Amino Acid Determination of the Dominant Protein Isolated from Fertilized, Corticated *Ascaris lumbricoides* Egg

De Guzman, Ana Isabella[*], Sevilla, Alyssa Denise[*], Bernabe, Adam Lesner[*], Baustista, Carmela[*], Yanga, Aedrick[*], De Ocampo, Yves Aaron[*], Martin I, Gregorio[*], Guevarra Jr., Leonardo[**]

[*] Department of Medical Technology, University of Santo Tomas
[**] Department of Biochemistry, University of Santo Tomas

***Abstract*:** Ascariasis is a neglected tropical disease (NTD) affecting one billion people worldwide. The most common laboratory diagnosis is still through the microscopic identification of *Ascaris lumbricoides* eggs from stool sample; thus, there is a need to establish a gold standard for its identification. In line with this, investigation of the amino acid content of the protein coat was done for possible development of rapid diagnostic tests. Micro-Bradford assay of the supernatants after decortication using Phosphate-Buffered Saline with Tween 20 revealed increasing absorbance suggestive of increasing protein content (r=0.71). Results of the High-Performance Liquid Chromatography exhibited peaks belonging to arginine and tyrosine. Moreover, these amino acids recovered are present in proteins which are inhibitors of mammalian trypsin and chymotrypsin.

***Index Terms*:** *Ascaris lumbricoides*, Decortication, High Performance Liquid Chromatography with FMOC-Cl derivatization, PBS-Tween 20, Rapid Diagnostic Tests

I. Introduction

Being the most common type of parasitic infection in the world, soil-transmitted helminthiasis is estimated to affect more than two billion people worldwide (Tchuem Tchuenté, 2011). The area most susceptible to the disease frequently includes tropical and subtropical regions and those with poor sanitation. Ascariasis is acquired through ingestion of the embryonated egg from food contaminated with soil, particularly when human feces is used as fertilizer or irrigated with ineffectively treated waste water. The eggs develop in the soil and become infective after two to three weeks but can remain infective for several months or years. Severe *A. lumbricoides* infection causes about 60,000 deaths per year, mainly in children since they often become infected when they place their contaminated hands into their mouths. (World Health Organization, 2015).

Laboratory diagnosis of ascariasis is done through the recovery of *Ascaris lumbricoides* eggs in stool. Although detection through microscopy is relatively inexpensive and simple, it has many limitations for it is time-consuming, labor-intensive, and it may include fluctuations in egg detection which contributes to its limited sensitivity and specificity (Lamberton & Jourdan, 2015). Thus, alternatives are currently studied and developed and one of these is the use of rapid diagnostic tests (RDTs). RDTs are known as diagnostic assays intended for use at the point-of-care which can be adapted for use in low-resource settings. An RDT is low-cost, requires simple operation, sensitive, specific, stable at high temperatures, and gives immediate results. RDTs are already in use for several neglected diseases and the majority that is in use today is based on immunoassays which involve the study of antigenic proteins (BIO Ventures for Global Health, 2015).

According to Florkin (2012), a layer of protein exists on the inner side of the chitinous "hard-shell" of *A. lumbricoides*. It is likely that this layer is made from protein granules in the primary ovocyte. Several studies have proven the role of protein in *Ascaris* egg as a marker used in molecular diagnostic, serologic, systematic, and biological studies of parasitic nematodes. The study done by Tormo and Chordi (as cited in Florkin, 2012), examined a number of purified protein-containing antigens. Although many of the studies have already shown the importance of proteins, ascariasis is still considered as a neglected disease and reliably approximating the scope of the problem is difficult because of inaccuracies and imprecisions in parasitological diagnosis and the lack of definite clinical signs (Brooker & Pullan, 2013). Its prevalence continues; therefore, further studies on the amino acids present in the dominant protein of *A. lumbricoides* egg should be conducted and performed. This can help and aid future researchers in developing a rapid diagnostic test that will be specific and highly sensitive for detecting *A. lumbricoides* infection.

II. Objectives

The general objective of this study was to determine the amino acid composition of the dominant protein isolated from the fertilized corticated *Ascaris lumbricoides* egg.

The specific objectives are as follows:

a) To obtain a pure isolate of the fertilized, corticated *Ascaris lumbricoides* egg;
b) To decorticate and isolate the dominant protein from the recovered eggs;
c) To determine which among 10%, 20%, 30%, 40%, and 50% is the optimum concentration of PBS-Tween 20 to be used in decortication; and
d) To determine the concentration of the amino acids present in the protein isolate.

