Extraction of Proteins from 30 Million Year Old Amber Using *The PCT Shredder* and Pressure Cycling Technology (PCT)

Introduction

The mass extinction of the dinosaurs, marked by the Cretaceous-Tertiary boundary, pales in magnitude compared to other lesser known mass extinction events, such as the Permian-Triassic boundary. As over 99% of all of the species that ever lived are now extinct, our understanding of biological processes has been limited by what we have learned from the fewer than 1% of species that have survived more than five major mass extinction events. Recently, collagen peptides were reportedly recovered from mineralized skeletal elements of *Tyrannosaurus rex* and *Brachylophosaurus canadensis* [1,2], indicating that proteins could be preserved over geological time spans.

Michael Crichton's novel *Jurassic Park* proposed the recovery of dinosaur DNA from the alimentary tracts of hemophagous insects preserved for millions of years in amber. Though Crichton's work was purely fictional, amber inclusions have shown remarkable preservation of organisms, at least at the tissue and cellular levels. Reptilian blood cells have actually been identified from partially digested blood meals in parasitic insects encapsulated in Cretaceous amber [3].

In its unfossilized form, the resins from which amber is derived contain diterpenoids which can rapidly dehydrate a "trapped" specimen, a prerequisite for preservation, and possesses anti-microbial properties that inhibit the usual decomposition processes. Such conditions have allowed DNA to be isolated from a 30 million year old fossil termite *Mastotermes electrodominicus* preserved in amber [4]. Similarly, scientists have isolated amino acids, peptides and proteins from organisms in amber.

Figure 1. *Preservation of fine structure in amber, including individual ommatidia of the compound eye of a formicine identified in Oligo-Miocene amber 20-30 million years old. Magnification bar equals 0.6 mm. (Courtesy of P.G. Righetti, Politechnico di Milano, Italy and G.B. Smejkal, Harvard University.)*

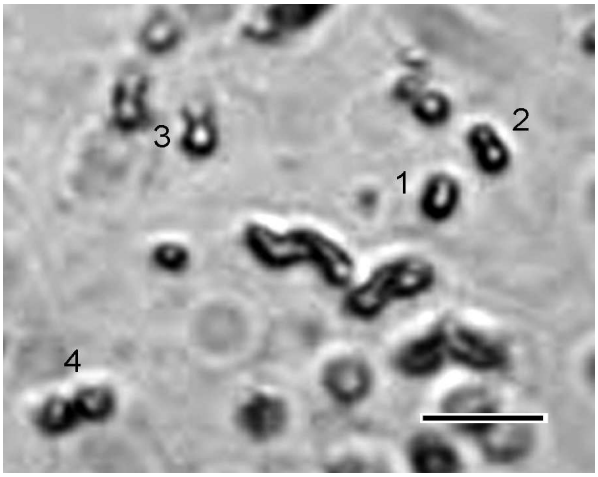

Figure 2. *Budding yeast cells in Dominican Republic amber 20-30 million years old. Growth stages are visible:* ***(1)*** *single cell;* ***(4)*** *progeny cell (nearly equal in size to its parent); and intermediary stages* ***(2, 3)****. Magnification bar equals 0.006 mm. (Courtesy of G.O. Poinar, Oregon State University.)*

Bada et al., [6] have isolated amino acids from 40-100 million year old insects in amber, representing the oldest unaltered amino acids reported to date. Compared to other fossils, the rate of amino acid racemization was retarded by four orders of magnitude in amber inclusions. Here we report a method of extracting of protein from yeast inclusions in amber which was developed by Smejkal et al. [7] using *The PCT Shredder* and pressure cycling technology (PCT).

