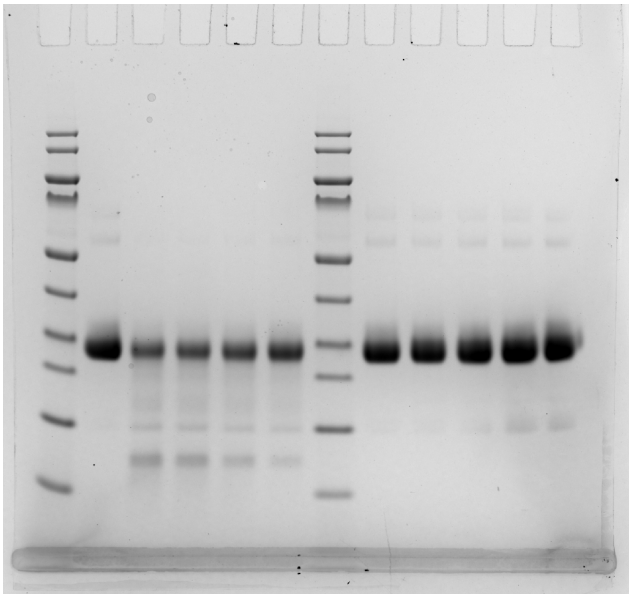


Fig 5A FLUC Control - *Ae. aegypti* midgut blood protein digestion extract analysis.

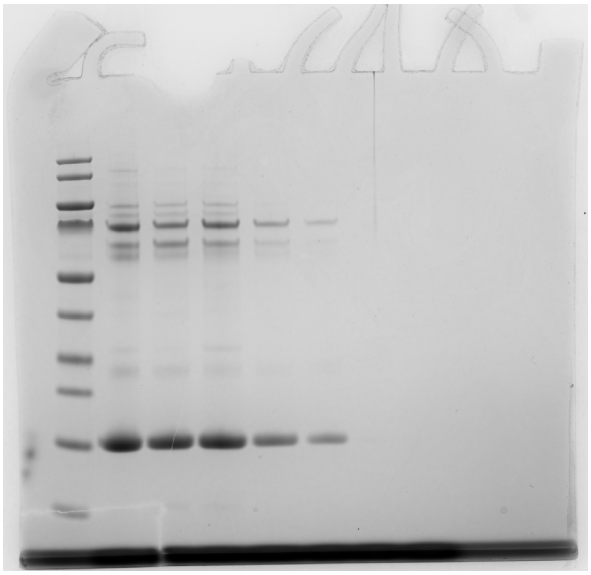


Fig 5A RNAi AaCHYMO – *Ae. aegypti* midgut blood protein digestion extract analysis.

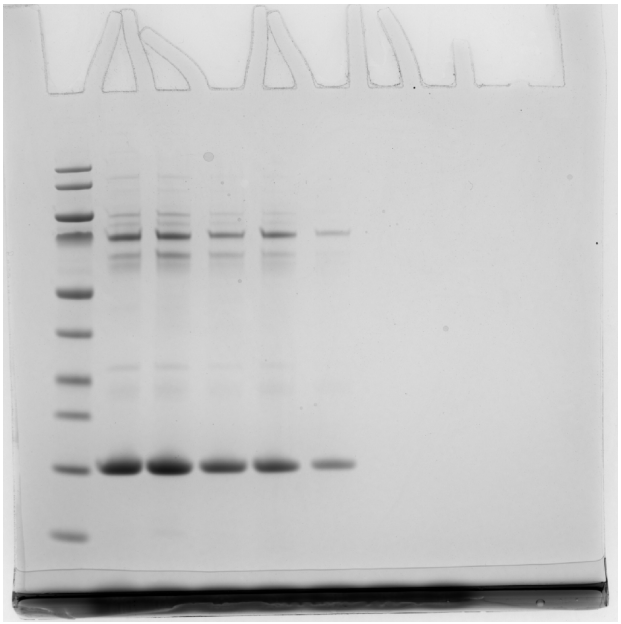
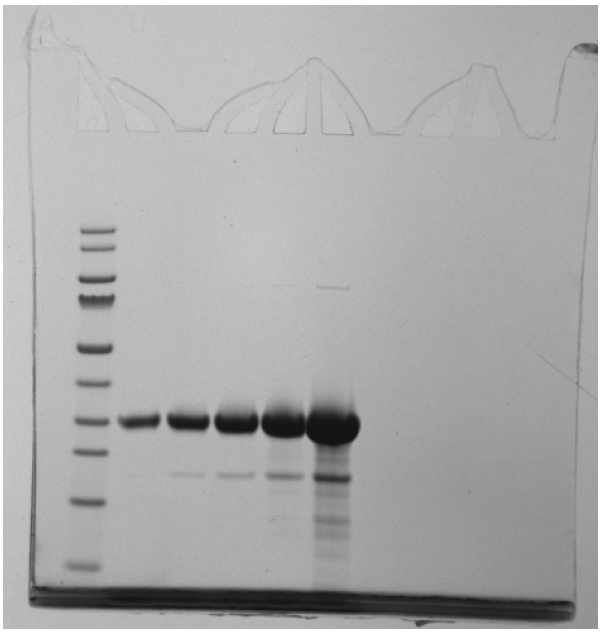