III. Materials and Methods

The sample used in the experiment was a formalinized stool positive for fertilized, corticated *Ascaris lumbricoides* egg

A. Isolation of Fertilized, Corticated Ascaris lumbricoides Egg

The stool sample was suspended in a brine solution with a specific gravity of 1.20; this was left undisturbed for 24 hours. The eggs were then aspirated on the surface of the solution, transferred to a slide, and examined microscopically. Several well-washings were performed before the eggs were transferred to a conical tube containing 1 mL of normal saline solution. Well-washing was done in order to isolate the eggs from the fecal debris. After which, the pooled eggs were washed three times using phosphate-buffered saline in order to remove the formalin.

B. Decortication of the Isolated Parasite Eggs

Aliquots of the sample containing 20 eggs each were prepared in Eppendorf tubes. The aliquots were decanted until 0.5 mL was left in the tubes. PBS-Tween 20 with concentrations of 10%, 20%, 30%, 40%, and 50% were added until it reached the 1.0 mL mark. Vortex-mixing of the tubes was done for 1 hour and 45 minutes. To confirm if decortication occurred, the supernatants before and after decortication were analyzed using 96-well Micro-Bradford Protein Assay. The supernatant with the highest increase in absorbance was then used for amino acid determination.

C. Amino Acid Determination

Microwave-assisted Hydrolysis

The supernatant after decortication was used for microwave-assisted acid hydrolysis. Fifty microliters of the sample was mixed with 100 µL of 6M hydrochloric acid, placed inside an industrial microwave oven along with 100 mL of distilled water; this was done for 5 minutes at medium heat. Two hundred microliters of distilled water was added to the hydrolyzed sample to increase the volume.

High-Performance Liquid Chromatography with FMOC-Cl Derivatization was used for the identification of amino acids. Preparation of the physiologic amino acid standard and the sample was done by the researchers. However, High-Performance Liquid Chromatography was done by ADIP Quality Control Product Research and Development Cooperative.

Sample and Amino Acid Standard Preparation

For the preparation of the sample solution, 50 µL of the sample was mixed with a 100 µL of acetonitrile. The solution was adjusted to appropriate pH by adding 50 µL of borate buffer (40 mM in water, pH 9.2). It was then derivatized using 50 µL FMOC-Cl (10 mM in acetonitrile).

For the preparation of the reference standard solution, 40 µL standard solution and 10 µL 0.5 M sodium hydroxide were mixed to reach a nearly neutral solution. After that, the solution was mixed with 100 µL of acetonitrile. The solution was adjusted to appropriate pH by the addition of 50 µL borate buffer (40 mM in water, pH 9.2); it was then derivatized using 50 µL FMOC-Cl (10 mM in acetonitrile). Sigma-Aldrich A9906 Physiologic Amino Acid Standard was used in this study.

Chromatographic Conditions

The separation was done on a C_{18} column. The mobile phase were acetonitrile (A) and 50 mM sodium acetate solution, with a pH of 4.15 (B). The gradient elution profile was 0 to 30 min A:B (28:72, v/v) to (50:50, v/v), 30 to 37 min A:B (50:50, v/v) to (58:42, v/v), 37 to 38 min A:B (58:42, v/v) to (68:32, v/v), 38 to 44 min A:B (68:32, v/v) to (76:24, v/v), 44 to 45 min A:B (76:24, v/v) to (85:15, v/v), 45 to 54 min A:B (85:15, v/v). The flow-rate was 1.0 mL/min, detection wavelength was 263 nm, the column temperature was 30°C, and the injection volume was 100 µL.

IV. Results and Discussion

A. Isolation of the Dominant Protein

Microscopic images of the *A. lumbricoides* eggs before and after treatment with PBS-Tween 20 are shown in Figures 1 to 6. Notable changes in the morphology of the parasite eggs are observed. This agrees with the results obtained from the micro-Bradford protein assay where the absorbance of the supernatant after decortication increased in all of the concentrations used (Refer to Table I). Moreover, among the concentrations used, 50% PBS-Tween 20 was the optimum concentration since it obtained the largest increase in absorbance. The results are parallel with the study conducted by Amirilargani *et al*. (2009) where in increasing concentrations of Tween 20, there was a notable change in the membrane morphology. In the study done by Jamur and Oliver (2010), saponin selectively removes membrane cholesterol but detergents such as Tween-20 extract proteins along with the lipids since they are nonselective; a study by Schuck *et al.* (2003) also proves the nonselectivity of Tween 20 in the solubilization of cell membranes.

