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Fluorescent method for detection of cleaved collagens using O-phthaldialdehyde (OPA)

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Abstract

Analysis of collagen degradation remains an important but cumbersome task. Traditional methods with dansyl chloride derivitatization of collagen have been used to quantify collagen damage. Fluorescent labeling reagents have been developed that offer advantages such as greater solubility in water and low background emission. One such reagent is o-phthaladehyde (OPA). In this study, we used OPA as a means of detecting small amounts of degraded collagen. Collagen samples isolated from skin or heart were used for OPA conjugation to exposed amino termini ("opalation"). Experiments utilizing small samples aliquoted in microtiter plates were performed to evaluate effects of increasing concentrations of OPA, varying concentrations of collagen, and effects of matrix metalloproteinase (MMP) digestion. Results indicate that within 10 minutes of reaction, OPA can be used to detect relative differences in cleaved vs. uncleaved collagen from skin or heart. Heart samples obtained from regions of high MMP activity correlated with increased OPA fluorescence relative to tissue with lower MMP activity. On the basis of these results, we conclude that OPA has valuable practical advantages for analytical use in detecting cleaved collagen in small tissue samples.

1. Introduction

Collagen, in particular fibrillar types I and III, is the most abundant protein type present in humans [1]. Collagen is the target of many pathological processes that alter collagen content or damages its structural integrity. Over the years, various biochemical methods that detect the presence of collagen fragments in the bloodstream have been developed [2]. However, these techniques, at best, yield an indirect read of the *in situ* level of collagen turnover. Assessment of tissue collagen damage via improved, reliable and well established biochemical techniques is thus, of great value to scientists and clinicians.

Collagen damage has been assessed by fluorescence detection using the fluorescent dye, dansyl chloride via conjugation to free primary amines that become accessible following collagen degradation [3]. This process is referred to as dansylation. Dansyl can be covalently attached to the amino terminus of a protein under specific conditions [4]. As more collagen is cleaved, more N-termini are made available for dansylation. However, since its original development, no further improvements in this technique have been implemented. Over the years, other fluorescent compounds that react with primary amines have been developed, such as fluorescamine and o-phthaldialdehyde (OPA, a.k.a. o-phthalaldehyde) [5,6]. OPA was shown to possess an approximate five-fold greater sensitivity vs. fluorescamine in detecting tryptic

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peptides [6]. Because it undergoes a strong fluorogenic reaction (in the presence of excess thiols) with α-amino acids such as are found at the N-terminus of peptide backbones, in principle, OPA can be used to estimate protein concentrations [5]. For the same reasons OPA can also be used to assay protein cleavage (i.e. degradation) where more N-termini are exposed. OPA is relatively non-fluorescent unless it is bound to a primary amine [7]. The low background fluorescence of OPA is predicted to yield a better signal-to-noise ratio than fluorometric techniques currently in use. OPA is also preferable over dansyl because the "opalation" reaction reaches completion within minutes and because OPA is soluble and stable in aqueous buffers [5,8].

The purpose of this work was to test the capacity of OPA to detect cleaved collagen. In addition, we show the utility of analytical-scale opalation reactions for use in rapid-throughput microplate assays using small amounts (micrograms) of collagen.

2. Materials and methods

2.1 Materials and Reagents

OPA was purchased from Alpha Aesar (Ward Hill, MA). OPA reagent was made fresh daily by dissolving 5 mg of OPA in 125 μl 95% ethanol, and adding 4.9 ml of 0.1 M phosphate buffer saline (PBS) pH 7.4 and 10 μl of β-mercaptoethanol. The final solution was 1 mg/ml of OPA in 0.1 M PBS pH 7.4, with 2.4% ethanol, and 0.2% β-mercaptoethanol.

