INTERNATIONAL JOURNAL OF CONSERVATION SCIENCE

ISSN: 2067-533X Volume 7, Special Issue 1, 2016: 239-246 www.ijcs.uaic.ro

COLD-ACTIVE MOLECULES FOR A SUSTAINABLE PRESERVATION AND RESTORATION OF HISTORIC-ARTISTIC MANUFACTS

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Abstract

In the last decades biotechnology research provides sustainable alternatives to traditional procedures for preventive preservation of cultural assets. Recently, bioactive molecules (BMs) isolated from marine invertebrate organisms have been isolated and tested for bioremoval of protein layers (BMP) or to controlling microbial colonization (BMA), acting at temperature lower than 30°C. The Protease or Antimicrobial activity was tested on ad hoc assembled specimens and on different historic-artistic manufacts. In bio-removing protocol BMP molecules were applied as gelled solutions, in order to guarantees a selective action, respectful of constitutive materials and manufact integrity. Peculiarity of Protease bioactive molecules is the temperature of action, lower than 30°C. Instead, BMAs molecules have been tested to control bacteria and fungi colonization in laboratory specimens. In our hypothesis these novel molecules provide an important contribution to the development of innovative protocols safe both for the environment and conservator health, representing a valid alternative to traditional methods according to the preventive conservation and "Minimal Intervention" concept in restoration procedures.

Keywords: *Biodeterioration; Biodegradation; Biocleaning; Protease; Antimicrobial molecules; Risk assessment*

Introduction

Cleaning represents a very complex issue, likened to artwork constitutive materials, layer/s to be remove, which removing protocol is more appropriate, past conservation treatments, the level selectively without damage the historical *patina*, etc. [1]. Nowadays, biotechnology has provided green methods with advantages over the traditional chemical or physical techniques, without (or deep reduced) negative impact for operators and environment [2-4].

Particularly, selected enzymes (hydrolases) or viable bacterial cells (non-pathogenic) have been identified and applied for biological removing of undesired matter (organic layers) or crusts (sulfate, nitrate) from art works surface. These approaches are based on the enzymatic hydrolysis of layer constitutive molecules or on utilize of crusts as carbon source or electron

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acceptors/oxidized compounds. These procedures, sharing high selectivity, acting on altered layers in more effective way than traditional techniques [3-11].

Commercial hydrolases are usually isolated from animal tissues or microbial sources: Amylase, from animal tissues (pancreas), plant tissues (seeds of oats and wheat); Lipase from microbial specie; Proteases can be extracted both from animal tissues (pancreas, stomach) and microbial cells [12]. Attempts to remove starch paste, animal glues or protein adhesives by Amylase or Protease, were initially performed by different restorers [13, 14]. Mixed enzymatic solution was tested to remove animal glue and protein/oily binder from painted on canvas surface [15]. Later R. Bellucci et al [16] showed the hydrolysis of aged acrylic coatings on painting by Lipase. Moreover, combining the metabolic activity of viable bacterial cells (*Pseudomonas stutzeri*) and the Protease action, G. Ranalli et al [17] cleaned the surface of frescoes.

In the last decades, biomedical and pharmaceutical research communities pay particular attention to marine organisms, in order to isolate and characterize bioactive molecules (from fish, sponges, jellyfish, marine invertebrates, micro-algae) useful in food industry and for biomedical application, the so-called Blue-Biotechnology [18-21]. These molecules are interesting in relation to their stability and activity at low temperature (e.g. cold-active enzymes, temperature < 30°C) or antimicrobial properties.

In this study bioactive molecules (BMs) isolated from marine invertebrate organisms (Cnidarian) are utilized to hydrolyse aged/altered protein layers or coatings (BMP) or to control bacteria/fungi growth (BMA). Cnidarians evolved 450 MY ago, using their bioactive molecules, in defence or predation mechanisms, for ensure survival in hostile and competitive environments, such as the seas and oceans. From benthic and pelagic species have been isolated a large number of compounds that can have an active role in the development of various antiviral, anticancer and antibacterial functions [22]. Peculiarity of BMP, in conservative restoration procedures, is the temperature of action < 30°C, instead of commercial proteases that are active at higher temperature (37°C). Moreover, the antimicrobial activity of BMAs was utilized to inhibit the growth of bacteria as *Enterobacter* spp. or *Micrococcus luteus* and fungi as *Aspergillus niger* or *Penicillium chrysogenum*.

