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A Minimally Destructive Method for Determining Protein Preservation

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Abstract

One of the problems associated with tissues following death and long term storage is that the component biomolecules degrade to a greater or lesser extent. As a direct result of this degradation it is necessary to use larger specimens for analysis compared to contemporary tissues. The ever-increasing scarcity of mummified tissue, coupled with the experimental difficulties encountered when working with ancient material, has made the development of minimally destructive techniques increasingly important. We are attempting to devise such a method for determining protein preservation based around infra-red spectroscopy using histological sections or small quantities of powdered tissue. The spectra obtained are compared to protein preservation as determined through immunohistochemistry and a protein extraction method also based around the use of histological sections. This method may be valuable in further reducing the number and size of samples taken for research.

Introduction

As mummified tissue is a scarce and irreplaceable resource, any destructive research carried out using it should have as high a probability of success as possible. The use of minimally destructive methods for assessing preservation before extraction of tissue components is therefore highly desirable. Amino acid racemisation, for example, is frequently used before genetic studies as the factors affecting racemisation also influence the major causes of damage to DNA (Poinar et al 1996).

Research on ancient DNA (aDNA) relies on the polymerase chain reaction to amplify trace amounts of DNA to detectable levels, a process that is notoriously vulnerable to contamination and easily affected by chemical alterations to the molecule under investigation. Therefore, a method that does not rely upon reproduction of a damaged biomolecule would provide extremely useful corroborative evidence for the preservation of genuinely ancient molecules.

For some studies, specific tissues are required. The diagnosis of schistosomiasis, for example, requires liver or bladder tissue. However, mummified tissues are difficult to distinguish from one another, which makes sampling a specific tissue type difficult. This is particularly the case for Egyptian mummies where the organs may have been removed from the body for preservation before being wrapped and returned to the body cavity or deposited in canopic jars, removing any chance of identifying them by their anatomical position. Histological analysis can be used to identify the tissue, but once processed through solvents and molten wax, the ancient material may well be unsuitable for many subsequent molecular analyses, even if the sample is completely dewaxed.

We are developing a minimally destructive protocol for protein analysis, all steps of which use histologically prepared tissue. It is hoped that this protocol will allow tissue identification, assessment of protein preservation using infrared spectroscopy, and identification of specific proteins using two complimentary techniques. As every stage uses sections cut from paraffin embedded samples, it should be possible to complete the analysis using small amounts of tissue and without the need for separate samples for molecular and morphological analysis. The analytical techniques used for protein identification (immunohistochemistry and Western blotting) use multi-layer antibody techniques that, when combined, will allow the location of the protein in the tissue to be demonstrated, and its molecular weight (and therefore the degree of fragmentation) to be determined. The results presented herein are the preliminary data from this protocol.

Materials and methods

Liver and skin samples were used from ten mummies from the Dakhleh Oasis, which were rehydrated in 10% formalin

with 1% washing-up liquid to restore the microscopic structure of the tissue. The samples were then dehydrated in alcohol and cleared in xylene before being embedded in paraffin wax. A number of standard histological stains were performed on 5µm thick sections according to the methods published in Kiernan (1998). Sections were also mounted onto calcium fluoride disks for IR microspectroscopy, which was performed at UMIST. For immunohistochemistry, 5µm thick sections were mounted onto APES-coated slides. A polyclonal antibody against collagen IV (catalogue number 10760, Progen Biotechnik) was used, as monoclonals may be more vulnerable to producing false negatives because of their reliance on the preservation of a single binding site on the molecule. Protease pre-treatment was used for antigen retrieval, as recommended by the antibody manufacturer. Protein extraction used a method based closely on Ikeda et al (1998), which uses 50µm thick sections of tissue from the block, which are then de-waxed and heated with an extraction buffer. A commercially available protease inhibitor cocktail designed for use with mammalian cell extracts (Sigma, catalogue number P8340) was used rather than one made in house, but otherwise the only difference to the published method was in the amount of tissue used per extraction, which was more reliant on the size of the tissue sample than any other parameter. The extraction yield was calculated using the Bio-Rad DC protein assay kit, which is based on the Lowry method.

