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ASSESSMENT OF PROTEASE IN CLEANING OF BAT BLOOD PATCHES FROM ANCIENT EGYPTIAN WALL PAINTINGS AND SURFACE INSCRIPTIONS

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Abstract

Wall paintings in ancient Egyptian tombs and temples, suffer many forms of damage. Protein-based patches is the most dangerous aspect which results from bats' wastes. These wastes are bat blood patches that cover large areas on the surface of wall paintings. The blood patches caused distorting of the surface of Egyptian paintings, drawings and inscriptions. The aim of the present research is to clean and remove these patches using an enzyme suitable for protein digestion. A variety of different analyses were used to evaluate the use of the protease enzyme in the cleaning and removal of blood patches. The study is conducted using the infrared absorption to assess the stability of the organic medium used before and after cleaning. The study also mad use of scanning electron microscopy, optical microscopy to determine the state of the surfaces of the wall paintings before and after cleaning, atomic absorption, elemental analysis units, examined by color change, to evaluate the effect of colored materials by enzyme cleaning. The results of the study confirmed the effectiveness of protease enzyme in the removal of these protein-based bat blood patches.

Keywords*: Wall paintings; Cleaning; Protease; Egyptian; FTIR; SEM; Optical Microscopy*

Introduction

The tombs and temples in various locations throughout Egypt suffer from a number of deterioration factors [1]. Perhaps the most prominent and recent factor is the presence of bat blood patches, of different colors; these patches cover the surfaces of Egyptian wall paintings and inscriptions, found in either tombs or temples.

It is well known that the surfaces of Egyptian murals and inscription in various archaeological sites reflect and present a wide range of damages. The manifestations of damage are due to internal or external damage factors. One of the most critical factors is the bats blood patches which are found in abundance on the surfaces of Egyptian tombs and temples, causing coverage of painted surfaces, inscriptions and wall paintings, as well as poor readability of the images [2-3].

The source of this blood spots is the wild bat, which prefers to live in closed and dark places, as the walls of tombs and temples are. The color of the blood of the bats alternates between light red and black. The accumulation of blood leads to the suppression of colors and the archaeological inscriptions within a few hours. The blood dries and become patches that are

difficult to remove from the surface of the murals because they merged and penetrated the painted layer. They may cause loss of pigment or the colors may become dim. These patches are characterized by proteins that stick to the surface after getting dry [5-10]. Therefore, it is difficult to remove them by normal and traditional ways such as using water or mechanical removal because it will cause the loss of the painted layer below it [4].

There have been many attempts to remove and clean these patches by the traditional methods of mechanical removal or with organic solvents or chemical poultices. However, these methods are not completely effective due to the intensity the decomposition of blood patches on the surface of the wall paintings. Another reason is that blood patches become an integral part of the surface that is difficult to be removed without leaving an impact and scratching of paintings or damage to pigment materials or organic media. Therefore, attention has been directed to the use of biotechnology by manipulating some types of enzymes to remove those patches. The removal depends on the following aspects: (i) the nature of the organic medium used in the painted layer, (ii) the type and nature of the patches on the painted surface, (iii) the bond between the patch and the painted surface. The nature of those patches is a protein-based material, and their proteinaceous chains can be broken down by proteases (enzymes). Hence, they are suitable for blood, egg, gravy, and other protein stains [11-22].

The main advantages of these enzymes are their specificity, efficiency and ability to stimulate the water division of polymers such as proteins, sugars and fats [23-34]. The first attempt of using enzymes in the treatment and restoration work was in the 1970's. Wendelbo [35] applied a proteolytic enzyme to some manuscripts.

It is known that the wall paintings and inscriptions are supported by limestone, sandstone and/or mud bricks. Above all, the wall is a preparation layer whose purpose is to enhance the stone surface and coat it with a white plaster layer of gypsum. The painted layer consists of pigments materials and organic medium used to dissolve, paste and link the granules of the pigments materials and then stick it to the surface. The sources of Egyptian pigment materials are numerous; among them are natural, organic and non- organic materials and synthesis. The Egyptian artist used some types of organic media such as Arabic gum and animal glue; however, the most common is egg yolk [36].

The protease enzyme has a major role in removing the blood patches from the surface of murals. The medium Arabic gum, the animal glue and the egg yolk are used in the process of coloring. Modern scientific research has turned to the sciences of microorganisms, including enzymes, as one of the main sources and methods of treatment and modern restoration where a group of enzymes are used to remove the products and aspects of damage on the archaeological surfaces. Since blood patches are protein, protease enzymes can be used as they are suitable for those patches and they have the ability to dissolve, clean and remove the patches without any damage. The effectiveness of removal of these materials is due to the amount of enzyme used, the duration of its application, the temperature in the environment, and the speed of removing the cleaning residues.