Materials and Methods

Amber triturates were extracted in 125 mM Tris-HCl pH 6.8 containing 2% SDS, 5 mM tributylphosphine, 20 mM aminoethylbenzene sulfonyl fluoride, 10 mM EDTA and 25 mM phenylacylthiazolium bromide by using a Shredder PULSE Tube specially fitted with a stainless steel serrated ram insert in stead of the commercially available serrated plastic ram. The samples were subjected to PCT (pressurized for 100 X 100 second cycles at 35,000 psi maximum pressure in the Barocycler NEP 3229). Each Shredder PULSE Tube was coupled to the insert of a sterile Ultrafree CL centrifugal filter (Millipore Corporation, Danvers, MA) and evacuated by centrifugation at 1000 RCF for one minute. Filtrates were applied directly to 8-16% PAGE (BioRad, Hercules, CA). Gels were stained using the mass spectrometry compatible SilverQuest Silver Stain Kit (Invitrogen, Carlsbad, CA). Following trypsin digestion, the resulting proteins were analyzed by mass spectrometry.

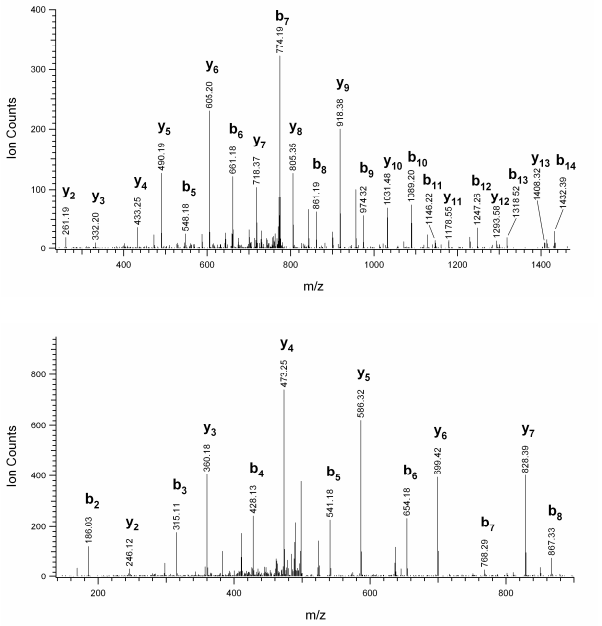

Figure 3. *Peptides with sequence homology to Saccharomyces enolase 1 (top) and alcohol dehydrogenase (bottom) isolated from 40 million year old amber inclusions. (Courtesy of F. Chu, University of New Hampshire.)*

Results and Discussion

Proteins isolated from the amber failed to penetrate a 4% polyacrylamide gel and accumulated as a single band on the surface of the gel, suggesting an extraordinary high molecular mass, possibly due to extensive crosslinking (a second band which penetrated the gel was identified as keratin). Thus, PAGE may prove to be an effective means of (i) concentrating trace proteins from paleontological samples, while (ii) removing substances that could otherwise interfere with later analysis by mass spectrometry, and (iii) also removing contemporary contaminants which have much lower molecular mass.

Trypsin digests of putative protein extracted from amber were analyzed by mass spectrometry. Through this method, 86 peptides with sequence homology to 20 *Saccharomyces* proteins were identified. Although we cannot yet rigorously prove that these proteins are derived from ancient yeast, the high degree of crosslinking of these proteins suggests that these are of prehistoric origin rather than the result of contemporary contamination**.**

Peptide sequences of the top five extracted proteins were further evaluated to verify species assignment. For example, enolase 1 sequences from the yeasts *S. cerevisiae* and the tree *Hevea brasiliensis* were compared. Although seven of the eleven identified peptides showed conserved regions with high sequence similarity, none of the peptides have identical sequence between these two species.

Over the course of millions of years, there is endless opportunity for the modification or even the complete destruction of proteins. However, for living organisms that become trapped in terpenous resins, subsequent dehydration could curtail indigenous proteolysis as well as the non-enzymatic hydrolysis of proteins. Because the amber environment is water-free, the hydrolysis of peptide bonds is inhibited. As a result, putative proteins extracted from amber may provide a reservoir of proteins for analysis which may reveal a wealth of information and insight into evolution. *The PCT Shredder* and pressure cycling technology (PCT) combine to provide an effective method for extracting proteins from organisms preserved in amber.

References

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14 Norfolk Avenue South Easton, MA 02375
TEL 508-230-1828 • FAX 508-580-1829
www.pressurebiosciences.com