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Mummy Proteins Tell a Different Tale

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By analyzing ancient proteins, researchers are not only learning more about history but also rewriting it. Andrew S. Wiecek reports on recent advances that are providing new details about the past.

At the age of 15, she was chosen to be sacrificed. The preparation for this had begun a year beforehand; she had been confined to a closed house within the Inca capital of Cusco and was fed a rich diet of maize and llama meat. Then, she traveled more than 1200 miles to the snowy summit of the volcanic Mount Llullaillaco. After consuming an intoxifying meal of caco leaves washed down with maize beer, she fell asleep. She was then covered with volcanic ash and left to die. She was chosen by her people to be sacrificed to their gods because she was an example of physical and spiritual perfection.

Well, at least, that's the story according to the Spanish explorers. But forensic anthropologist Angelique Corthals isn't so sure. To piece together what was really happening during the last days of this young Inca girl's life, Corthals is pioneering new techniques and applications to analyze ancient proteins from her mummified remains.

Mount Llullaillaco was the site of Inca ritual sacrifices. Source: Wikipedia

500 Years Later

Corthals is part archeologist, part biomedical researcher. Think Indiana Jones in a lab coat. While she spends most of her time in her lab at the Stony Brook University School of Medicine using molecular biology techniques to study evolution, infectious disease, and climate change, she also gets into the field often. For example, one of her current projects is to help researchers from the University Libre de Bruxelles in Brussels, Belgium, identify human remains at a tomb in southern Egypt.

In 1999, about 500 years after that 15-year-old Inca girl was sacrificed, a group of archaeologists uncovered her body along with two younger children—a boy and a girl—that were also sacrificed at that time. The cold temperature and volcanic ash protected her body from decomposition. They nicknamed her "the maiden," and performed a genomic analysis to learn more about her (1).

But Corthals knew that analysis only scratched the surface of what those remains could tell us. Ancient DNA analysis can provide a list of genes in a given individual, but the problem is that not all of those genes are expressed at the same time. In contrast, protein analysis could tell us precisely what that individual was expressing at the time of their death.

“That’s the beauty of proteins. Proteins are basically what the individual is expressing at the time of sampling,” says Corthals. “So, if you sampled my proteins today and then resampled my proteins in a month, you wouldn’t get the same profile because my DNA would be expressed differently because of a response to disease or to diet or something else.”

For instance, if researchers discover the DNA of a pathogen in an ancient sample, they compare the ancient and the modern versions of the pathogen. But the presence of the pathogen doesn’t necessarily mean that the individual was infected by that pathogen or died because of it.

To figure that out, scientists need to analyze the immune system response of the host. By studying the interaction between the pathogen and the host’s immune system, you can gauge the severity of the infection. And to do that, you need tools and methods that can analyze the ancient proteins behind those interactions.

Angelique Corthals is part archeologist, part biomedical researcher. Source: Millsaps College

Further Back in Time

At the Natural History Museum of Denmark, postdoc Enrico Cappellini studies both ancient DNA and proteins. Like Corthals, he believes ancient proteomics nicely complements ancient DNA studies.

For instance, some proteins don’t degrade as quickly as DNA. While it is currently difficult to recover DNA for analysis from samples that are more than 500,000 years old, collagen—an abundant protein that makes up connective tissue in vertebrates—has been recovered from samples that are 1 million years old.

Of course, not all proteins are as thermostable as collagen in resisting degradation or as abundant as collagen. And not all samples are preserved in conditions that reduce environmental damage

to proteins. So, while researchers have been using this workflow to study ancient proteins for more than 20 years, success has been limited to analyzing only a small number of abundant proteins.

But now, recent advances in sample preparation, instrumentation, and software are improving ancient protein studies. For example, in a paper published last year in the *Journal of Proteome Research* (2), Capellini and colleagues analyzed 126 unique proteins from a 43,000-year-old woolly mammoth. “We showed it was possible to go from very few proteins of the most common, something boring like collagen, to something more interesting like albumin,” says Capellini. Albumin is a protein that helps transport other biomolecules through the bloodstream of a mammal.

“This is a very exciting time to get into ancient proteins because the technological developments that are coming out on a yearly basis are opening up a lot of possibilities,” says Capellini.

A typical proteomics workflow includes three steps: sample preparation, mass spectrometry, and data analysis. Protein samples are purified by liquid chromatography. Then, these proteins are fragmented into smaller peptides whose mass can be accurately measured by a mass spectrometer. Finally, these masses are then compared with a database of known peptide masses to identify the proteins in the sample.