Protease enzymes can be used from two sources: namely protease from a bacterial source, or protease from a fungal source. The aim of this study is to assess the use of biological cleaning, with protease, in cleaning and removing the bat blood patches. The role biological cleaning is playing in monitoring the blood stains of the bat, their shapes, thickness, adhesion to the wall paintings and inscriptions surfaces and its damaged impact on the surface is also investigated here.

Different methods are employed to identify and evaluate the results of the use of bacterial enzymes in the cleaning and removal of bat blood patches. These methods include the use of infrared absorption spectra to determine the type of medium used, as well as knowledge of the stability of the organic medium used before and after the biological cleaning. The color change measurement is investigated so as to determine whether or not the color tone is stable. The scanning electron microscopy and optical microscopy were used to examine the surfaces of

the wall paintings before and after cleaning. The results of the study confirmed the efficiency of the protease enzyme in removing the protein patches (blood of the bat.).

Materials and Methods

Sample of the study

In this study, the following samples were collected:

(a) Bat Blood Patches

Samples from blood patches of bats were collected from the surfaces of ancient Egyptian wall paintings and inscription for the analysis and identification of the elements and the composition of blood patches.

(b) Experimental Wall Paintings

Experimental samples were prepared in the same manner of the archaeological one. All samples were covered with bat blood and they were left to dry. They were divided into groups as follows:

1.Group one included experimental wall painting samples with Arabic gum as organic binder.

2.Group two encompassed experimental wall paintings with animal glue.

3. Group three included experimental samples that were prepared with egg yolk binder.

(c) Enzyme

Protease from Bacillus Licheniformis, Type VIII, lyophilized powder, 15units/mg solid, unite definition, one unit will hydrolyze casein to produce color equivalent to 1.0μmole (181μg) of tyrosine per min at pH = 7.5 at 37°C (color by Folin-Ciocalteu reagent), solubility in 10mM NaAc (pH = 7.5) and 5mM CaAc: soluble, clear, It is active between pH = 6.5 and 8.5 and has an optimum temperature of 37°C.

The experimental studies were conducted on those samples which are similar to those murals by using protease enzyme in the cleaning and removal of bat blood patches. Protease was applied with a soft brush to clean and remove blood from the experimental samples (mock ups). Significant and crucial results were obtained, and then applied in removing the blood patches of bats from the surfaces of archaeological murals both colored and non-colored in some Egyptian archaeological sites.

Methods

In this study, the following methods were applied: Microscopy examination, Atomic Absorption Spectrometry, CHNS Analysis, Fourier Transform Infrared Spectroscopy, Scanning Electron Microscopy (SEM) investigation and color change measurements using CIE L*a*b* colorimetry. Each method is explained in detail below.

Microscopy examination

The examination of wall painting samples using optical microscopy provides valuable information about the number of layers, its thickness and the current state of the painted surface, the condition and the extent of the damage to each layer [37-46]. The study was carried out using optical microscope on the experimental wall paintings samples, which were prepared in Fayoum University, Faculty of archaeology, Restoration Department. The study was conducted by the wild MRI stereomicroscope, supported with the Olympus BX51. Examination for each experimental sample was viewed in the normal ranking light under multi magnification and evaluated for the enzyme's effectiveness at removing the bat blood patches.

Atomic Absorption Spectrometry

The atomic absorption spectroscopic analysis (AAS) is the most generally used technique nowadays due to its fast and quantitative nature [47, 48]. The detection limit in AAS analysis technique is up to zero. 1.0μg/kg beneath optimum check conditions [49, 50]. The sample atoms absorb ultraviolet or actinic radiation and create transitions to higher electronic energy levels. Concentration is set from the quantity of sunshine absorption [50, 51]. It is a very

accurate and sensitive method for the quantitative decision of metals and metalloids down to absolute amounts as low as picograms for some component [52, 53]. It cannot be used directly for the determination of nonmetals, it is utilized to dissect both significant components and follow components in archeological articles plans of investigation are portrayed for old metals and silicate-based materials, in view of tests of material penetrated or rubbed from the item, it is an investigative procedure that measures the centralizations of components. It has numerous utilizations in various territories of science [54, 55].

