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Novel proteases from marine organisms with potential interest in restoration procedure

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ABSTRACT: In the last decades, molecular biology allowed the development of innovative protocols in the field of conservation/restoration of cultural assets. In this work new hydrolyses, isolated from marine invertebrate organisms, are applied to remove protein layers form works of art surface. Proteolytic zymography assay evidenced that these enzymes are active in a broad temperature range, between 4° and 37°C. The enzymatic cleaning by these proteases, tested on wooden furniture of the second half of the eighteenth century showed positive results, without needing to heat the enzyme solution or the surface on which they were applied. The present report proposes novel proteases more appropriate than other, which usually are active at temperature ≥37°C, for a controlled removal of protein layers from wooden painted artifacts.

1 INTRODUCTION

Enzymatic cleaning dates back to 1970 and was initially utilized on paper and later on canvas and polychrome works of art, but they were isolated experiences. Wendelbo (1970) reported the enzymatic hydrolysis (Trypsin in phosphate buffer—pH 8.0, for 10 minutes at 40°C) of animal glues, on glued book pages. Segal & Cooper (1977) described the removal of starch and protein adhesives from parchment by Amylase (phosphate buffer pH 7.0, at 38°C up to 60 minutes) and microbial protease (phosphate buffer pH 7.5, at 40°C). Bio-remediation on painted canvas was first described by Makes to remove glue paste (1982) or protein/oily binder (1988). Bonomi (1994), described the application of a protease solution heated at 40°C, to remove the protein patina from a polychrome ceramic sculpture. Bellucci & Cremonesi (1999) described the enzymatic cleaning (by Lipase) of an acrylic resin coating. The combined action of the metabolic activity of viable bacterial cells (*Pseudomonas stutzeri*, strain A29) and purified enzyme (Protease), was described by Ranalli et al. (2003, 2005), for the bio-cleaning of frescoes (Camposanto Monumentale di Pisa). The application was performed keeping the bacteria solution onto the fresco surface for 10–12 hours, at 28–30°C, followed by using the Protease (Type XIX), to remove the glue residues. Recently, bio-remediation of mural paintings by *P. stutzeri*, has been described by Bosch Roig et al. (2011).

The rational use of an enzyme or of an enzyme mixture requires information on their hydrolytic activity and on the specificity of action, on the nature of the material to eliminate (proteins, starches, oils, fats), on temperature, pH and salt concentration.

In this study, hydrolases isolated from marine organisms allow us to remove protein layers (mainly animal glue) at "room temperature" (19–24°C), without heating the enzyme solution or the artwork surface on which they have been applied.

2 MATERIALS AND METHODS

2.1 *Sampling on protein layers and samples preparation*

Micro-fragments were carefully collected from the surfaces of wooden furniture of the 18th century (MUDIPA), homogenized by Ultra-Turrax in dH_20 (W/V) for 5 minutes in ice, then centrifuged (Eppendorf microfuge) at 13000 rpm for 20 minutes and the supernatant recovered for successive analysis.

2.2 *Characterization of the protein layers samples*

The characterization of each sample was carried out by size exclusion High-Performance Liquid Chromatography (Waters: BioSuite 250-10 Tm, SEC-7.5 × 300 mm, stable pH 2.5–8.0); the pressure was equal to 350 psi (24 atmospheres). 200 µl aliquots of each sample were inserted in a silica column by a rheodine manual injector, and the reading was carried out simultaneously at 280 nm and 220 nm (mAU) for 30 minutes in T.B.S. solution (150 mM NaCl, 10 mM TRIS-HCl pH 7.4).

Quantitative analysis was performed according to the Bradford method using the bovine serum albumin (BSA, concentration 0.1–10 mg/ml) as standard (Salamone et al., 2012).

2.3 *Marine organisms proteases*

Proteases were isolated from tissues or organs of two marine invertebrate species, *Palinurus* and *Anemonia*, and their molecular weight was determined by Sodium dodecyl sulphate–polyacrylamide gels, SDS-PAGE (Laemmli 1970), using 5% (w/v) stacking and 15% (w/v) separating gels. After running (190 V for 45 minutes) the gel was stained in Coomassie solution (2 gr *C*oomassie *B*rillant *B*lue, 500 ml methanol, 100 ml acetic acid, 400 ml distilled water), and destained by DS (10% acetic acid, 40% methanol, 50% dH_2O) The molecular weight was estimated by Bioloabs marker.

2.4 *Protease activity*

The protease (gelatinolytic, caseinolytic) activity was tested by:

- SDS-PAGE containing 0.1% Gelatin as substrate. After running, the gel was incubated in 50 mM TRIS-HCl pH 7.4/1.5% Triton X-100/0.02% Na azide/2 mM $CaCl_2$ solution. Staining was performed by Coomassie Brillant Blue R250 to detect the proteolytic activity (Salamone et al., 2012).
- Kembhavi method (1993), using 1% Casein as substrate. 0.5 ml aliquot of protease mix was dissolved in 0.5 ml of 100 mM TRIS-HCl (pH 8.0) and incubated at 20°C for 15 min. The reaction was stopped by addition of 0.5 ml of 20% Trichloro-Acetic Acid (TCA). After centrifugation at 13,000 rpm for 15 minutes, the precipitate was removed and the absorbance values (280 nm) of the solution determined.

The activity of each single protease was defined as the amount of enzyme hydrolyzing 1 mg per milliliter of Thyroxine, in 1 minute at room temperature (20°C).

2.5 *Bio-cleaning essays*

Proteases at the final concentration of 1 mg/ml have been applied onto 4 cm^2 test surface, by 5% Tylose (Methyl Hydroxyethyl Cellulose) gel as carrier, in order to ensure a selective and controllable cleaning.