One of the main keys to Capellini’s success was to limit sample loss at every step of sample preparation. In contrast to samples in modern protein studies in which more cells can be grown or experiments can be repeated, ancient samples are always limited. For example, the team eliminated precipitation steps in the sample preparation to reduce the loss of cross-linked proteins.

In 1999, archeologists found the preserved remains of three children who were sacrificed on Mount Llullallaico. Source: PLoS One

Techniques Age Quickly

Another thing that researchers are constantly concerned about when working with ancient samples is contamination. One of the most common contaminants that has plagued proteomics has been keratin, a structural protein that makes up human skin, hair, and nails.

As a result, proteomics labs follow strict protocols to reduce the introduction of keratin and remove the slightest traces of contamination. For example, Corthals’ lab performs peptide fractionation by liquid chromatography rather than gel electrophoresis. With a gel-based approach, technicians remove proteins from a solution after lysis, run them on a gel to resolve

them, and then return them to a solution for **MS analysis**. Liquid fractionation keeps the proteins in solution, reducing the possibility of contamination.

In addition, data analysis software used to identify the peptides and proteins from the MS data have improved over the years. Now, these programs make fewer false positive identifications, thanks to mathematical biologists and programmers who have developed new algorithms. "That's not trivial," says Corthals. "It's paramount to the field. Those people are the unsung heroes because no one wants to hear about the programming."

Furthermore, **ancient proteomics** has another data analysis challenge: what do you search your ancient MS data against? For the woolly mammoth study, Cappellini and colleagues searched against a protein analysis of its modern descendant, the elephant. While a search against a high-quality mammoth genome could have identified new isoforms or similar enzymes with different functions, the genome that was published in 2008—one of the first examples of sequencing ancient DNA—was only done at 0.7-fold coverage (3).

"We weren't able to do anything with that," says Capellini. "But no one is going to embark on the mammoth genome sequencing because it's already been published. It's not rewarding from a publication exploitation point of view. So, we had to rely on modern stuff."

And all these published techniques to study ancient proteins are already, well...ancient. Since the woolly mammoth study, Capellini's team has replaced the mass spectrometer that they used with a newer one that has significantly improved the number of peptides and proteins identified.

"At the moment, we changed almost everything. I can tell you something, but I can't tell you much. Not everything is published at the moment," says Capellini. "In terms of sample preparation, I have to be more vague...we continue to improve ways to minimize losses and improve recovery. Demineralization steps and digestive steps, we... the main point here is we're not throwing anything away."

Not So Perfect After All

So, with these recent technical advances in the ancient proteomics, Corthals prepared herself to uncover what was really happening the final days of the 15-year-old Inca maiden. She convinced the archeologists who had found the remains to let her take a sample.

The remains of the maiden and her two younger companions are stored at -20 degrees at the Museum of High Mountain Archaeology in the city of Salta, Argentina, close to Mount Llullaillaco where the bodies were discovered. Corthals was allowed to take three samples: a piece of blood-soaked cloth from the boy and a cotton swab from the lips of the maiden and the boy. Back at her lab, Corthals and her team handled these samples with the strict protocols in place in her lab as they prepared them for LC-MS.

In a paper published earlier this year in the *Public Library of Science ONE*, Corthals and colleagues reported the first use of **shotgun proteomics** to detect the immune response of an ancient sample. As a result, her team discovered that these two Inca children—children selected

because they represented an idealized state of humanity, according to Spanish explorers—were battling a chronic lung infection. Needless to say, these findings have opened up new questions about the past.

“It puts into question, first of all, the idea of perfection, in terms of that these children need to be perfect,” says Corthals. “And also it puts into question these secondary sources, which are our only sources we have of the Inca empire because they didn’t have writing. And it starts opening a whole new field of questions of what was happening, and makes things a little more exciting than we thought they were.”

In the end, Corthals believes that there might be some plausible explanations for their findings. For instance, these children might have acquired their infections sometime during the months of preparation for the ritual sacrifice. It could have been a result of being confined to a closed house with an open fire during this time period. This smoky environment could have irritated the lungs, allowing for an opportunistic infection. Another possibility could have been the 1200-mile journey from Cusco to Mount Llullaillaco. “It’s the equivalent of walking from Mexico to the Canadian border. For a 15 year old, that’s pretty tough,” says Corthals.

The next step is to test the shotgun proteomics technique to study the immune system response in older, more damaged samples. And she already has a target in mind: the frozen corpse of a flu victim from the 1918 Spanish flu epidemic. “Everybody’s been studying the Spanish flu virus to death, but nobody’s been actually looking at the immune system response,” says Corthals. “But that’s just a pipe dream.”

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