CHNS Analysis

A CHN Analyzer is a scientific instrument which helps to determine the elemental concentrations in the sample [56, 57]. It was used to measured carbon, hydrogen and nitrogen. Natural examinations of all out nitrogen and carbon and sulfur is performed to give carbonate and natural carbon and to get some thought of the arrangement of the natural matter [58]. A CHN Analyzer known as a carbon hydrogen and nitrogen analyzer, it is a scientific instrument used to accurately measure elemental concentrations of carbon, hydrogen and nitrogen in a given sample [59]. Sample sizes are usually just a few milligrams, but may vary depending on the system. Because of sample heterogeneity, larger mass is preferred for some sample matrices. These are fit for dealing with a wide assortment of test types, including solids, fluids, unstable and gooey examples, in the fields of pharmaceuticals, polymers, synthetic substances, condition, sustenance and energy [60].

Fourier Transform Infrared Spectroscopy

Fourier-transform infrared spectroscopy (FTIR) analysis is used to identify the organic materials used in archaeological assets, such as organic media (coloring media) used in the coloring process of archaeological wall paintings. It is known that the Egyptian artist used a number of organic media in the coloring process of murals, such as gum Arabic, animal glue and egg yolk medium [61-66].

The infrared analysis was carried out in the infrared unit of the Faculty of Graduate Studies at Beni Suef University, Egypt, using the VERTEX 70 system. The German Bruker optics industry and the model of the device was Version 7.2 Build: 7, 2.

Samples were analyzed by grinding the sample well then using KBr and was compressed to form a thin film and then tested. The analysis were done on two samples from blood of bat which were collected from the archaeological wall paintings surface, one was dry and other one was fresh one.

Scanning Electron Microscopy (SEM) investigation

The surfaces of mock-ups were examined using the scanning electron microscopy to examine the surface before and after protease cleaning in Fayoum University, Faculty of Science using Scanning Electron Microscope (SEM), Zeiss FE-SEM, Gemini, Sigma 500 VP [67, 68].

Color change measurements

The characteristics of the measurements of pigment are as follows: (i) the color strength (K/S) values of experimental samples before and after cleaning with protease were examined and evaluated at the maximum wavelength of the natural colorant using a color matching system (Color Eye 3100) spectrophotometer, SDL, England; (ii) to evaluate the change in color as a result of the ageing factor, spectrophotometric measurements were used; (iii) the most used color models are the perceptually uniform CIE L*a*b* (where: L* color lightness, color coordinates ±a* - reddish/greenish and ±b* - yellowish/bluish). The color difference (ΔE*) between a sample and standard in this system is given by [69-73]:

$$\Delta E = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \quad (1)$$

where: ΔL* = L* sample - L* standard; Δa* = a* sample - a* standard and Δb* = b* sample - b* standard.

As for the yellowness index samples, they were detected as calcite white origin, were measured and evaluated according to ASTM D 1925 by using a Color Eye 3100 Spectrophotometer SDL, England. The mean value of three measurements was recorded for each sample before and after cleaning with protease enzyme.

Results and discussion

Analyses of original samples of mural paintings

For bat blood patches identification, tow samples were collected previously from the remains of the bat blood stains from the surfaces of murals in the various Egyptian tombs and temples have been analyzed by multi scientific techniques, to identify the nature of these bat blood patches and its components

Atomic analysis results of blood patches

The Atomic Analysis of bat blood consists in Fe 0.248, Na 2.111, Ca 2.100 and K 3.482. Patches which showed the proportions of iron, sodium, calcium and potassium respectively indicate the presence of bat blood, which is one of the most damage products that stick to the surface of wall paintings, penetrating inside the layers and needing modern and safe solutions to clean and remove them from the colored surfaces.

The results of the CHNS analysis of blood patches

The CHNS analysis of the bad blood consists in: C 10.38, H 1.81, N 5.10, S 1.24 and Cl 0. Carbon has been oxidized and transformed from red to dark red – black. Nitrogen appears to be high and this high ratio helps to grow certain types of nitrogenous bacteria. Sulfur, although the ratio is not high in sample size, it increases the acidity of the medium, which leads to corrosion in the stone and the entry of blood into the pores.

FTIR Spectroscopy results of blood patches

Two blood patches samples were collected for analysis with FTIR, one sample was wet one, means still fresh blood, and another was dry blood patches. The figure 1 shows an infrared spectrum of an old sample with dry bat blood showing the total disappearance of the OH due to its complete dryness, that led to the full adhesion of blood stains to the surface, another spectrum shows an infrared analysis of the bat's blood sample (fresh blood stain) showing a total of OH group, means that blood still wet. It also shows a total amino acid component of protein as it is the primary compound of bat blood.