2.2 Collagen Experiments

Collagen samples used in these experiments were either purchased from commercial sources (SIGMA Chemicals; rat type I collagen) or total fibrillar collagen extracted and purified from canine heart tissue. Rat type I collagen was dispersed in 0.1 M acetic acid at 1 mg/ml and dialyzed (12,000-14,000 MWCO, Fisher Scientific) against a 500-fold volume of 0.1 M acetic acid. This step was necessary to remove free amino acids, which when combined with OPA, fluoresce brightly [8] and could generate high background fluorescence. The total collagen extraction method for samples of myocardium was modified from Garcia et al. [9]. Approximately 50-100 mg of frozen (-80° C) transmural cardiac tissue was dissected from canine heart ventricle. Samples were collected from regions in which low and high matrix metalloproteinase (MMP) activity was detected [9]. Tissue was minced, then sonicated on ice 5-6 times for 15 second bursts (resting 45 seconds in between to prevent overheating) in 1 ml of 10 mM Tris-HCl pH 7.7, 0.1% Triton X-100, 0.2 mM NaN_3. After centrifugation for 2 min at 14,000 g the supernatant was discarded and the pellet resuspended in 1 ml of 1.0 M NaCl, then centrifuged 1 min at 14,000 g. This NaCl extraction was repeated twice more. The pellet was extracted three times with 1 ml of 0.07 M disodium phosphate, then three times with 1 ml of 0.15 M sodium citrate pH 3.7, with resuspension and centrifugation each time (saving the pellet each time). The insoluble collagen fraction was collected by centrifugation for 1 min at 14,000 g and the supernatant discarded. The pellet was washed 5 times with 1 ml of de-ionized water to remove any residual salts, and resuspended in 0.5 M acetic acid to disperse the collagen into solution. This was dialyzed overnight at 4° C (12,000-14,000 MWCO) in 0.5 M acetic acid. The collagen solution was then centrifuged for 20 min at 14,000 g to spin out any residual tissue, and the supernatant saved to a clean tube. NaCl was added to a final concentration of 2 M NaCl to precipitate the collagen. After centrifugation for 5 min at 14,000 g, the collagen pellet was washed 5 times with 1 ml de-ionized water and resuspended in 0.1 M acetic acid for subsequent use. The total time for completion of collagen sample preparation from tissue is typically 2 days. The isolated total heart collagen samples contain both types I and III fibrillar collagen. A 10 μl sample of solubilized total heart collagen was taken and processed as described below for opalation reactions. To normalize OPA fluorescence readings to collagen

concentrations a hydroxyproline assay (requiring ~ 3 days) was performed as described in Garcia et al. [9].

2.3 Opalation Reactions

Opalation reactions were carried out in 300 μl total volume in white, 96-well plates (Nunclon #136101) that are made from white polystyrene to optimize fluorescence detection. A 100 μl volume of fresh OPA reagent was added to 200 μl of sample (containing 0-40 μg of 1 mg/ml collagen in 0.1 M acetic acid) diluted in 0.1 M PBS, pH 7.4. The reaction was read on an FLX 800 microplate fluorescence reader (Biotek Instruments, Inc.) with excitation at 360 nm and emission at 460 nm. Readings were taken every 60 seconds for 15 min. Fluorescence intensity was measured as relative fluorescence units (RFU). The final pH of all reactions was 7.4, unless otherwise noted. To determine whether there is optimum OPA-to-collagen ratio at low collagen levels, 0.1 or 0.2 mg of OPA reagent were reacted with 10 μg of collagen and read as above.

To determine the range of concentrations at which opalation reactions detect intact collagen 5 μg, 10 μg, 20 μg, and 40 μg of protein were reacted with 100 μl of OPA reagent. The development of fluorescence was plotted over time and analyzed for the timing of peak and plateau for each concentration. A single time point was selected that coincided with the maximum plateau for all the concentrations tested. This was used to create the standard curve of fluorescence intensity versus intact collagen concentration.

2.4 Collagenase and MMP Experiments

Initial experiments were performed to analyze the time dependent generation of fluorescence by degraded collagen. Subsequently, 10 μg of pure type I and *ex vivo* extracted total collagen were digested with various concentrations of bacterial collagenase (0.25 –1.0 U; Worthington Biochemical Corp., Lakewood, NJ) for 5 min at 37°C. The digestion was stopped by placing the reaction tubes on ice for 5 min. OPA reagent was added directly to the digestions, and read as above. Reactions were performed in triplicate. To determine the correlation between myocardial regions of known low and high MMP activity and levels of collagen degradation, samples of purified total collagen were used. As outlined in Garcia et al., [9] myocardial samples were selected from one animal that was subjected to pacing induced abnormal wall motion from regions with high MMP activity (as determined using fluorescent peptide probe assays, n=3) and contrasted with those of normal wall motion (low MMP activity regions, n=3).