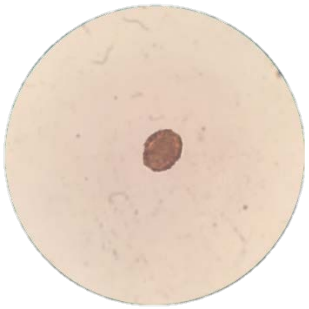

Figure 1: Microscopic Image of Fertilized, Corticated Egg of *Ascaris lumbricoides* Before Treatment with PBS-Tween 20

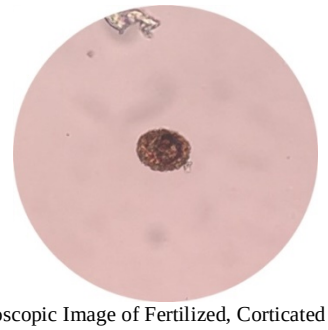

Figure 2: Microscopic Image of Fertilized, Corticated Egg of *Ascaris lumbricoides* After Treatment with 10% PBS-Tween 20

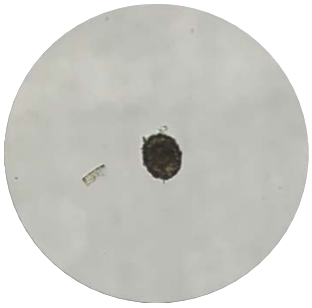

Figure 3: Microscopic Image of Fertilized, Corticated Egg of *Ascaris lumbricoides* After Treatment with 20% PBS-Tween 20

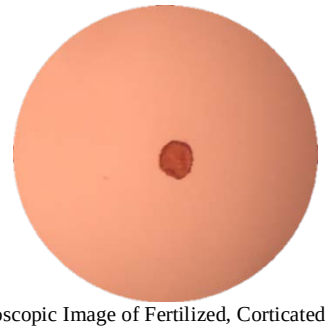

Figure 4: Microscopic Image of Fertilized, Corticated Egg of *Ascaris lumbricoides* After Treatment with 30% PBS-Tween 20

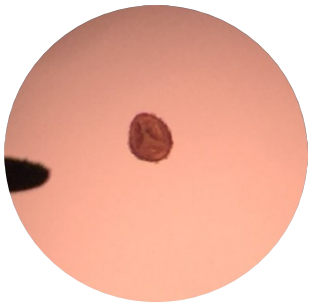

Figure 5: Microscopic Image of Fertilized, Corticated Egg of *Ascaris lumbricoides* After Treatment with 40% PBS-Tween 20

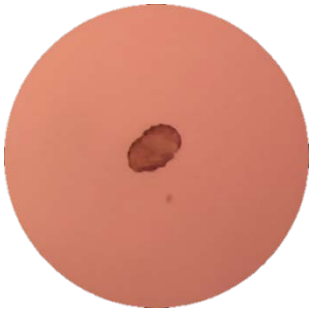

Figure 6: Microscopic Image of Fertilized, Corticated Egg of *Ascaris lumbricoides* After Treatment with 50% PBS-Tween 20

Table I. Absorbance difference of supernatant before and after decortication

Supernatant Fluid	PBS-Tween 20				
	10%	20%	30%	40%	50%
Before Decortication	0.783	0.790	0.860	0.794	0.813
After Decortication	0.821	0.857	0.912	0.843	1.903
$\text{Absorbance}_{\text{after}}$ - $\text{Absorbance}_{\text{before}}$	0.038	0.070	0.052	0.049	1.090

B. Determination of the Amino Acid Composition

Figures 7 and 8 are chromatogram of the samples for Trial 1 and Trial 2. After comparison of these chromatograms to that of the blank, Components 1 and 34 were identified as the content of the sample. The specific identification of amino acids was done by comparing the chromatogram of the standard (Refer to Figure 9) to the chromatogram of the standard used in the study conducted by Zhou *et al.* (2011) due to the same chromatographic conditions applied. Component 1 was identified as arginine, with a concentration of 1.627 µmol/mL and Component 34 was identified as tyrosine, with a concentration of 1.678 µmol/mL.