Wall paintings mock-ups results

Model samples were prepared for experimental studies: the first group was colored by Arabic gum as an organic medium. The second group was colored with egg yolk, and the last group was colored with animal glue. Their surfaces were covered with bat blood patches, and then protease was applied in the process of cleaning of blood patches. The paintings surfaces were investigated before and after protease application by multi-analytical approach.

Investigation with optical microscope results

Investigation with Optical Microscope was used for experimental samples to characterize the paintings surfaces before and after cleaning with protease. Figure 5 showed the state of paint before and after blood patches cleaning with protease, for the gum Arabic group. The pictures provide us with information on the surface state after the protease cleaning process where the surface condition is good and no change has been observed in the colored surface. We notice the surface of group two with egg yolk binder is stable even in its color, in the third group with animal glue as a binder, the state of the surfaces is not stable, there is some change in color hue, and it can be seen that the chromatic layer has fading of color tone.

FTIR results of model samples

FTIR analysis were carry out for the experimental samples before and after protease application to study the organic medium of the three groups of gum Arabic, egg yolk and animal glue samples, before and after the application of protease, and notice any changes or effects that may have been caused by the application of protease during blood cleaning.

The FTIR results of gum Arabic group before and after protease application can be showed in figure 2 which indicated that there is not any change in the organic functional groups of gum Arabic even before or after protease application. FTIR results of egg yolk experimental samples group before and after protease application can be seen in Figure 3 that indicated the stability of the egg yolk binder before and after cleaning with protease. As for the animal glue groups, Figure 4 showed the instability of the organic medium. The results showed the effect of protease on the organic functional groups of the animal glue medium.

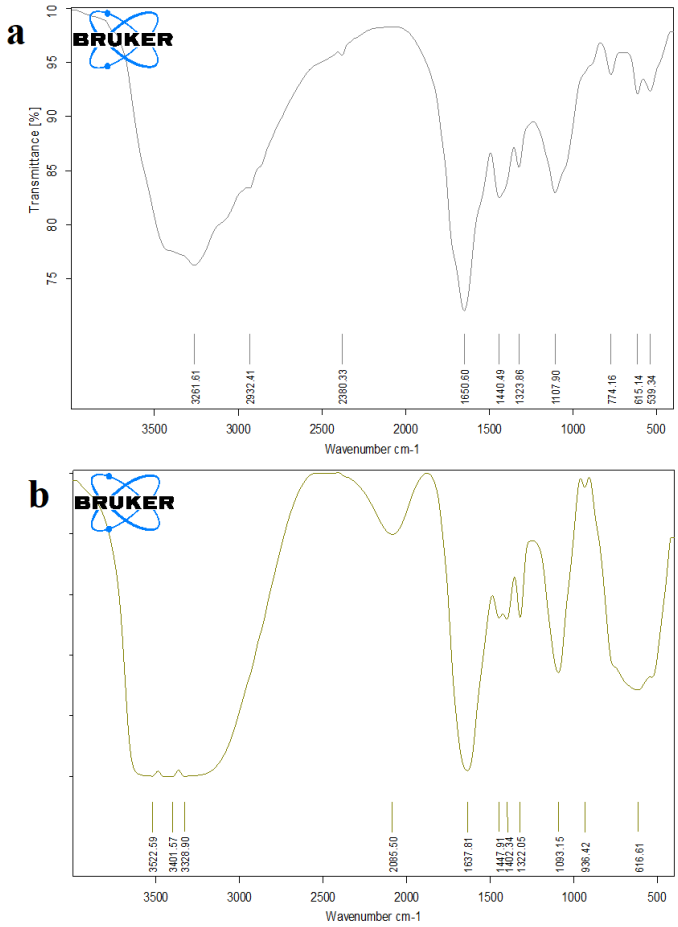

Fig. 1. Infrared analysis of dry bat blood sample:
a. showing the total disappearance of the OH due to its complete dryness, this led to the full adhesion of blood stains to the surface, it also shows a total amino acid component of protein as it is the primary compound of bat blood;
b. FTIR analysis of the bat blood sample (wet one) showing a total of OH where it is still wet and fresh. It also shows a total amino acid component of protein as it is the primary compound of bat blood