Fig S1A



Purified recombinant AaCHYMO (arrow). Different amounts of purified AaCHYMO were loaded onto a NuPAGE 4-12% BIS-TRIS gel (Invitrogen) to assess purity, but for clarity only 2.5 and 5 μg is shown. Uncropped original protein gel.

Supplementary Table S1. Commercially available and AaCHYMO-specific AMC substrates used.

Substrate (Commercially Available)	*Company*	*Catalog #*	*Substrate (AaCHYMO-Specific Designed)*	*Company*	*Catalog #*
Suc-Ala-Ala-Pro-Phe-AMC	Biosynth	MAA-3114-V	Ac-Ala-His-Val-Phe-AMC	Biomatik	1169025
Suc-Ala-Ala-Phe-AMC	Biosynth	FS110519	Ac-Ala-His-Leu-Phe-AMC	Biomatik	1169029
MeO-Suc-Arg-Pro-Tyr-AMC	Biosynth	SAP-3885-PI	Ac-Ala-His-Val-Tyr-AMC	Biomatik	1169023
Suc-Leu-Leu-Val-Tyr-AMC	Biosynth	MLL-3120-V	Ac-Ala-His-Leu-Tyr-AMC	Biomatik	1169027
Suc-Arg-Pro-Phe-His-Leu-Leu-Val-Tyr-MCA	Biosynth	MRP-3110-V	Ac-Ala-His-Thr-Trp-AMC	Biomatik	1169037
Suc-Leu-Tyr-AMC	Enzo Life Sciences	ALX-260-054-M005	Ac-Ala-His-Asp-Trp-AMC	Biomatik	1169039
Suc-Ile-Ile-Trp-MCA	Biosynth	MIW-3150-V	Ac-Ala-His-Val-Trp-AMC	Biomatik	1169038
Ac-Ala-Asn-Trp-AMC	AdipoGen	AG-CP3-0037	Ac-Ala-His-Val-Leu-AMC	Biomatik	1169024
Z-Leu-Leu-Leu-MCA	Biosynth	MLL-3177-V	Ac-Pro-His-Val-Met-AMC	Biomatik	1169036
Ac-Pro-Ala-Leu-AMC	AdipoGen	AG-CP3-0036	Ac-Ala-His-Val-Met-AMC	Biomatik	1169026
Z-Gly-Gly-Leu-AMC	Thermo Scientific	J64796LB0			
Z-Val-Lys-Met-MCA	Biosynth	MVM-3156-V			
Z-Arg-Arg-AMC	Biosynth	MRR-3123-V			
Boc-Val-Leu-Lys-AMC	Biosynth	MVL-3104-V			

Supplementary Table S2. Gene-specific primers used for RNAi and qPCR

Gene names		Primer sequence (5' to 3')	PCR size
Gene-specific primers used for RNAi			
Chymotrypsin, AaCHYMO	Forward	CTCACGGTAGCTTTCCTGCT	587 bp
	Reverse	CTCTTCGCTAGAGCACTGTC	
Ecdysone receptor, EcR	Forward	GGTTCTGGAGGAACAACGAC	544 bp
	Reverse	GTGTAGGTGTGGCTCTGCGA	
Late trypsin, LT	Forward	GCATCTCTGATGGCTTTGGCTTC	516 bp
	Reverse	GCATTCGTTGTTACTGATCACCGT	
Firefly Luciferase, Fluc	Forward	AGCACTCTGATTGACAAATACGA	548 bp
pGL3-Basic Vector	Reverse	AGTTCACCGGCGTCATCGTC	
Gene-specific primers used for qPCR			
Chymotrypsin, AaCHYMO	Forward	GAACAATGTGACACCCTGTGG	116 bp
	Reverse	ACCAACGGGCCACCTGAATC	
Ecdysone receptor, EcR	Forward	TGGATTTCCTCTCCTAGTCC	113 bp
	Reverse	GACGAGTAGCCATTGGACAT	
Late trypsin, LT	Forward	GGAAGTGATACCTTTACCGACCG	150 bp
	Reverse	GATCACCAACGGGCTGTAGGC	
Ribosomal protein S7, RPS7	Forward	ACCGCCGTCTACGATGCCA	131 bp
	Reverse	ATGGTGGTCTGCTGGTTCTT	

T7 promoter sequence (5' TAATACGACTCACTATAGGGAGA 3') was added in 5' of each RNAi primer.