3. Results

3.1 OPA reaction kinetics and OPA/collagen Ratios

We first determined the kinetics of opalation reactions by tracking time-dependent changes in intact collagen-mediated-fluorescence (Figure 1a). Background values for OPA fluorescence (i.e. no collagen added) were obtained and used to generate corrected values (data not shown). Results indicated that OPA fluorescence increased over time and was stable ~10 min after reacting with collagen. Given these results, in subsequent experiments OPA generated fluorescent readings were always taken at 10 min for subsequent data analysis. We also determined if the ratio of OPA:collagen yielded a difference in background fluorescence readings by varying the amount (0.1 or 0.2 mg) of OPA reacted with 10 μg of intact collagen. Figure 1b results demonstrate that comparable amounts of fluorescence are obtained irrespective of doubling the amount of OPA used, indicating that increasing the amount of unreacted OPA does not increase background fluorescence. Thus, 0.1 mg of OPA is sufficient to yield a robust signal for the collagen concentration tested.

Experiments were also performed to assess changes in OPA (0.1 mg) fluorescence as a function of increasing intact collagen concentrations. As clearly demonstrated in figure 2a, fluorescence

levels increased, reaching a plateau by ~10 min in proportion to the amount of collagen present in the reactions. Substantial fluorescence emissions were detected using at least 5 μg of collagen (fig. 2a). Figure 2b plots 10 min fluorescence values obtained from figure 2a and demonstrates a linear relationship between the collagen mass and OPA fluorescence.

3.2 In situ collagen cleavage experiments

Experiments were designed to test the effects of exposing a fixed amount of intact collagen (10 μg) to increasing amounts of collagenase. Purified type I skin collagen or total fibrillar collagen from heart tissue were used. Collagen samples were allowed to react with collagenase for 5 min at 37°C. As shown in figure 3, exposure of intact collagen to increasing amounts of collagenase led to increases in OPA fluorescence.

Samples of dog heart total collagen with constitutive low or high MMP activities were reacted with OPA to detect collagen cleavage. OPA fluorescent readings (kinetic reads from 0-10 minutes) were normalized per milligram of collagen included in the assay. Results shown in figure 4 indicate that tissues samples with high levels of myocardial MMP activity showed significant increases in fluorescence-emission, suggesting that MMP-mediated collagen cleavage was greater in samples with high MMP activity.

4. Discussion

Our study provides evidence for the suitability of using small-volume opalation reactions as a means to conveniently quantify relative levels of enzyme-mediated collagen degradation.

OPA has been used to quantify proteins, peptidase activity and to detect lysine residues within a protein's active site [5,8,10]. The latter method takes advantage of the ability of OPA to bind the α-amine on the side chain of lysine. In our experiments, OPA reactions at neutral pH were used to preferentially label the terminal amine, as the pK_a of the amino-terminal amine is lower than that of the lysine α-amino groups [6,7]. The ability to detect increases in amino-terminal amines with OPA resulting from collagen breakage is helpful for assessing activities of collagen-remodeling enzymes in a range of both physiological and pathological conditions. When OPA's properties are contrasted to those of the currently used dansyl chloride method OPA appears superior on the basis of its well characterized applications for peptides and protein analysis, stable fluorescence, fast reactivity, water solubility and low background emission (i.e. better sensitivity) [5-8]. OPA is also an affordable reagent. However, it is worth noting that newer, more costly fluorogenic substrates (e.g. cy3) are available which are likely also suitable to detect collagen cleavage.

As OPA fluorescence is dependent on the number of exposed N-termini, it can be used as a reliable means to quantify collagen degradation. Our results indicate that exposure of intact collagen to increasing amounts of collagenase leads to linear increases in fluorescence. This result was independent of the source of fibrillar collagen used (skin or heart). Although both curves increased as a function of collagenase concentration, their slopes were different. It is possible that the organ/tissue source of collagen could affect labeling efficiency. We conjecture that unlike purified rat skin type I collagen, dog heart collagen samples used in these experiments likely possessed residual structure that imposed steric hinderance for labeling. In addition, the collagen extracted from the heart is a mixture of types I and III. However, collagen degradation differences correlated with measured levels of low and high MMP activity present within these tissues (figure 4).