The results are parallel with Kreuzer's (1953) study which identified amino acids present from the outer layer of the primary egg envelope, and it included the presence of arginine. Meanwhile, Jaskowski (1962) determined the presence of tyrosine and arginine on the middle, chitinous coat. As stated by Yanagisawa (1955), a layer of protein may exist on the inner side of the chitinous shell of *Ascaris lumbricoides*. These granules on the inner side move to the surface and become associated with the layers of the primary egg shell. Moreover, Green (1955), Peanasky and Laskowski (1960, as cited in Florkin, 2012) stated that there are low-molecular weight proteins present in *A. lumbricoides* that inhibit the activity of mammalian trypsin and chymotrypsin. In at least four of the inhibitors, tyrosine and arginine were present; the said inhibitors showed stability to heat and pH changes.

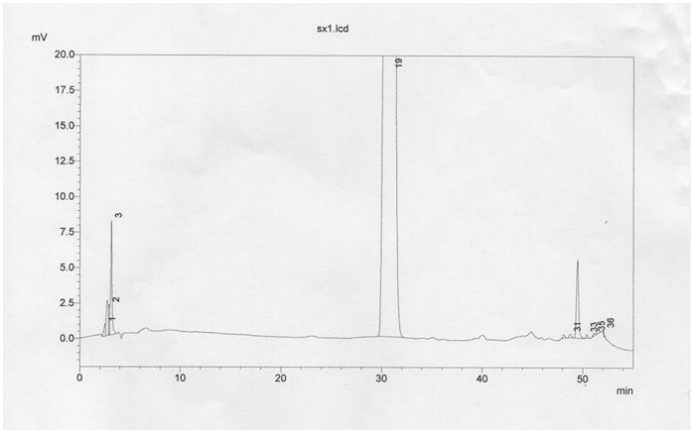

Figure 7: Chromatogram of Sample (Trial 1)

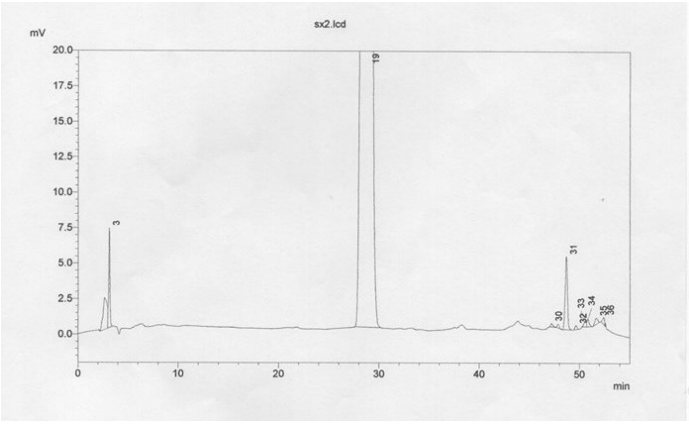

Figure 8: Chromatogram of Sample (Trial 2)

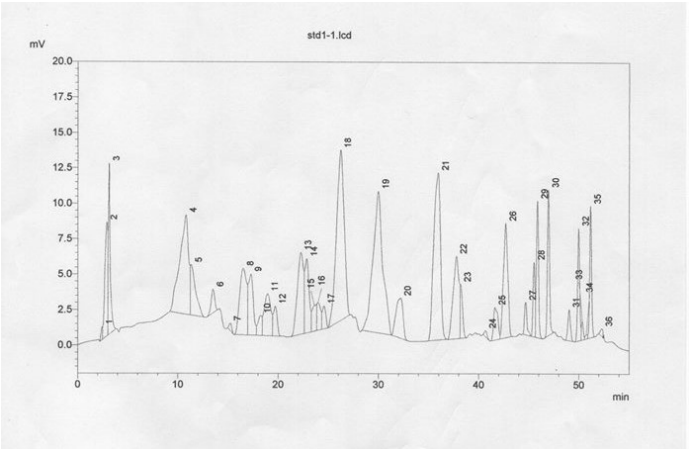

Figure 9: Chromatogram of Physiologic Amino Acid Standard

V. CONCLUSION AND RECOMMENDATIONS

Conclusion

The amino acids identified and quantified as a result of the experimentation were arginine and tyrosine with the concentrations of 1.627 µmol/mL and 1.678 µmol/mL, respectively. These amino acids are found in low-molecular weight proteins present in *Ascaris lumbricoides* which inhibit the activity of mammalian trypsin and chymotrypsin. Decortication using 50% PBS-Tween 20 obtained the greatest change in absorbance; thus, it is the optimum concentration to be used for the isolation of the protein coat as compared to the other concentrations used in this study. Furthermore, based on the data obtained, the use of increasing concentrations of PBS-Tween 20 also increased the amount of protein recovered from the supernatant (r=0.71).