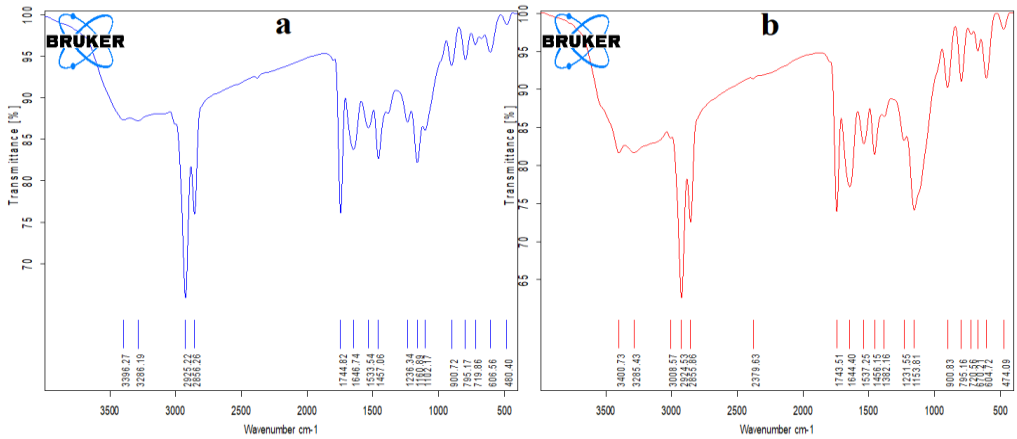

Fig. 2. FTIR spectra of the experimental samples of the egg yolk group:
a. prior to the use of protease in the cleaning of the bat blood patches;
b. after cleaning - we can observe the stability of functional groups of egg yolk before and after application

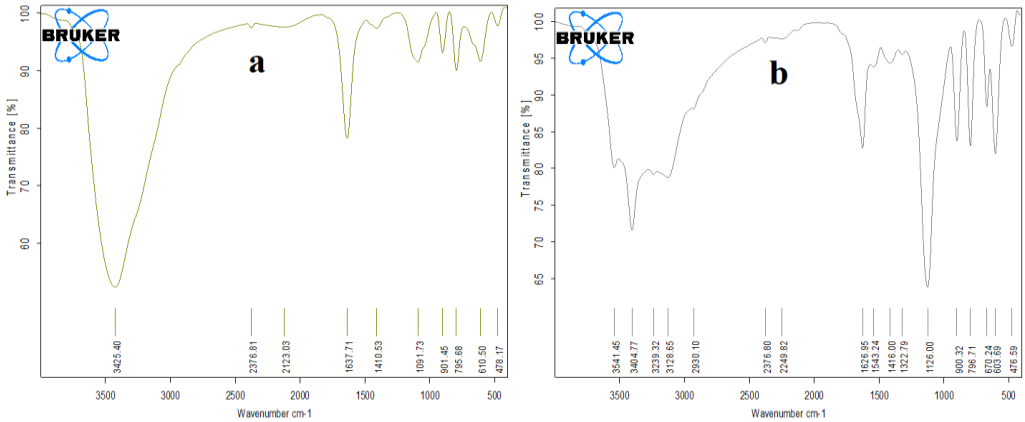

Fig. 3. FTIR spectra of the model samples of the gum Arabic group:
a. refer to the use of protease in the cleaning of the bat blood patches;
b. after cleaning - observing the relative stability of functional groups of gum before and after application

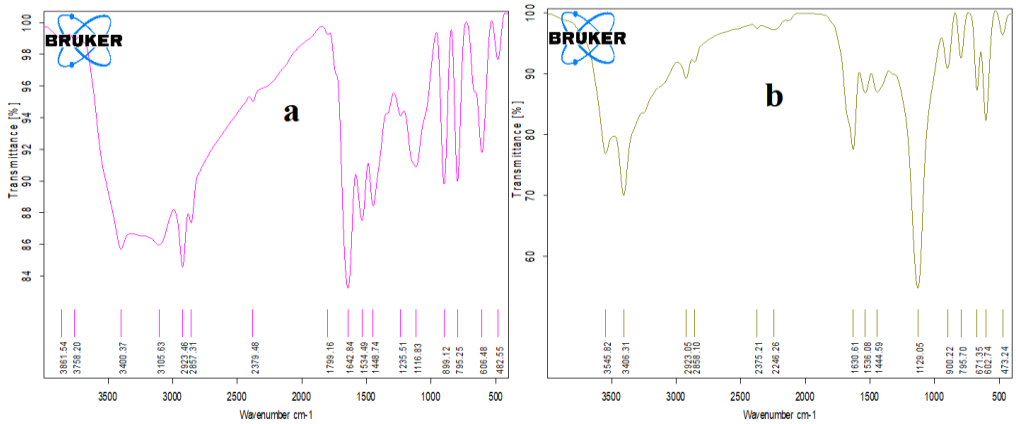

Fig. 4. FTIR spectra of the model samples of the animal glue group:
a. prior to the use of protease in the cleaning of the bat blood;
b after cleaning - we can observe the instability of functional groups of animal glue
as an organic intermediary before and after application