The opalation technique reported here for assessment of collagen breakage has potential application for use with small analytical samples such as that obtained from mouse organs/

tissues. The ability to use this method with mouse tissues allows analyses of protease degradation of collagen in a wide range of genetically altered backgrounds.

5. Simplified description of the method and its applications

The opalation technique described allows for the improved quantitation of collagen degradation in biological samples. Analytical-scale opalation reactions are performed in a physiological buffer using microgram samples of purified collagen dissolved in 0.1 M acetic acid. This method works optimally when collagen is highly pure since non-collagenous peptide impurities can affect baseline fluorescent readings. The reaction is complete by 10 min and fluorescence can be quantified using a microplate reader. The technique takes advantage of the superior properties of o-phthaldehyde such as excellent solubility in water, rapid reactivity and low background fluorescence yielding an improved signal/noise ratio. Once pure collagen samples are available, this technique requires in total (including fluorescent reading time) less than 1 hour for completion. This optimized method should be useful to life-science researchers and clinicians who wish to detect levels of collagen degradation using small tissue sample sizes.

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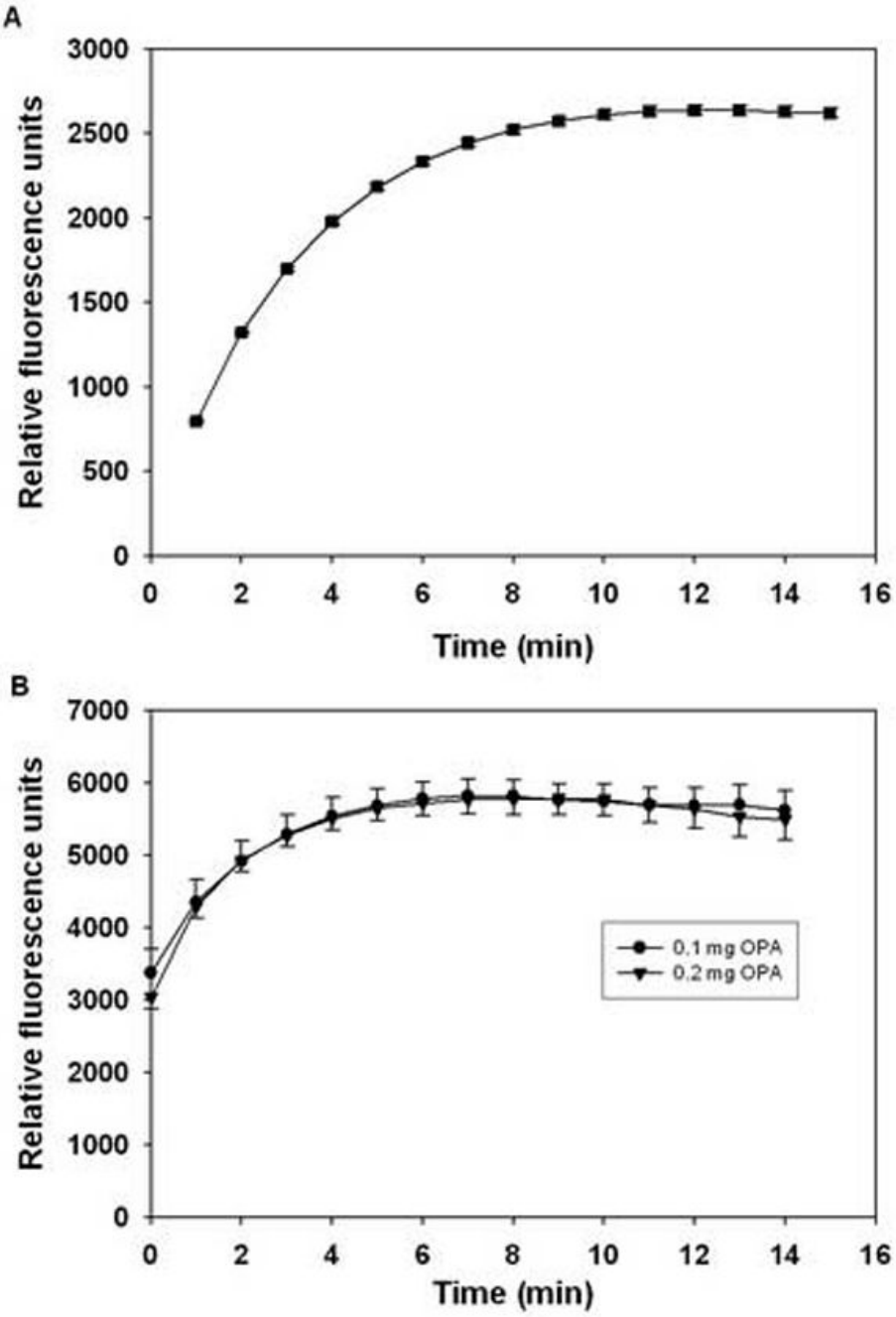