Results

The majority of the histological and histochemical stains used performed poorly on mummified tissue, though this is perhaps unsurprising considering that the biochemistry of the ancient tissue will be very different to modern samples. Loss of nuclei and other intracellular structures will also have an effect on the staining success, as will alterations to the charge distribution. Masson's trichrome, however, worked very well, as the different dyes used allowed tissue components to be easily distinguished. The skin samples were on the whole very well preserved, with fat cells, nerves and blood vessels easily visible in many cases, such as figure 1. The liver samples, whilst also showing generally good morphological preservation, were often not liver but some other, nearby tissue, such as diaphragm. Two of the 20 samples could not be rehydrated sufficiently for thin sections to be cut, but the rest were easily sectioned using a disposable steel microtome blade. Optimising the technique for infrared microspectroscopy of the mummified tissue unfortunately took so long that to date only a single sample has been scanned. The spectrum for the ancient tissue showed a great resemblance to a modern sample, as seen in figure 2. The protein peaks in particular were very similar. Although the peak area is dependant on the amount of substance present, the regions identified for specific classes of biomolecule are chosen based on their predominant chemistry, so other types of molecules will contribute some signal to the peak area. Nevertheless, it is hoped that further analysis will reveal a correlation between peak area and the protein yield which will enable an approximate yield to be calculated for other samples from their spectra alone. If this is the case, it will be possible to identify the tissue type, the morphological preservation, and its suitability for protein extraction using only two 5µm thick sections. Immunohistochemistry was positive in eight of the samples. Six of the positive results came from pairs of samples, i.e. both liver and skin from the same mummy. The positive staining was limited to the basement membranes surrounding blood vessels, as expected for collagen IV. The protease pre-treatment had very little effect on the tissue matrix, and in some cases (mainly for the skin samples) it lightened the background colour of the tissue, making the

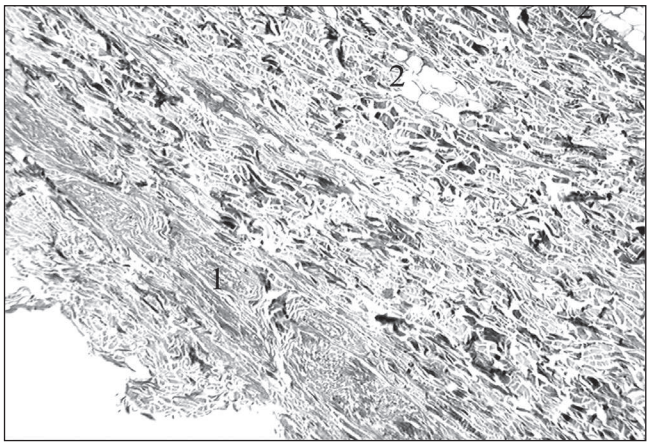

Fig. 1 - A section of mummified skin stained with Masson trichrome. A large nerve fibre (1) and the remains of fat cells (2) are visible. Original magnification χ50.

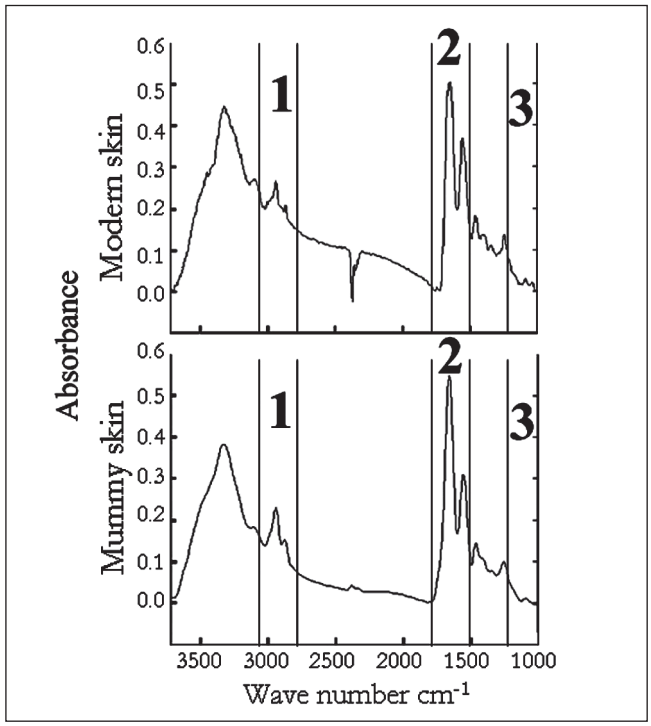

Fig. 2 - IR spectra for modern and ancient skin. The regions for fatty acids (1), proteins (2), and polysaccharides (3) are shown.

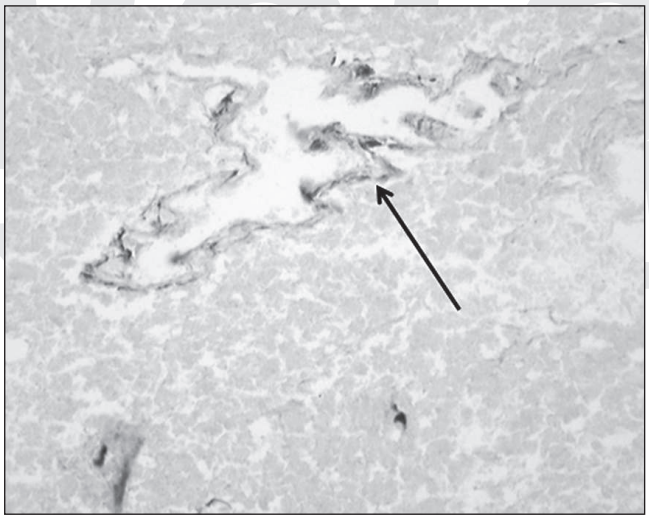

Fig. 3 - Positive immunostaining for collagen IV in mummified liver. Specific staining is seen in the basement membrane surrounding a blood vessel (arrow). Original mummification χ200.