These “green methods” represent innovative procedures that minimize the exposure to harmful solvents and chemicals compounds, of both employers and environment.

Experimental

Protein layers

The presence of protein matrix was investigated by Fourier Transform Infrared (FTIR) Spectroscopy in *velinatura* fragments [23]. Samples collected from polychrome wood manufact or wax sculpture were analysed by High Performance Liquid Chromatography (HPLC) [24] or by Sodium Dodecyl Sulphate-Polyacrylamide gel (SDS-PAGE) electrophoresis [25] respectively.

Bioactive Molecules with Protease activity (BMPs)

Bioactive molecules were extracted from the body of invertebrate marine organisms (Cnidaria) in TBS solution (150mM NaCl, 10mM TRIS-HCl pH = 7.4) and the total amount was estimated by Bradford method, using bovine serum albumin (BSA) as standard. The Bradford method is the more common simple and sensitive colorimetric test for the quantitative analysis of the total content of proteins, essential for follow the yield during all the steps of bioactive molecules enrichment and isolation [26]. As showed in Fig. 1B, the related electrophoretic profile was resolved on 7.5% SDS-PAGE, according to Laemmli method [25]; a high protein molecular marker (Sigma-Aldrich) was utilized as weight standard (Fig. 1A). Gelatin-degrading enzymatic activity was tested using 2mg/mL co-polymerized gelatin in 7.5% SDS-PAGE. After migration, the gel was washed in TBS buffer (150mM NaCl, 10mM Tris-

HCl, 10mM $CaCl_2$, pH = 8.0) containing 2% of Triton X-100 and incubated overnight in TBS buffer. Finally, washed in 50% Methanol and stained in Blue Coomassie (Fig. 1C).

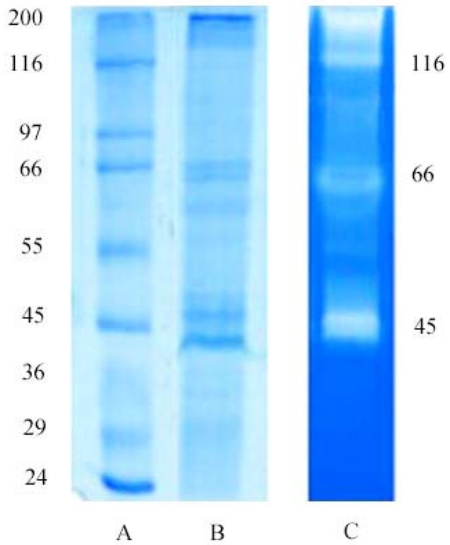

Fig. 1. Bioactive Molecules on Polyacrylamide Gel Electrophoresis (7.5% in SDS): A) marker; B) SDS - PAGE pattern of extracted; C) zymogram on 2mg/ml gelatin, BMP activity generates three mainly protein bands of 116, 66 and 45kDa

Bioremoving assays

Tests were performed using BMP to remove the *velinatura* (Japanese paper + animal glue) or protein layers (animal glue) from the surfaces of different works of art (Table 1). The enzyme was applied in 10mM Tris–HCl pH = 7.5 and as gelled solution, in order to guarantee stable reaction conditions, improving the cleaning procedure [5, 27]. Particularly, Gellano (Gellan gum) or Klucel-G (hydroxypropylcellulose) was utilized in *velinatura* or polychrome wood removing tests respectively. Instead, due to the three-dimensional structure of the wax sculpture surfaces, the enzymatic solution was in 1% Klucel-G and applied by paintbrush through a delicate mechanical action. All tests were performed applying the enzyme solutions for 5 or 10 minutes; a Control test (10mM Tris-HCl pH = 7.5 solution, lacking of protease) was carried out for 10 minutes per each assay. Other controls, using distilled water, were also done on *velinatura*.

In order to remove any residues, the test-areas (approx. $3cm^2$) were finally cleaned by dry and distilled-water soaked swabs.

Table 1. Bioremoving of protein layers performed applying BM Protease gelled solution at environmental temperatures (19-25°C).

Removal from	Protease solution applied by	Temperature (°C)
Painting on canvas (velinatura)	*2.5% Gellano*	24
Polychrome wood (animal glue layer)	*5% Klucel- G*	25
Wax sculpture (animal glue layer)	*1% Klucel- G (soft- brush)*	19

Bioactive Molecules with Antimicrobial activity(BMAs)

The BMAs molecular weight (2.4 - 2.8kDa) was determined by mass spectrometry (Ultraflex TOF/TOF, Bruker Daltonics). Their antimicrobial activity was tested against *Enterobacter* spp. and *Micrococcus luteus* or *Aspergillus niger* and *Penicillum chrysogenum*. These microbial colonies were isolated from canvas + animal glue specimens and identified combining microscopy analysis and molecular biology investigations [28, 29].