Figure 1. Opalation reactions of purified collagen and effects of OPA concentrations

Panel A illustrates the time dependence of opalation reactions on intact purified type I skin collagen (10 μg). Fluorescence emission is maximal at ~10 minutes. Panel B illustrates the effects of increasing concentrations of OPA on collagen (10 μg) fluorescence readings. Since panel B experiments were executed independently from those in panel A, Y axis arbitrary unit values are distinct. Values shown are mean ± SD. If no error bars are evident they were within the height of the symbols shown.

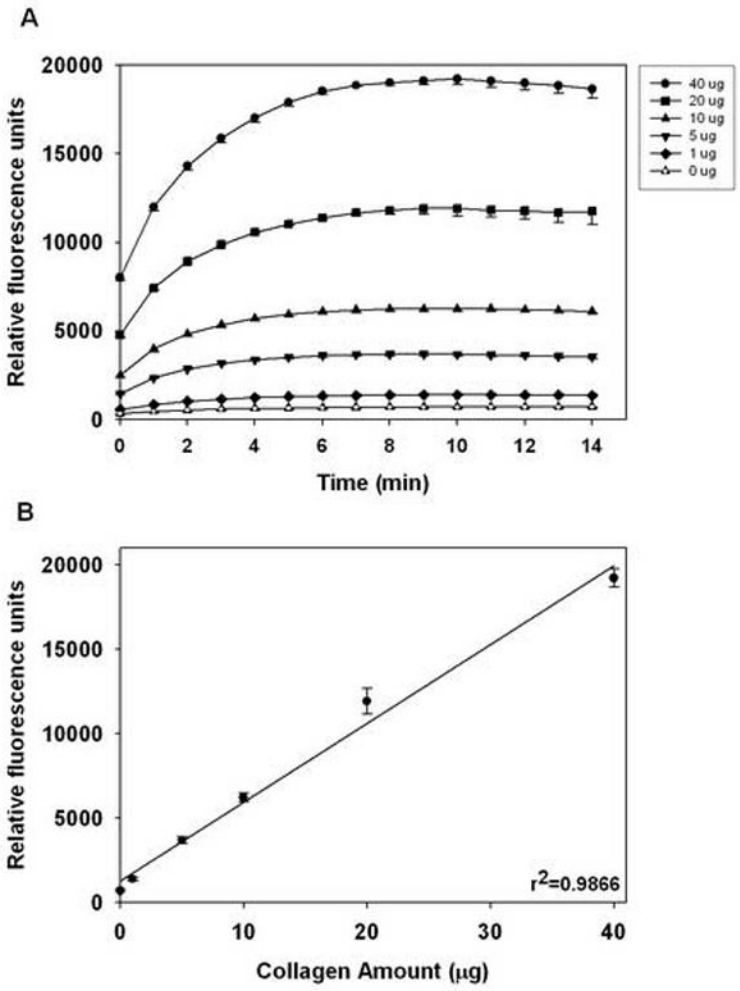

Figure 2. Effects of increasing collagen concentrations on OPA fluorescence emission

Panel A illustrates the effects that increasing collagen has on fluorescence readings. Panel B plots these values and illustrates the linearity of the relationship between increasing amounts of collagen and fluorescence emission. Values shown are mean ± SD. If no error bars are evident they were within the height of the symbols shown.