As it can be observed in Figure 5, the first group from the top is the Arabic gum model samples group, before, during and after protease cleaning. Protease efficiency is shown in removing and cleaning the blood stains of bat from the experimental samples used by gum Arabic as an organic intermediary in coloring. The second group is followed by the experimental group of egg yolk samples before, during and after protease cleaning. Protease efficiency is demonstrated by removing and cleaning the bat blood samples the stability of the chromatic surface is evident and not affected. The third group below is the animal glue experimental group before, during and after protease cleaning. Protease efficiency is shown to remove and clean the bat blood stains from experimental sample. The surface is unstable and affects the application of protease.

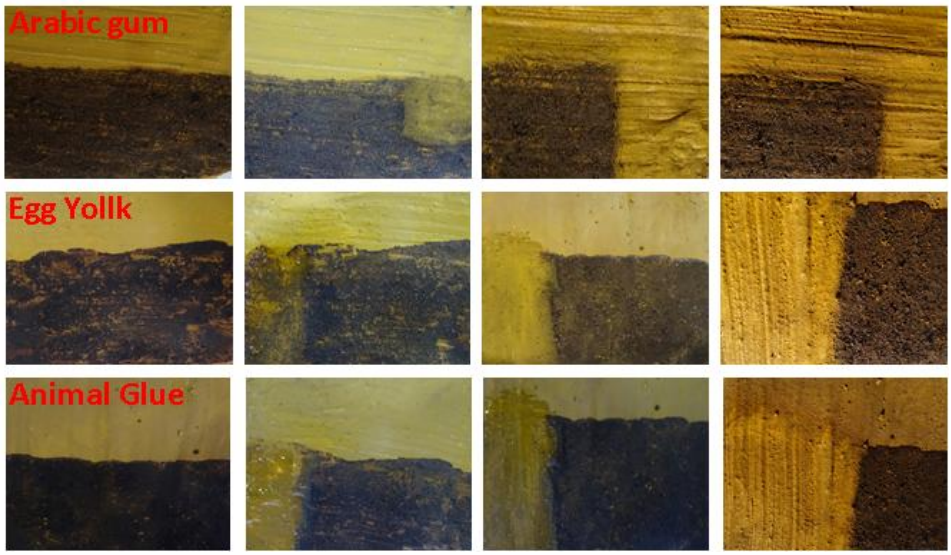

Fig. 5. Optical microscopy images of the surfaces of the three experimental group samples before, during and after cleaning with protease

Scanning Electron Microscopy (SEM) investigation results

The investigations were carried out by the scanning electron microscopy of the 3 models sample groups to show the extent of the changes that occurred to the colored surface before and after the protease cleaning. The results of model samples of gum Arabic showed that the colored surface was stable and did not undergo any significant changes with the stability of the chromatic layer before and after the protease cleaning the blood of bats (Fig. 6).

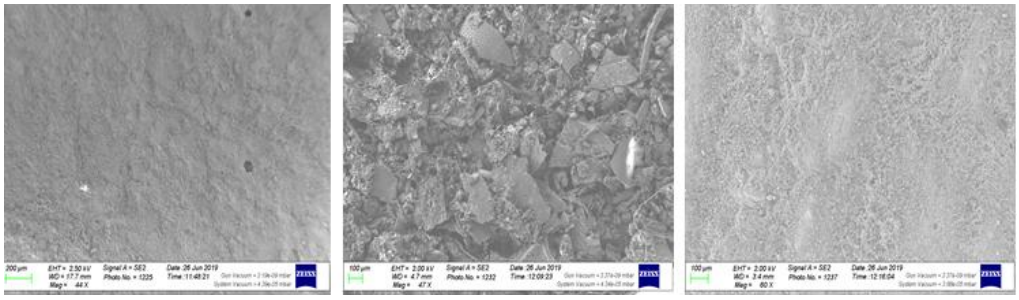

Fig. 6. SEM images for the model samples with gum Arabic as organic medium, from left the surface of the experimental sample before any cleaning, the middle one the surface covered with bat blood, the right one the surface after cleaning bat blood with protease

The results of the examination of the model samples of the egg yolk group confirmed the stability of the chromatic layer and that there was no change. As well as the stability of the colored surface before and after cleaning the blood of bats by protease (Fig. 7). The results of the examination of the model sample group with animal glue medium the test showed the instability of the chromatic layer and its effect on protease cleaning, as well as noticeable change in the chromatic surface (Fig. 8).