Antimicrobial assay

The antibacterial and antifungal activity of three bioactive molecules (BMA_1, BMA_2, BMA_3) was investigated by the micro-dilution technique [30]. The assay was performed by the serial dilution technique using 96-well micro-titer plates. The BMAs molecules and commercial biocide (Nipagin-M, Methylparaben, 2.5mg/mL) were dissolved in bacterial or fungal liquid (Nutrient Broth, Difco) cultures. The BMA concentrations that completely inhibited microbial growth (MICs) was defined as the lowest concentrations without visible growth after incubation at 30°C for 24h (bacteria) or 48h (fungi). The Minimal Bactericidal or Fungicidal Concentrations (MBCs and MFCs) were determined by serial sub-cultivations into fresh medium lacking of biocide molecules.

Results and Discussions

Enzymatic removal

In this study, bioactive molecules with proteolytic activity (BMPs) extracted from invertebrate marine organisms were tested in removing of protein layers from laboratory specimens or artworks surfaces. Preliminary to artworks application, the BMP proteolytic activity was valued through zymography, performed at 24°C (Fig. 1).

The bioremoval of both *velinatura* (Japanese paper bonded by animal glue) on ancient oil on canvas (Fig. 2) and animal glue layer from polychrome wood (Fig. 3), was successfully performed after 5 and 10 minutes of application at temperature < 30°C.

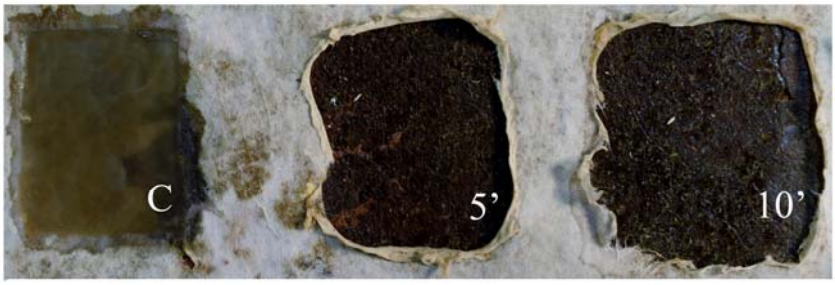

Fig. 2. *Velinatura* (Japanese paper + animal glue) from oil painting surface: C = Control (Gellan gel in the reaction solution); enzymatic removal performed by application of gelled enzymatic solution applied for 5 and 10 minutes

Fig. 3. Polychrome wood: C = Control (reaction solution without BMP, gelled by Klucel-G); bioremoval performed by application of gelled enzymatic solution for 5 and 10 minutes

An altered protein layer was removed from wax sculpture surface after 5 and 10 minutes of application at 19°C (Fig. 4). Control test, performed applying the reaction solution without BMP, was also performed; results are showed in Table 2.

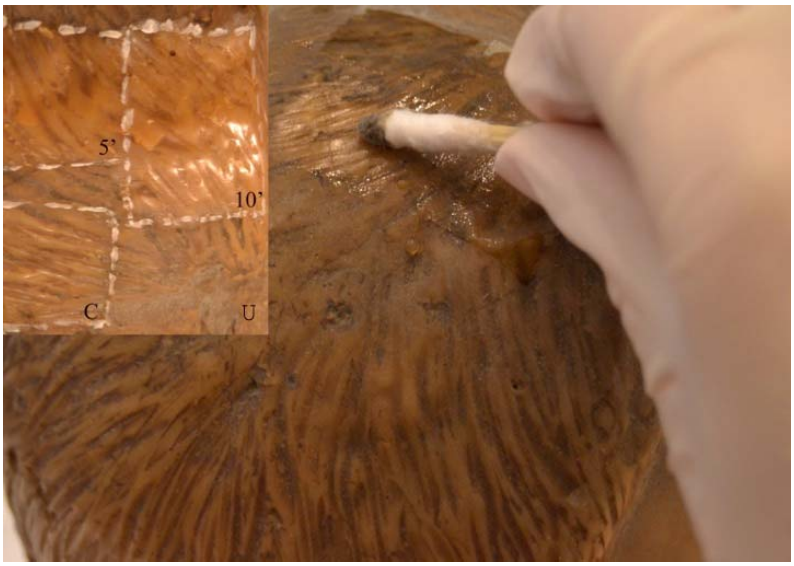

Fig. 4. Wax sculpture. U - Untreated area. C - control, reaction solution without BMP, gelled by Klucel G. 5'-10' - application of gelled enzymatic solution, by paintbrush, for 5 or 10 minutes. Right side = removal of gelled solution by cotton swab.