The cleaning solution (100 µg/ml protease + 5% gel) respectively *Palinurus* Protease + Tylose gel (Fig. 1) or *Anemonia* Protease + Tylose gel (Fig. 2) were applied for 5 and 10 minutes at the environmental temperatures (22 ± 3°C); a control test was performed for each experiment using 5% Tylose gel without enzyme. After application times the gel was immediately removed by a dry swab first, then with some swabs moistened with dH_2O.

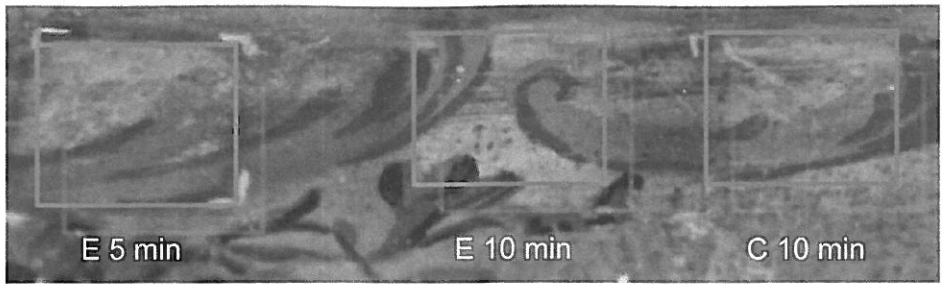

Figure 1. Bio-cleaning test by *Palinurus* protease, stratified by 5% Tylose gel, for 5 and 10 minutes (E). Negative control (C) corresponds to the gel alone applied, for 10 minutes.

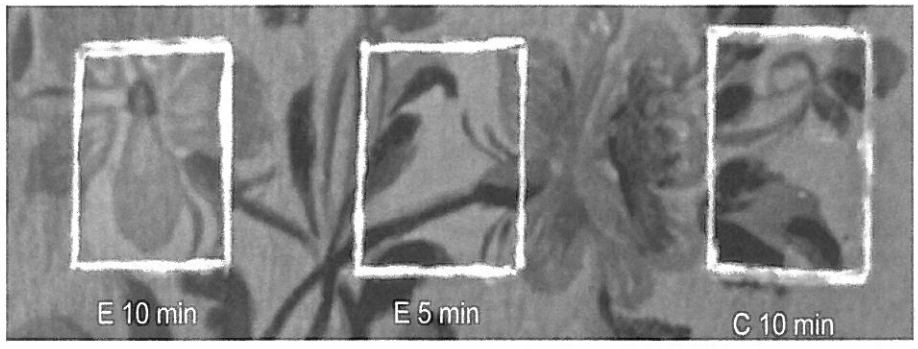

Figure 2. Bio-cleaning test by *Anemonia* protease, stratified by 5% Tylose gel, for 5 and 10 minutes (E). Negative control (C) corresponds to the gel alone, applied for 10 minutes.

3 RESULTS

The SEC-HPLC surveys, conducted on micro-fragments (few milligram) collected from the wood artifacts surfaces, revealed the presence of protein molecules at the concentration of 24–110 micrograms/ml with a molecular weight ranging 20–35 kDa and 60–90 kDa.

The enzymatic activity of the proteases isolated from marine organisms, *Palimurus* and *Anemonia*, was previously tested in laboratory by zymography, through the ability of these enzymes to digest gelatin or casein substrates, respectively at 4°C and 20°C.

The application of these proteases on the wooden artifacts surfaces allowed the hydrolysis of protein layers selectively, in a short time (5–10 minutes), especially in a temperature range which coincided with that for restoration or storage environments (22 ± 3°C).

4 CONCLUSIONS

In the last decades molecular biology has provided both diagnostic protocols and innovative molecules successfully applied in the field of conservation and restoration of cultural assets (Palla et al. 2002, 2006, 2010, Ranalli et al. 2005, Gonzalez 2003).

The cleaning assays reported in this study, were carried out to test the use of protease extracted from marine organisms *Palinurus* sp. (Fig. 1) and *Anemonia* sp. (Fig. 2), to hydrolyze and easily remove animal glue layers, from the surface of wooden furniture (18th century).

They fulfill the criteria of the modern restoration: compatibility with the original constitutive materials; minimal intervention; selectivity and controllability of the cleaning (reduced invasive action); applicability at environmental temperature.

Considering the results we hypothesize that these enzymes will implement the efficiency of bio-cleaning protocols, according to the conservative procedures.

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From 2nd to 5th October 2012 an International Congress on Science and Technology for the conservation of Cultural Heritage was held in Santiago de Compostela, Spain, organized by the Universidade of Santiago de Compostela on behalf of TechnoHeritage Network. The congress was attended by some 160 participants from 10 countries, which presented a total of 145 contributions among plenary lectures, oral, and poster communications. The congress was dedicated to eight topics, namely (1) Environmental assessment and monitoring (pollution, climate change, natural events, etc.) of Cultural Heritage; (2) Agents and mechanisms of deterioration of Cultural Heritage (physical, chemical, biological), including deterioration of modern materials used in Contemporary Art and information storage; (3) Development of new instruments, non invasive technologies and innovative solutions for analysis, protection and conservation of Cultural Heritage; (4) New products and materials for conservation and maintenance of Cultural Heritage; (5) Preservation of industrial and rural heritage from the 19th and 20th centuries; (6) Security technologies, Remote sensing and Geographical Information Systems for protection and management of Cultural Heritage; (7) Significance and social value of Cultural Heritage; and (8) Policies for conservation of Cultural Heritage. This volume publishes a total of ninety three contributions which reflect some of the most recent responses to the challenge of cultural assets conservation.

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