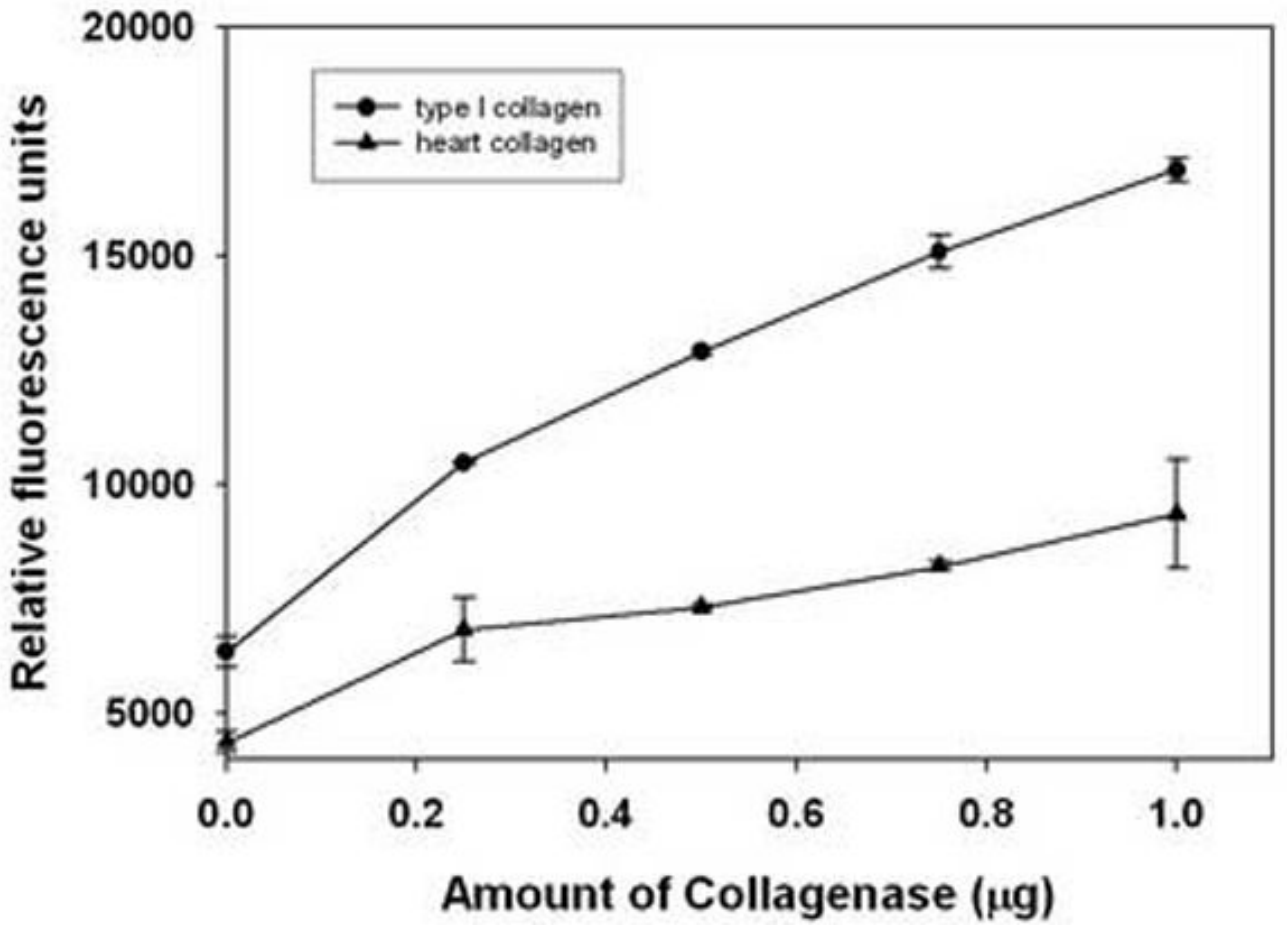

Figure 3. Effects of collagenase digestion of purified type I skin or total heart collagen on OPA generated fluorescence emission

Purified type I skin collagen or total dog heart collagen (10 μg) were exposed to increasing amounts of bacterial collagenase. OPA-fluorescence was obtained and plotted as a function of the amount of collagenase used. Values shown are mean ± SD. If no error bars are evident they were within the height of the symbols shown.