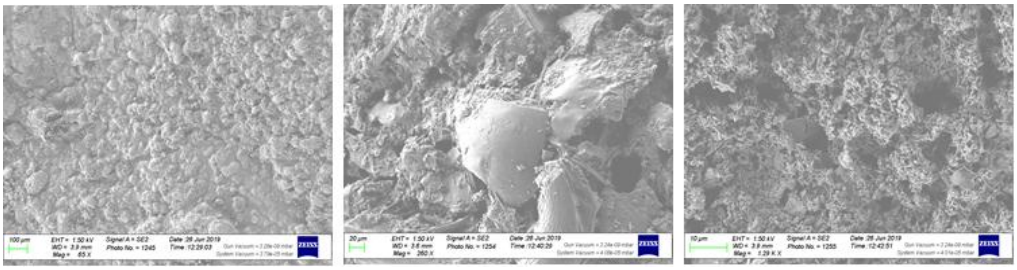

Fig. 7. SEM images for the model samples with animal glue as organic medium,
from left the surface of the experimental sample before any cleaning,
the middle one the surface covered with bat blood,
the right one the surface after cleaning bat blood with protease

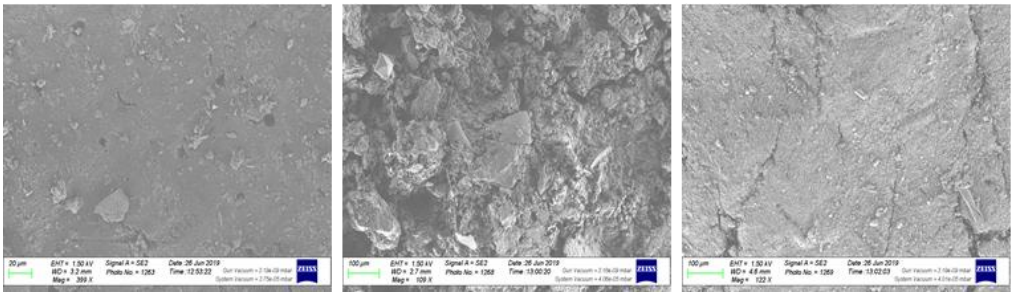

Fig. 8. SEM images for the model samples with Egg Yolk as organic medium,
left one showed the surface of the experimental sample before any cleaning,
the middle one refers to the surface covered with bat blood,
the right one, the surface after cleaning bat blood with protease

Color change measurements results

Based on Table 3 of colorimetric results, it can be noticed that color parameters of the model samples investigation were estimated before and after the application fo the enzyme. The overall color change of the three model samples groups is calculated from the difference of the L, A and b coordinates, before and after protease cleaning of bat blood stains, indicating the extent of color change that occurred to the chromatic surface due to enzyme cleaning.

Table 3 Colorimetric coordinates of the prepared model samples before and after applying protease enzyme under the effect of different binders

Color coordinates	The organic binder					
	Egg yolk		Arabic Gum		Animal Glue	
	Before enzyme	After enzyme	Before enzyme	After enzyme	Before enzyme	After enzyme
L*	60.26	60.41	66.27	65.07	60.41	58.80
a*	10.55	10.77	12.68	13.15	10.92	11.77
b*	21.47	22.17	30.40	30.31	20.38	23.35
ΔE*	**0.74**		**1.29**		**3.48**	

Three model samples were made to imiatte samples from original the painted layers. These samples were prepared using the most common color in the ancient wall paintings; goethite or yellow ochre FeO(OH) and different organic binders, i.e. egg yolk, gum Arabic and animal glue. Color parameters (L*, a* and b*) of each prepared sample were measured spectrophotometrically and recorded twice. The first measure was done on the native samples without any treatment, while the second measure was done after applying protease enzyme on each sample individually to assess the effect of the enzyme on the behavior the wall painted layers. Moreover, the color difference (**ΔE***) of each sample was calculated as the difference between the sample before and after treating it with the protease enzyme. The obtained data are listed in Table 1.

The data listed in the table reveals that, there is slight change in the color coordinates (L*, a* and b*) values before and after enzyme treatment for each sample individually. The most obvious change was observed for the samples with glue binder followed by those of Gum and Egg samples. Regarding to the b* (yellow-blue) component it is clear that, gum samples gained the highest b* values after the enzyme treatment. On the other hand, glue samples showed higher change in the b* values after enzyme treatment more than that of the egg sample.