The proteins removal was valued detecting the molecules presence on cotton swabs, utilized in the final cleaning procedure, by *Amido Black Staining Solution* (Sigma) [31].

Table 2. Results obtained by cleaning tests with BMPs and respective controls.

	Efficiency in Removal		
	BMP		**Controls**
***Application time* (min)**	**5'**	**10'**	**10'**
Velinatura	+ +	+ + +	-
Polychrome wood	+	+ + +	-
Wax sculpture	+ +	+ + +	+

(+) - low; (+ +) - good; (+ + +) - high; (-) - no activity

Antimicrobial activity

The MIC and MBC/MFC, the biostatic or biocide activity against fungi or bacteria taxa were defined and summarize in Table 3. The antimicrobial molecules tested by micro-dilution method, showed inhibitory effects against bacteria as *Enterobacter* spp. and *Micrococcus luteus* or fungi as *Aspergillus niger* and *Penicillum chrysogenum*.

Table 3. BMAs antimicrobial activity. Minimal Inhibitory Concentration and Biocide/Biostatic activity.

	MIC (mg/mL) and Antimicrobial activity							
	Enterobacter spp.		*Micrococcus luteus*		*Aspergillus niger*		*Penicillium chrysogenum*	
BMA_1	(0.35)	BC	(0.35)	BC	(2.80)	FC	(1.40)	FC
BMA_2	(0.15)	BC	(0.15)	BS	(1.20)	FC	(0.30)	FC
BMA_3	(0.10)	BS	(0.10)	BS	-	-	(0.40)	FC

(BC) = bactericidal; (BS) = bacteriostatic; (FC) = fungicidal; (-) = microbial growth

Conclusions

In this study, bioactive molecules isolated from marine invertebrate organisms (Cnidaria) were tested in order to remove protein layers or to control microbial colonisations. Concerning proteolytic enzyme (BMP), the removal of undesired layers under "room temperature" (19°C - 25°C) conditions, without heating the enzyme solution or the artwork surface on which it was applied was tested. Agreeable results were obtained after application of gelled enzymatic solution for 10 minutes, in removing coherent protein layer both from the surface of polychrome wood (Fig. 3) or wax sculpture (Fig. 4). In both cases the complete removal of the protein layer, without producing whitening phenomena was observed. In the removal of *velinatura* the solubilisation of the adhesive layer (animal glue) without altering the painting surface plays an important role, since the traditional methods based on use of warm water, applied in a free form, acts in a not controllable way [32, 33].

Promising results were obtained from antimicrobial bioactive molecules (BMAs) in order to control the growth of bacteria and fungi. In Table 3 are summarized the results concerning the Minimal Inhibitory Concentration and Biocide or Biostatic activity, versus bacteria (*Enterobacter* spp*., Micrococcus luteus,)* and fungi (*Aspergillus niger, Penicillium chrysogenum).* While BMA_1 and BMA_2 showed an antimicrobial action against all microorganisms, BMA_3 didn't show antimicrobial activity against *Aspergillus niger*. More microbial species will be investigated in order to implement the information.

These molecules are totally safe for works of art, restores and environment, requiring short time of application. We hypothesize that these bioactive molecules represent a valid alternative to the traditional procedures in sustainable restoration projects.

Moreover, we are studying and characterizing other bioactive molecules from marine invertebrate organisms with esterase activity. Preliminary results encourage us to test these enzymes to remove wax or oily layers from works of art surfaces.

Acknowledgements

Authors thank the restorers Mauro Sebastianelli, Adriana Richiusa and Maria Rosalia Teri, Diocesan Museum of Palermo.

Funding Body

This study was developed within the research project It@cha, "Ricerca e Competitività 2007-2013", PON 01_00625 (FP) and partially supported by FFR-UNIPA research grant (MC).

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Received: September, 24, 2015
Accepted: February, 25, 2016