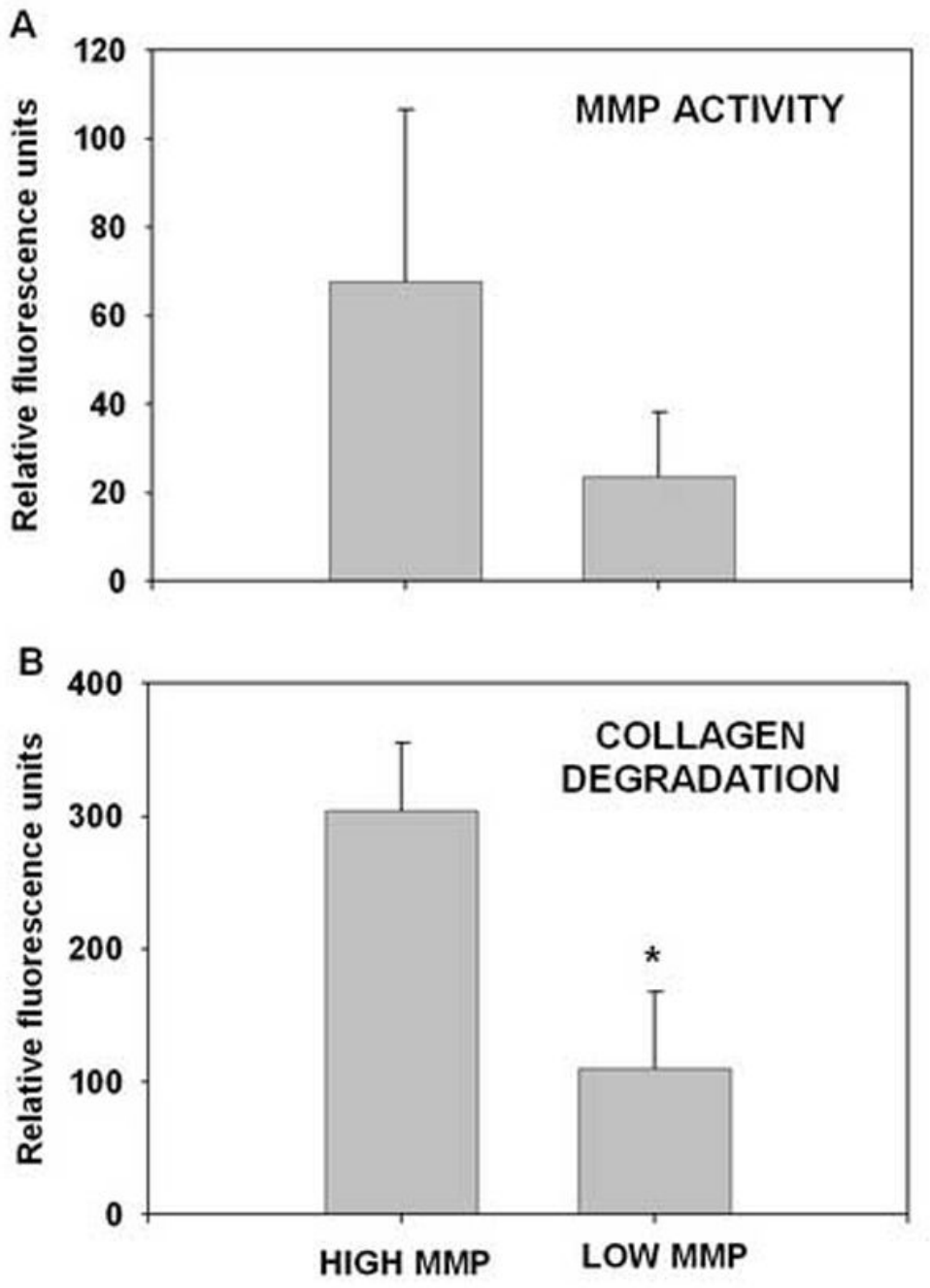

Figure 4. MMP and OPA generated fluorescence readings obtained from total collagen heart samples in regions with low or high MMP activity

Panel A illustrates MMP activity results as derived from the use of fluorescent peptide substrates assays obtained from one dog in samples with low (n=3) or high (n=3) MMP activity. Panel B illustrates the results derived from the opalation of extracted total collagen samples obtained from the same region. Results demonstrate a corresponding relationship between MMP activity levels and collagen cleavage (t-test, p=0.03, opalation of low vs high MMP activity regions). Values shown are mean ± SD.