This may be due to the fact that, different proteinaceous substances cannot be expected to show similar physico-chemical characteristics, as egg is considered as lipid proteinaceous substance, while gum is monosaccharide substance, and glue is highly viscous protein substance. Moreover, Protease which is a nontoxic and environmentally friendly enzyme consisting of a group of enzymes that assist the hydrolysis of peptide bonds in proteins into peptone, polypeptides, dipeptides, and finally amino acid. For all these reasons, egg yolk as binding medium (contains lipids 66% in terms of mass and smaller amount of proteins) becomes extremely resistant when applied and treated because of the polymerization of its lipid components [1-5].

Regarding to the color difference ΔE values of the prepared and treated samples before and after applying the enzyme, it was found that, the binder glue samples had the highest ΔE* values after treatment followed by that of gum treated sample and finally come the egg treated samples, taking the ranking order:

$$\Delta E^*\ \text{glue} > \Delta E^*\ \text{gum} > \Delta E^*\ \text{egg} \qquad (2)$$

This order complies well with the above-mentioned constitution of the different media and their behavior with the enzyme. So, it can be said that, the glue treatment had the highest harshening effect on the tested samples while the egg treatment showed the tenderest effect, and the gum is the moderate effect.

Through the results obtained through the previous experimental studies, which confirmed the possibility of using protease enzyme in the removal and cleaning of protein spots, the blood stains of bats from the surfaces of murals and inscription. Protease was used in the cleaning operations to remove the blood stains of the bat from the surfaces of murals in several and different places and has been given good results in cleaning and removing those stains completely from the surfaces of murals and engravings. Figure 9 shows the effectiveness of the protease application in the cleaning of bat blood stains.

The use of enzymes (biotechnologies) in the cleaning of archaeological surfaces, especially murals, whether colored or not colored, of different stains, deposits, soil and plankton, which have been deposited on the archaeological surfaces has become important in conservation field. Its advantages are, for example, a safe, easy and satisfactory application, especially with some types of hard and difficult spots such as bat blood patches which spread heavily on the surfaces of Egyptian murals, especially in closed and dark places. These patches are difficult for the restorer to remove even with traditional techniques such as dry cleaning, that because it will scratch and remove the painted layers. The use of chemical cleaning is not easy and has adverse effects on painted surfaces and organic media, cleaning with different

organic solvents did not produce any results and did not have the ability to remove and clean those dry and penetrating protein spots inside the pores of the mural layers.

Fig. 9. The application of protease, the cleaning and removal of bat blood stains from the surfaces of some Egyptian murals in different locations, every group shows the surfaces before cleaning then during application of enzyme and last one shows the surface after application enzymes and completely removal of blood

The use of protease enzyme has the ability to remove and disassemble protein materials because the blood spots produced by the bats are protein-based substances. Protease has succeeded in removing these spots and cleaning them completely from the surfaces of Egyptian murals in different locations. In the previous study, which was intended to evaluate the application of protease in cleaning bat blood stains, it was necessary to evaluate this according to the organic medium used in the coloring process (gum Arabic, animal glue and egg yolk, adopted and used as organic media in the coloring process).

Conclusion

The study, which confirmed the effectiveness of protease in the cleaning and removal of bat blood patches, brings an important result for the use of restorers in Egyptian sites to restore the surface of the murals and revive archaeological scenes.

The efficacy of protease application depends on its nature and ability to decompose, break, clean and remove those spots without affecting the chromatic surface, taking into account the quality of the medium used.

Protease proved to be efficient in removing blood stains from the surfaces of model samples of murals made gum Arabic medium without affecting the organic medium and without affecting the color surface. Also the protease had good results in removing the blood stains from the surfaces of the model samples of murals, which use the egg yolk as binder without affecting the organic medium or the color surface. On the model samples where animal glue was used as binder in the coloring process, protease has already succeeded in the removal of blood stains, but with an effect on the organic medium, and the occurrence of the substitution of the medium, as well as the occurrence of color changes in the color surface.

It is therefore possible to say that the use of enzymes, especially protease, has been selectively successful in removing and cleaning bat blood stains from the surfaces of Egyptian murals, taking into account the type of organic medium used. The study recommends the use of protease enzyme in the cleaning of the blood stains of bat from the surfaces of Egyptian murals with a previous test to know the type of organic medium used before the application of protease in cleaning.

Future study will include additional tests for protease enzyme in cleaning of other types of stains on painted mural painting. Such as improvements in the properties of enzymes to enhance their cleaning ability, and to work on a more secure and effective on painted archaeological surfaces, especially the surfaces of murals. This study will include application of analytical techniques and evaluate the efficiency of protease in cleaning of mural painting.

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