Recommendations

For further improvement of the study, the researchers recommend the following:

1. A study on the amino acid sequence
2. Development of Rapid Diagnostic Tests for the identification of *Ascaris lumbricoides*
3. Increased exposure time with 50% PBS-Tween 20 and increased amount of sample to be hydrolyzed
4. Use of concentrations higher than 50% PBS-Tween 20
5. Analysis of the presence of other amino acids

REFERENCES

[1] Amirilargani, M., Saljoughi, E., & Mohammadi, T. (2009). Improvement of permeation performance of polyethersulfone (PES) ultrafiltration membranes via addition of Tween-20. *Journal of Applied Polymer Science*, 504- 513. doi: 10.1002/app.

[2] BIO Ventures for Global Health (n.d.). Retrieved from http://www.bvgh.org/Current-Programs/Neglected-Disease-Product-Pipelines/Global-Health-Primer/Targets/cid/ViewDetails/ItemID/16.aspx

[3] Brooker, S. J., & Pullan, R. L. (2013). Chapter 13-*Ascaris lumbricoides* and ascariasis: Estimating numbers infected and burden of disease. *Ascaris: The Neglected Parasite*, 343-362. doi:10.1016/B978-0-12-396978-1.00013-6.

[4] Florkin, M. (2012). Chemical Zoology V3: Echinodermata, Nematoda, and Acanthocephala. London: Academic Press.

[5] Jamur, M.C., & Oliver C. (2010). Permeabilization of cell membranes. *Methods in Molecular Biology, 588*, 63-66. doi:10.1007/978-1-59745-324-0_9.

[6] Lamberton, P. H. L., & Jourdan, P. M. (2015). Human ascariasis: Diagnostics update. *Current Tropical Medicine Reports*, *2*(4), 189–200. http://doi.org/10.1007/s40475-015-0064-9.

[7] Schuck, S., Honsho, M., Ekroos, K., Shevchenko, A., & Simons, K. (2003). Resistance of cell membranes to different detergents. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(10), 5795-5800. doi: 10.1073/pnas.0631579100.

[8] Tchuem Tchuenté, L. A. (2011). Control of soil-transmitted helminths in sub-Saharan Africa: Diagnosis, drug efficacy concerns and challenges. *Acta Tropica*, *120*, S4-S11. doi:10.1016/j.actatropica.2010.07.001

[9] *Water related diseases (2015). Retrieved from World Health Organization website:http://www.who.int/*water_sanitation_health/diseases/ascariasis/en/.

[10] Zhou, W., Zhang, X., & Duan,G. (2011). Liquid-chromatography quantitative analysis of 20 amino acids after derivatization with FMOC-Cl and its application to different origin *Radix isatidis*. *Journal of the Chinese Chemical Society*, *58*, 509-515.

AUTHORS

First Author: De Guzman, A., Department of Medical Technology, University of Santo Tomas. aisabelladg@gmail.com
Second Author: Sevilla, A., Department of Medical Technology, University of Santo Tomas. ailee_1275@yahoo.com
Third Author: Bernabe, A., Department of Medical Technology, University of Santo Tomas. adamlesnerbernabe@yahoo.com
Fourth Author: Bautista, C.,Department of Medical Technology, University of Santo Tomas. aaamielbautista@gmail.com
Fifth Author: Yanga, A., Department of Medical Technology, University of Santo Tomas. yaedrick@yahoo.com
Sixth Author: De Ocampo, Y., Department of Medical Technology, University of Santo Tomas. yves1113@gmail.com
Seventh Author: Martin I, G., Department of Medical Technology, University of Santo Tomas. uklid_greg@yahoo.com
Eighth Author: Guevarra, L., Department of Biochemistry, University of Santo Tomas.

Correspondence Author: De Guzman, A., aisabelladg@gmail.com