positive staining more apparent. The absence of staining in some samples may be caused by damage to or loss of collagen IV from the tissue, whether from chemical reactions, decomposition, or the pre-treatment. That some samples remained positive argues against the antigen retrieval process being responsible, but it is impossible to state with absolute certainty that it had no effect.
Protein extraction yields varied widely between samples

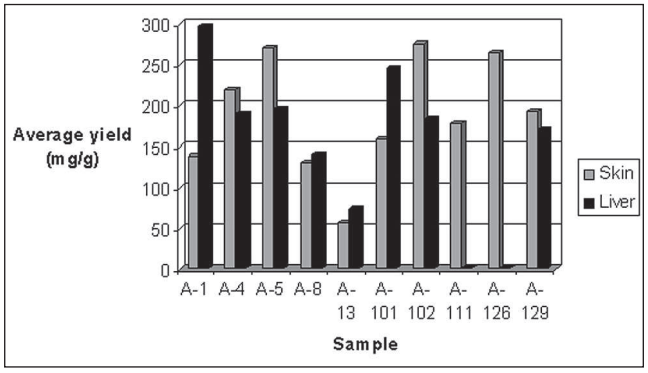

Fig. 4 - Protein yields for the ten skin and eight liver samples successfully rehydrated and suitable for extraction. Skin yields are shown in grey, liver yields in black.

(Fig. 4), with no obvious correlation between the yield and the morphological preservation or positive immunohistochemical staining. The average yields for both mummified skin and liver (188.5mg/g and 187.6mg/g respectively) were higher than the average for modern skin (~50mg/g), a discrepancy which could be caused by a loss of other tissue constituents in the ancient samples increasing the proportion of protein in the tissue.
The extracts themselves appeared yellow, and this discolouration was not removed by acetone precipitation, but had no effect on the quantitation. Coloured Maillard products have been found in mummified tissues before, and are commonly seen in aDNA extracts, where they inhibit the PCR. If these are present in the protein extracts, they may interfere with subsequent analysis as they can cause protein-protein crosslinks (Vasan et al 1996) that would affect their separation by electrophoresis. If this is the case, then addition of *N*-phenacylthiazolium bromide should reduce the extent of crosslinking, as was seen in the aDNA analysis of a ground sloth coprolite (Poinar et al 1998).
The amount of tissue used per extraction was very low, with three or four 50µm sections used per extraction depending on the cross-sectional area of the sample. In almost every case, this amounted to less than a milligram of tissue, and, although on this scale small errors in weighing can have a large effect on the subsequent calculations of yield, each extraction produced enough protein for at least one electrophoretic separation.

Discussion

Although this data represents the preliminary work in designing a minimally destructive method for the assessment of protein preservation and the subsequent analysis of specific proteins, it is sufficient to show that each individual stage is viable. It is hoped that further work will produce a protocol suitable for use by tissue banks on new samples which will, at the very least, identify the tissue type with a greater degree of certainty than is possible macroscopically. Infrared spectroscopy will almost certainly be able to show which samples are then suitable for taking on to molecular analysis. Although unsuitable for determining the preservation of DNA or, because of the solvents used during processing, lipids, it should also be able to indicate the preservation of polysaccharides. Even if the sample proves unsuitable for molecular analysis, it will still be suitable for histological experiments and, by re-embedding the tissue in resin, electron microscopy.

Whilst the protein extraction method produces extracts of sufficient yield for Western blotting, their suitability for this has yet to be assessed. However, the ability to extract proteins from such small samples is highly desirable, especially as this technique does not require taking additional samples from the stock supply, reducing the chances of contaminating it. Embedding the sample in paraffin wax should also improve the resistance of the tissue to any further degradation, so no special conditions for storage are required. Given that sections can be cut and proteins extracted as and when they are needed, there is little need to store the extracts for further use, removing the vulnerability to freeze-thaw degradation and freezer breakdowns.
The development of a screening technique for ancient tissues will help to lessen the needless destruction of this valuable resource in experiments with a low probability of success. Furthermore, by using a number of corroborative techniques, confidence in the results obtained by molecular analysis can be greatly improved. This is especially true for aDNA analysis, where additional verification of the results will be most welcome. A combination of DNA sequence

analysis and the identification and localisation of specific proteins will provide powerful evidence of, for example, parasitic infection in preserved remains. It is hoped that the protocol described herein will prove suitable not only as a method to investigate the preservation of ancient proteins before extraction, but will also be useful for confirming the results of other experiments by studying several aspects of the preserved molecules.

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